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Regulation of thyrotropin-releasing hormone (TRH)-degrading enzymes

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REGULATION OF THYROTROPIN-RELEASING HORMONE (TRH)-DEGRADING ENZYMES

by

Chen-Shian Suen

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ABSTRACT :

REGULATION OF THYROTROPIN-RELEASING HORMONE (TRH)-DEGRADING ENZYMES

by

Chen-Shian Suen

Adviser : Professor Sherwin Wilk

Thyrotropin releasing hormone (TRH, pGlu-His-Pro-NH_2) is degraded by three enzymes. The pGlu-His bond is cleaved by pyroglutamyl peptidases I and II separately and the Pro-NH_2 bond is cleaved by prolyl endopeptidase. Pyroglutamyl peptidase I (EC 3.4.19.3) and prolyl endopeptidase (EC 3.4.21.26) are widely distributed cytosolic proteases and have a broad substrate specificity. Pyroglutamyl peptidase II (EC 3.4.19.-), a membrane-bound metalloprotease, is a highly specific TRH degrading enzyme. It has been known that thyroid hormone negatively regulates TRH action at the pituitary gland. The molecular mechanism of TRH action in GH_3 cells has been extensively studied. Activation of TRH receptors leads to the hydrolysis of phosphatidyl inositol, generating the dual signals inositol triphosphate (IP_3) and diacylglycerol (DG). DG is an endogenous activator of protein kinase C. The effects of thyroid hormone, the protein kinase C activator (TPA), and sodium butyrate on TRH degrading enzymes was investigated. These studies were carried out either in cell culture (GH_3 Cells and Y-79 retinoblastoma cells) or in vivo.

Exposure of GH_3 cells to thyroid hormone (T_3) led to a time-and concentration-dependent increase in pyroglutamyl peptidase I activity (EC_{50}= $5X10^{-10}M$). The increase in this enzyme activity was not due to a decrease in the K_m for the substrate but rather to an increase in V_{max}. Experiments with cycloheximide indicated that the increase in V_{max} was due to induction of new enzyme synthesis. In in vivo studies, we have also found an increase in the activity of pyroglutamyl peptidase I in a number of brain regions and in the pituitary following chronic administration of T_3. This effect was most marked in the hypothalamus and pituitary gland where pyroglutamyl peptidase I activity was increased two to three fold.

Acute treatment with T_3 produced a three fold increase in pituitary pyroglutamyl peptidase II activity, and a 70% increase in the activity of this enzyme in frontal cortex. These studies indicate that control of TRH degradation by thyroid hormone is one of the mechanisms in the negative feedback regulation of thyroid hormone by T_3.

Sodium butyrate, a short chain fatty acid, can exert a variety of effects in GH_3 cells, such as increasing both growth hormone (GH) and prolactin (PRL) synthesis 2- to 4-fold, changing cell morphology, and decreasing TRH receptors. We found that the specific activity of pyroglutamyl peptidase I increased upon exposure to sodium butyrate in a time- and dose-dependent manner, whereas the specific activity of prolyl endopeptidase was unchanged. The maximal effect occurred at a concentration of 1 mM sodium butyrate and at 16 h after exposure. Cycloheximide totally blocked this stimulation, indicating that the increased enzyme activity was due to new protein synthesis. These studies suggest that sodium butyrate can mimic the action of T_3 in regulation of pyroglutamyl peptidase I in GH_3 cells.

The Y-79 human retinoblastoma cell, derived from a primitive neuroectodermal cell, contains relatively high amounts of pyroglutamyl peptidase II activity. Exposure of these cells to 12-0-tetradecanoyl phorbol 13-acetate (TPA) caused a biphasic inactivation of pyroglutamyl peptidase II but did not affect pyroglutamyl peptidase I or prolyl endopeptidase. The rapid and marked inactivation of pyroglutamyl peptidase II occurred after 5 min, with only 10% enzyme activity remaining after 15 min treatment. The enzyme activity returned to 70% of the control at 1 h. A delayed phase of inactivation occurred upon longer exposure of the cells to TPA. Both phases of these phenomena were mediated by TPA activated protein kinase C. Pretreatment of the cells with protein kinase C inhibitors abolished the TPA mediated decrease of pyroglutamyl peptidase II activity. Protein kinase C was rapidly translocated from cytosol to membrane as determined by measuring the enzyme activity and by quantitatively immunoblotting PKC molecules. Immunoblot and immunoprecipitation analysis of pyroglutamyl peptidase II demonstrated that inactivation of pyroglutamyl peptidase II at the early phase was not due to dissociation and/or internalization of this enzyme but rather due to phosphorylation by TPA-activated protein kinase C.

Measurement of new protein synthesis by ^{35}S-methionine labelling showed that the decreased pyroglutamyl peptidase II activity after longer exposure of the cells to TPA resulted from a decreased de novo synthesis of this enzyme. It has been suggested that polypeptide hormone levels may be controlled not only by their rate of synthesis, but also by their rate of enzymatic degradation. We propose that up- and down-regulation of TRH degrading enzymes are of physiological significance in biological actions of TRH.

FORMAT OF THESIS

This thesis is prepared according to the new guidelines of the City University of New York which permit the direct incorporation of published research articles as chapters. The thesis has a general introduction, several papers as chapters and a general discussion. Each chapter contains a specific introduction, materials and methods, results, discussion and references. The references for the general introduction and discussion follow the discussion. Copyright permission for each chapter has been obtained from the respective publishers.

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INTRODUCTION :

The idea that the thyrotropic function of the pituitary was under the control of the brain derived from earlier physiological experiments. In the late 1940s, several workers had shown that the pituitary stalk section modified thyroid activity, and by the mid-1950s, it had been shown that section of the pituitary stalk, or lesions of the hypothalamus, inhibited pituitary-thyroid function. On the basis of these results, the existence of a hypothalamic TSH-releasing factor was postulated (1). The first significant efforts to isolate a thyrotropin-releasing factor (TRF) were reported by Shibusawa and colleagues in a series of papers beginning in 1956 (2,3,4). Guillemin and Schally begun to work on isolating TRF in 1961 and 1962 separately, and almost simultaneously reported the structure of native TRF in 1969. Thyrotropin releasing hormone (TRH), the first hypothalamic factor isolated, is a weakly basic tripeptide (5,6). The chemical structure of TRH is pyroglutamyl-histidyl-proline-amide (pGlu-His-Pro-NH_2). The presence of both the pyroglutamic acid and the carboxyl terminal amide are important for the biological activity and metabolic stability of the compound. The synthesis of TRH is carried out in the hypothalamus, from which it is secreted into the portal venous system to reach its target cells, the thyrotropes of the anterior pituitary.

Distribution studies, using both radioimmunological and immunohistochemical techniques, have revealed a wide distribution of this tripeptide in the central nervous system (CNS) and peripheral tissues (7). In the hypothalamus, extensive TRH positive-cell groups were seen mainly in the parvocellular part of the paraventricular nucleus and in the periventricular area. Cells were also seen overlying the optic chiasm. The supraoptic nucleus also contained some TRH positive cells. Other hypothalamic nuclei containing TRH positive-cell bodies were the dorsomedial nucleus, the posterior arcuate nucleus and the basolateral hypothalamus. In addition to the hypothalamus, TRH-immunoreactive (IR) cell bodies were seen in the glomerular layer of the olfactory bulb. In

cortical areas, particularly high numbers of TRH-positive cells were seen in the piriform cortex, and entorhinal cortex. In the hippocampal formation, TRH-positive cells were seen both in the pyramidal and granular cell layers and in the ventral aspects of the dentate gyrus. TRH-IR cells were found in the amygdaloid complex. In the spinal cord, numerous small TRH-IR cell bodies were observed in lamina II-III of the dorsal horn. In peripheral tissues, TRH has been shown to be present in the pancreas with a marked postnatal decline in TRH levels (8,9). In the rat, the TRH-positive cells were present in the central part of the islet, presumably representing insulin-producing beta cells, in agreement with the results showing that streptozotocin treatment markedly lowers TRH levels in pancreatic islets (9.10). It has been demonstrated that TRH enhances arginine-induced glucagon release from the pancreas of the adult dog (11). and that TRH can inhibit somatostatin release (12). Release of TRH concomitantly with insulin would therefore counteract somatostatin-induced inhibition of insulin release and result in increased insulin serum levels.

Although the structure of TRH (pGlu-His-Pro-NH_2) was determined nearly two decades ago, only recently has information been obtained about the synthesis and the gene of this regulatory peptide. The TRH cDNA was cloned from a rat hypothalamic lambda gt11 expression library using an antiserum directly against a synthetic peptide containing the TRH progenitor sequence, Gln-Pro-His-Gly, flanked by paired basic residues and cysteines (13). The nucleotide sequence of the TRH cDNA contains an open reading frame that encodes a 255 amino acid protein with a molecular weight of approximately 30 kD_a (14). The deduced protein contains a hydrophobic leader sequence characteristic of a secreted protein and five copies of the TRH precursor sequence, Gln-His-Pro-Gly, flanked by paired basic amino acids. This structure suggested that five TRH molecules are generated from a single molecule of the precursor. The identity of the isolated cDNA clone was confirmed by *in situ* hybridization analysis using radiolabeled antisense pro-TRH RNA as the probe (14). TRH mRNA is localized to the parvocellular part of the paraventricular nucleus of the hypothalamus, corresponding to the known distribution of TRH cell bodies detected by immunohistochemistry. In the

initial study of hypothalamic control of pituitary-thyroid function, Shibusawa et al (15) reported that cells in the paraventricular nucleus showed increased neurosecretory activity in hypothyroidism, and showed decreased activity after thyroxine replacement. This finding was recently confirmed by using specific molecular probes for TRH mRNA and antiserum reacting with TRH prohormone. A subpopulation of paraventricular neurons (PVN) was shown to be activated in hypothyroidism (16).It is now well-accepted that the dominant stimulatory role of the hypothalamus in the control of TSH synthesis and release is mediated by TRH. The direct dose-related action of TRH on TSH release has been demonstrated both in vivo and in vitro. Decreased TSH release and hypothyroidism are consequences of hypothalamic-pituitary dissociation and hypothalamic lesions (17,18,19). The effect of TRH on TSH release is achieved at nanomolar concentrations (20) and is mediated by specific high affinity receptors (21). In addition to stimulating TSH release, TRH also stimulates TSH synthesis. A biphasic pattern of TSH release is seen after prolonged intravenous infusion of TRH in man. The early phase may reflect the release of a readily releasable pool of stored TSH within the thyrotropes, whereas the later phase could be due to release of newly synthesized TSH produced under the influence of increased TRH drive (22).

The actions of TRH are initiated by interaction with a specific membrane receptor. [^{3}H]Me-TRH binding has been studied extensively using tumor cell line models of thyrotropes and mammotropes (23,24). Equilibrium binding of [^{3}H]Me-TRH to intact cells and isolated membranes has shown that [^{3}H]Me-TRH appears to bind to a specific class of high affinity binding sites with an apparent dissociation constant of 10 nM. This concentration is close to the concentration of TRH causing half-maximal stimulation of hormone secretion (2-3 nM), when measured under the same conditions (25). The TRH receptor exhibits strict structural specificity in all three amino acid positions (23). Substitution of the N-terminal pGlu with a noncyclic Gln, for example, causes a 200 fold decrease in binding affinity. The receptor likewise has stringent requirement at the His moiety. The N-1-methyl-His derivative is relatively inactive, whereas the N-3 methyl-His compound is 3-10 times more avidly bound than the parent compound. The TRH receptors appear to be restricted to responsive cells.

Autoradiography reveals that only a fraction of anterior pituitary cells apparently bind radioactive TRH, consistent with restriction of receptors to thyrotropes and mammotropes (26). TRH receptors were also found in the CNS by either ligand binding or receptor autoradiography (27,28). The biochemical properties of TRH receptors from pituitary gland and brain have been compared and found to be quite similar. Receptors from both tissues bind [^{3}H]Me-TRH with similar affinity and competition curves for TRH analogues are similar . The effect of monovalent cations and reagents capable of covalently modifying proteins are also in agreement (29). Although these receptors are similar in many respects, some evidence has shown that (a) binding sites from pituitary and pituitary tumor cells are regulated by guanyl nucleotides, whereas no guanyl nucleotide effect can be demonstrated for the receptors from brain (30), (b) in isoelectric focusing studies of TRH receptor solubilized with Triton X-100, brain receptors exhibited an isoelectric point (pI) of 5.5, whereas the pI for pituitary receptors was only 4.9 (31). The most obvious interpretation is that receptors in brain and pituitary differ in amino acid sequence due to expression of different receptor genes or from differential RNA splicing.

The number of TRH receptors is regulated both homologously and heterologously. Occupancy of TRH receptors by TRH leads to a loss of TRH binding sites, i.e. homologous desensitization. This occurs with both mammnotropes (32) and thyrotropes (33). The process of down-regulation of TRH receptors is slow, requiring 24 hours to reach a maximum, and it requires ongoing protein synthesis. The concentration of TRH receptors can also be modulated by other hormones in what appears to be physiologically significant heterologous receptor regulation. Thyroid hormones cause a slow and fully reversible decline in TRH receptor density, in parallel with a diminished TRH response (34). The regulation of TRH receptors by thyroid hormone occurs in vivo and in normal pituitary cells in culture, as well as in mammotropic and thyrotropic tumor cells. Estrogens, by contrast, can increase TRH receptor levels in vitro and in vivo, which may account for the heightened sensitivity of pituitary cells to TRH in the presence of elevated estrogen (35). Glucocorticoids also lead to stimulation of TRH receptor density (36). The mechanism of regulation of TRH receptor density by these hormones is not known. It has been known

that steroid and thyroid act on their own receptors, transcriptional factors, to either turn on and/or off gene transcription (37). It is likely that the regulation of concentration of TRH receptors by these hormones can be accounted for by changes in receptor synthesis.

GH cells, clonal prolactin (PRL)-secreting cells derived from rat pituitary tumors, have been chosen as the experimental system to study the mechanism of TRH action during the last several years (38). It was well established that a burst phase of secretion at a rate 6-10 times higher than basal secretion lasts approximately 2 minutes followed by a sustained phase at a rate 3-4 times basal lasting at least 30 minutes . By 1987, it was clear that hormone-regulated phospholipid hydrolysis constituted an important transduction process for hormones (39). Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a minor plasma phospholipid that undergoes hydrolysis on addition of TRH to GH_3 cells (38). Direct TRH receptor-regulated stimulation of membrane-associated PIP2 phospholipase C has been demonstrated in vitro and is mediated through activation of a GTP-binding factor (G protein). The two products of PIP_2 hydrolysis each serve second messenger functions and are involved in mediating the biphasic PRL-releasing action of TRH in GH_3 cells. Inositol 1,4,5 triphosphate (IP_3) activates the release of Ca^{+2} from an intracellular, nonmitochondrial sequestered pool. The subsequent cytoplasmic Ca^{+2} rise may underlie the rapid first phase increase in PRL release. Simultaneously and in equimolar amounts, there is the beginning generation of 1,2-diacylglycerol (1,2-DG). Sustained formation of 1,2-DG, however, is from persistent hydrolysis of PI, and not PIP_2, allowing for generation of a lipid-soluble messenger in the absence of a Ca^{+2} signal (40). 1,2-DG activation of protein kinase C apparently mediates the sustained phase of PRL secretion. Protein kinase C presumably acts via phosphorylation of proteins involved in the exocytotic process.

TRH is rapidly degraded in body tissues by enzymatic breakdown (22). The half-life of TRH in human and rat plasma is 6.5 and 3 min, respectively

(41). It is now recognized that three enzymes can catalyze the initial degradation of TRH at either the pGlu-His or Pro-NH_2 bonds (Fig.1). The Pro-NH_2 bond of TRH is cleaved by prolyl endopeptidase (EC 3.4.21.26). Two distinct enzymes, pyroglutamyl peptidase I (EC 3.4.19.3) and pyroglutamyl peptidase II (EC 3.4.19.-), can cleave the pGlu-His bond (42). These three enzymes will be described separately:

(1) Prolyl endopeptidase cleaves peptide bonds on the carboxyl side of proline residues within a peptide chain. In addition to TRH, this enzyme also cleaves a variety of peptides such as substance P, LHRH, neurotensin, bradykinin and angiotensin II. Prolyl endopeptidase is a serine protease and contains a sulfhydryl group whose integrity is required for maximal expression of enzymatic activity. Within the brain, prolyl endopeptidase exists predominantly as a cytosolic enzyme. It can be potently inhibited in vitro by the peptide aldehyde Cbz-Pro-Prolinal, the first transition state analogue inhibitor of prolyl endopeptidase to be synthesized (43). This compound inhibits prolyl endopeptidase noncompetitively with a Ki of 14 nM. Cbz-Pro-Prolinal is also an effective inhibitor in vivo. This lipophilic compound readily crosses the blood-brain barrier, where it produces a long-lasting inhibition of brain prolyl endopeptidase (44).

(2) Pyroglutamyl peptidase I (EC 3.4.19.3) is a cysteine protease and cleaves all pyroglutamyl amino acid bonds except pGlu-Pro (45). Neuropeptide substrates of this enzyme in addition to TRH include LHRH, neurotensin and bombesin but not eledoisin which has a pGlu-Pro bond. Pyroglutamyl peptidase I is primarily a cytosolic enzyme with a widespread tissue distribution. Within the brain, the enzyme is fairly uniformly distributed. Purified pyroglutamyl peptidase I is virtually inactive in the absence of a thiol-reducing agent and is highly sensitive to thiol-blocking reagents. Pyroglutamyl peptidase I can be potently inhibited by pyroglutamyl diazomethyl ketone (PDMK), an active site-directed alkylating agent, and reversibly by 5-oxoprolinal, a transition state-analogue inhibitor (46). PDMK is an excellent inhibitor of pyroglutamyl peptidase I in vivo. One hour after intraperitoneal administration of 0.1 mg/kg of PDMK to male Swiss albino mice, pyroglutamyl peptidase I is virtually totally inhibited in all tissues studied. 5-Oxoprolinal, however,is a much less effective inhibitor in vivo. Injection of 50 mg/kg of 5-oxoprolinal produced greater than 60% inhibition in all organs analyzed 10 min later. After 30 min the degree of inhibition decreased but was still significant. Metabolic inactivation may

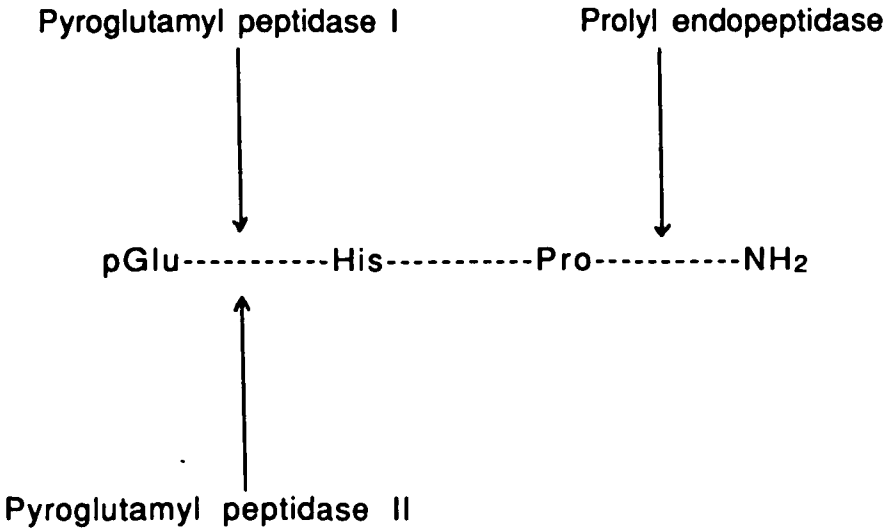
account for the relatively weak in vivo inhibition by this reversibly bound compound.

(3) Pyroglutamyl peptidase II (EC.3.4.19.-) is a highly specific TRH degrading enzyme. This enzyme was first detected in serum and termed " thyroliberinase". Unlike pyroglutamyl peptidase I, pyroglutamyl peptidase II is a metalloenzyme which is not inhibited by -SH blocking reagents such as iodoacetamine and N-ethylmaleimide but is inhibited by metal chelators such as o-phenanthroline and EDTA. This enzyme is found predominantly in brain with only trace activity in other tissues. Within the brain, it is present as an ectoenzyme on synaptosomal membranes. Pyroglutamyl peptidase II has been purified from guinea pig (47) and rabbit (48) brain. The solubilized form has a molecular weight of 230,000, one order of magnitude greater than the molecular weight of pyroglutamyl peptidase I. Pyroglutamyl peptidase II will cleave the pGlu-His bond of TRH but will not cleave the pGlu-His bond of LHRH. LHRH is actually a potent competitive inhibitor of TRH hydrolysis (K_i=20 uM). Specificity studies conducted with natural peptides and with pyroglutamyl peptidyl naphthylamides indicate that pyroglutamyl peptidase II acts on pGlu-His tripeptides. CPHNA (N-[1-(R,S)-carboxy-2-phenylethyl] N-imidazolebenzyl histidyl-beta-naphthylamide), inhibits pyroglutamyl peptidase II with an IC_{50} of approximately 6 uM. Recent studies showed that CPHNA enhances the recovery of basal and K+ stimulated TRH released from brain slices (49). Its unique substrate specificity and localization suggest that pyroglutamyl peptidase II plays a critical role in the termination of TRH action.

It is well documented that thyroid hormones can act on the pituitary to inhibit both TSH secretion and synthesis (50,51). An inverse relationship between plasma TSH and thyroid hormone was revealed by radioimmunoassay (52). In vivo administration of thyroid hormone not only suppressed the basal plasma TSH level but also blunted the TRH-induced stimulation of TSH release in both animals and humans (53,54). In fact, the degree of inhibition of the TSH response to TRH was shown to be a more sensitive parameter of thyroid hormone activity than the inhibition of basal TSH secretion. Thyroid hormones also affect the synthesis of TSH in vitro. This was shown by following the incorporation of labeled amino

acids into cultured thyrotropic tumors or normal pituitary cells (51). Administration of T_3 resulted in a reduced incorporation of labeled amino acid into immunoprecipitable TSH in the thyrotropic tumor cells; the lowest inhibitory concentration was 10 pM, and the minimal exposure required for a significant inhibition was 48 hrs (55). When this was compared with the inhibition of TSH released by T_3, the latter required a much shorter incubation (6-24 hrs), suggesting that the inhibition of TSH release precedes the inhibition of its synthesis. Thyroid hormones have also been shown to regulate a number of enzyme activities. For instance, pyruvate carboxylase activity from hyperthyroid rat liver is twice that of normal rat liver (56), and the activity of malic enzyme in liver cells from chick embryos is increased 23-fold by thyroid hormone (57). Enzymes involved in the metabolism of catecholamines are also affected by thyroid hormones. Tyrosine hydroxylase and monoamine oxidase A are decreased under hypothyroid conditions in two neuroblastoma cell lines (58). Several investigators have measured the total activities of the enzyme metabolizing TRH in human and rat serum or plasma and reported that TRH degrading activity is significantly increased in hyperthyroidism and decreased in hypothyroidism (59,60). Age-dependent changes in total TRH-degrading activity in both rat and human serum have also been reported (61,62). TRH-degrading activity is absent in the serum of 2 to 15-day-old rats, but reaches up to 75% of the adult level by the age of 40 days. These results provide good background to study whether TRH degrading enzyme activities in responsive cells are under regulation in order to either down regulate or potentiate TRH action.

FIG. 1. Sites of enzymatic cleavage of TRH :



CHAPTER 1

Endocrinology **121** 770-775, 1987.

REGULATION OF A THYROTROPIN-RELEASING HORMONE-DEGRADING ENZYME IN GH_3 CELLS : INDUCTION OF PYROGLUTAMYL PEPTIDASE I BY 3,5,3',-TRIIODOTHYRONINE.

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SUMMARY:

The effect of exposure of GH_3 cells to T_3 on the TRH-degrading enzymes pyroglutamyl peptidase I (EC 3.4.19.3) and prolyl endopeptidase (EC 3.4.21.26) was studied. T_3 produced a dose-dependent increase in the specific activity of pyroglutamyl peptidase I after 3 days of exposure. The EC50 for T_3 was $5X10^{-10}$ M. The specific activity of prolyl endopeptidase was unaffected by exposure to T_3. The increase in pyroglutamyl peptidase I activity was dependent upon the time of exposure of the cells to this hormone. A maximal effect occurred at 72 h. The stimulation of pyroglutamyl peptidase I by T_3 was totally blocked by cycloheximide, indicating that this enzyme is induced in GH3 cells by T_3. The effect of T_3 on the two TRH-degrading enzymes was also studied in the ACTH-secreting cell line AtT20. T_3 had no effect on these enzyme in the AtT20 cell, suggesting that the effect of T_3 on pyroglutamyl peptidase I may be cell specific. These studies indicate that the induction of pyroglutamyl peptidase I by T_3 may contribute to the negative feedback regulation of T_3 levels.

INTRODUCTION :

It is well documented that thyroid hormones can act on the pituitary to inhibit both TSH secretion and synthesis (1-4). Thyroid hormones directly inhibit the transcription of the gene for both TSH subunits and decrease the levels of alpha and beta mRNA (5.6.) RIA has revealed an inverse relationship between plasma TSH and thyroid hormones (7). T_3 also down-regulates TRH receptors in pituitary tumor cells, leading to a decreased responsiveness to TRH (8-11).Enzymatic inactivation of TRH by rat serum also appears to be regulated by thyroid hormone status. In rat serum, TRH-degrading activity is significantly increased in hyperthyroidism and decreased in hypothyroidism (12,13). The deamidation of TRH in plasma from hyperthyroid patients was reported to be greater than in plasma from hypothyroid patients (14).

We have reported that the TRH-degrading enzymes pyroglutamyl peptidase I (EC 3.4.19.3) and prolyl endlpeptidase (EC 3.4.21.26) are present in GH_3 cells, a clonal line of rat anterior pituitary tumor cells (15). We have also shown that exposure of these cell to 5-oxoprolinal, a specific inhibitor of pyorglutamyl peptidase I (16), followed by removal of inhibitor, produces a 3-fold elevation in pyroglutamyl peptidase I activity (17). These experiments suggested that the activity of pyroglutamyl peptidase I is regulated in GH_3 cells. In this study we report that pyroglutamyl peptidase I is induced in GH_3 cells by T_3. Elevation of enzymatic activity is dependent upon the dose and the time of exposure of cells to this hormone. This increase is totally blocked by cycloheximide. The regulation of pyorglutamyl peptidase I activity by T_3 may play a role in the control of TRH degradation in the pituitary.

Materials and Methods :

T_3, cycloheximide, TRH, and TRH-free acid (TRH-OH), were obtained from Sigma Chemical Co. (St.Louis, MO). L-Pyroglutamyl beta-naphthylamide (pGlu-NA) was obtained fron the U.S. Biochemical Corp. (Cleveland, OH). N-Benzyloxycarbonyl-Gly-Pro-sulfamethoxazole (Z-Gly-Pro-SM) and pyroglutamyl diazomethyl ketone (PDMK) were synthesized as previously described (18.19). All medium components were obtained from Gibco (Grand Island, NY). GH_3 cells were a kind gift of Dr. Marvin Gershengorn of the Cornell University Medical Center (New York, NY). AtT20 cells were kindly donated by Dr. Gwen Acker of our department.

Measurement of enzymatic activities :
Pyroglutamyl peptidase I was determined with the substrate pGlu-NA. The incubation mixture (final volume, 250 ul) contained 10 ul of a 10-mM solution of substrate prepared in dimethylsulfoxide, 20 ul 20mM dithiothreitol (DTT), 20 ul 20mM EDTA (pH=7.2), 50 ul cell homogenate, and 150 ul 50mM Tris-HCl, pH=7.5. Tubes were incubated for 2 h at 37C, and the reaction was stopped by the addition of 250 ul 10% trichloroacetic acid (TCA). Prolyl endopeptidase activity was determined with the substrate Z-Gly-Pro-SM . The incubation mixture (final volume, 250 ul) contained 50 ul 5mM substrate in 0.1M Tris-HCl (pH=8.3), 10 ul 10 mM DTT, 10 ul cell homogenate, and 180 ul 0.1 M Tris-HCl (pH=8.3). Tubes were incubated for 1 h at 37 C,and the reaction was stopped by the addition of 250 ul of 10% TCA. The activities of both enzymes were measured by determination of release of the aromatic amine with the diazotization procedure of Bratton and Marshall (20), as modified by Goldbarg and Rutenberg (21). The chromogen formed from NA was read in an LKB Ultrospectrophotometer (LKB, Rockville, MD) at 580nm, and the chromogen formed from SM was read at 540 nm. The amount of amine liberated was calculated from standard curves prepared from known amounts of chromogen. Specific activity was expressed as nanomoles of amine per mg protein/h. Protein was measured by the method of Lowry et al(22).

Effect of T_3 on TRH-degrading enzymes in GH_3 cells :
GH_3 cells were grown in 25-cm^2 tissue culture flasks. The medium (Ham's F-10 medium supplemented with 12.5% horse serum, 2.5% fetal bovine serum, pH=7.2) was changed every three days. Nine days after plating, various concentrations of T_3 were added to the flasks. After 3 days of incubation at 37C, the cells were harvested by adding 2.5 ml of a solution composed of 0.2g/liter EDTA, 0.8g/liter NaCl, 2g/liter KCl, 1.15g/liter Na_2HPO4, and 0.2g/liter KH_2PO_4. The suspension was centrifuged for 15 min at 359Xg. The cells were washed with 5 ml PBS and recentrifuged. The cell pellet was placed on ice and homogenized in 250 ul 0.1M Tris-HCl (pH=7.5) with a Potter-Elvejhem homogenizer. Enzymatic activities were measure as described above .

Effect of time of continuous exposure to T_3 on the activity of pyroglutamyl peptidase I:
The time course of the effect of T_3 was studied by incubating GH_3 cells with $1X10^{-9}$ M T_3 for times ranging from 4 h to 5 days. At varying time intervals, cells were harvested, washed, homogenized, and assayed for pyroglutamyl peptidase I activity.

Determination of the minimum time of exposure of GH_3 cells to T_3 necessary to activate pyroglutamyl peptidase I:
GH_3 cells grown in 25-cm^2 flasks were exposed to $1X10^{-9}$M T_3 for time intervals varying from 1-48 h. After different times of exposure, T_3 was removed by replacement of the medium. All flasks were incubated for a total of 3 days, after which time the cells were harvested, washed, homogenized, and assayed for pyroglutamyl peptidase I, as described above. Control flasks received no T_3 and were carried through the procedure.

Cycloheximide experiments :
GH_3 cells grown in 25-cm^2 flasks were incubated for 24 h with cycloheximide (200ng/ml medium). Three flasks served as control (no

additions), three flasks contained cycloheximide, three flasks contained T_3 ($1X10^{-9}M$), and three flasks contained cycloheximide plus T_3. The cells were harvested, washed, homogenized, and assayed for pyroglutamyl peptidase I and prolyl endopeptidase as described above.

Identity of the pGlu-NA-hydrolyzing activity induced by T_3:
GH_3 cells grown in 25-cm^2 flasks were incubated for 3 days with T_3 ($1X10^{-9}M$). A parallel set of control flasks was similarly incubated. The cells were then harvested, washed, and homogenized. Homogenates were preincubated for 15 min with 1 uM PDMK, a specific pyroglutamyl peptidase I inhibitor. Pyroglutamyl peptidsae I was assayed as described above.

Kinetic studies :
Cells were incubated in the presence and absence of $1X10^{-9}$ M T_3 for a period of 3 days. The cells were harvested, and pyroglutamyl peptidase I was measured with the substrate pGlu-NA over a concentration range of 0.05-3.2 mM. After a 1-h incubation period, the reaction was stopped by the addition of 2 ml 1 M acetate buffer, pH=4.2. A blank was prepared by adding substrate at the end of the incubation . NA release was determined by fluorometry. Fluorescence was recorded on a Perkin-Elmer LS-5 fluoresence spectrophotometer (Perkin-Elmer, Palo Alto, CA) at an excitation wavelength of 285 nm and an emission wavelength of 420 nm. Km and maximum velocity (Vmax) values were obtained by a computerized analysis of the direct linear plot (23).

RESULTS :

The effects of different concentrations of T_3 on the activities of pyroglutamyl peptidase I and prolyl endopeptidase in GH_3 cells were studied. Cells were exposed to T_3 for 3 days. The activity of pyroglutamyl peptidase I was elevated by T_3 in a dose-dependent manner. Half-maximal elevation occurred at a hormone concentration of approximately $5X10^{-10}M$(Fig. 1A). A maximal effect occurred at $10^{-8}M$. At $10^{-7}M$, the response to T_3 decreased. The activity of prolyl endopeptidase was not significantly altered at any of the concentrations of T_3 (Fig.1B).

To rule out the possibility that the effect of T_3 was mediated by release of a serum factor, the experiment was repeated in a serum-free medium. Elevation of the specific activity of pyroglutamyl peptidase by T_3 was still observed. Activity in the presence of $5X10^{-9}M$ T_3 (29.7/-+1.5 nmol/mg.h; n=3) or $5X10^{-10}M$ T3 (22.5+/-1.6 nmol/mg.h;n=3) was significantly greater than the control value (11.1+/-1.7 nmol/mg.h;n=3).The effects of T_3 ($1X10^{-9}M$) on the activities of pyroglutamyl peptidase I and prolyl endopeptidase were studied as a function of time of continuous exposure of GH_3 cells to this hormone. The specific activity of pyroglutamyl peptidase I reached a maximal value after 72 h of exposure, attaining a level approximately 4 times greater than the control value (Fig.2). Longer periods of exposure led to a diminished effect. Thus, after 5 days exposure enzyme activity returned to control levels. The specific activity of pyroglutamyl peptidase I in cultures not exposed to T_3 was unaltered at any of the time points. The specific activity of prolyl endopeptidase was not altered after different times of exposure to T_3 (Fig.3).

It was of interest to determine whether continuous exposure of the cells to T_3 was required to elevate pyroglutamyl peptidase I activity or whether

this effect could be attained by brief exposure to the hormone. GH_3 cells were incubated with T_3 (1X10-9M) for varying periods of time. The hormone was removed by decanting the medium, washing the cells twice with PBS, and replacing the hormone-containing medium with fresh Ham's F-10 medium. Enzyme activity was then measured on day 3 of culture. A near-maximal effect was produced by 24-h exposure to T_3 (Table1).

Since T_3 has been reported to stimulate the activities of other enzymes (24,25), a study was performed to determined whether the increased pGlu NA-hydrolyzing activity was indeed due to pyroglutamyl peptidase I. We have previously reported on the synthesis and properties of PDMK, a potent and specific active site-directed inhibitor of pyroglutamyl peptidase I (19). This compound does not inhibit aminopeptidase M, dipeptidyl peptidase IV, papain, or prolyl endopeptidase (19), nor does it inhibit the membrane-bound enzyme pyroglutamyl peptidase II (26). GH_3 cells were grown for 3 days in the presence or absence of T_3 ($1X10^{-9}M$). At the end of this time period, cells were harvested for determination of pGlu-NA-hydrolyzing activity. This activity in cells exposed to T_3 (24.0+/- 2.5 nmol/mg.h; n=3) was almost 3 times greater than in cells not exposed to this hormone (8.6+/-1.0 nmol/mg.h; n=3). When homogenate from the T_3-stimulated cells were preincubated with $1X10^{-6}M$ PDMK for 15 min, and the pGlu-NA-hydrolyzing activity was measured, greater than 90% inhibition was found (1.7+/- 0.3 nmol/mg.h; n=3).

To determine whether the increased enzymatic activity could be attributed to an increased affinity of the enzyme for its substrate or to an increase in the number of active sites, the K_m and V_{max} for pGlu-NA hydrolysis were determined in control and stimulated preparations. As shown in table 2, exposure of cells to $1X10^{-9}$ M T_3 did not alter the K_m, but increased the V_{max} approximately 3-fold.The kinetic parameters (K_m and V_{max}) of TRH hydrolysis by prolyl endopeptidase were determined with the use of HPLC for the measurement of TRH-OH. The values obtained were : K_m=5.3 mM, and V_{max}=236 nmol TRH degraded /mg protein.h.

Experiments were performed to determine whether the increase in Vmax was due to de novo synthesis of pyroglutamyl peptidase I. Flasks containing GH_3 cells were divided into four groups. One group served as the control. The second group was incubated with T_3 ($1X10^{-9}M$) for 24 h, the third group was incubated with T_3 ($1X10^{-9}M$) for 24 h, and the fourth group was incubated with T_3 plus cycloheximide for 24 h. The cycloheximide was not cytotoxic, as judged by trypan blue exclusion. As shown in table 3, the basal activity of pyroglutamyl peptidase I was reduced upon exposure to cycloheximide, presumbly as a consequence of decreased protein synthesis. The increase in pyroglutamyl peptidase I produced by T_3 was totally blocked by cycloheximide. These results indicate that T_3 increases specific activity of pyroglutamyl peptidase I by inducing new enzyme synthesis. In a final experiment, we investigated whether this effect could be replicated in another pituitary cell type. Studies were conducted on AtT20 cells, an ACTH-secreting cell line derived from a mouse pituitary tumor (27). These cells were cultured under the same conditions as described for GH_3 cells. The specific activities of pyroglutamyl peptidase I and prolyl endopeptidase in AtT20 cells were similar to the corresponding activities in GH_3 cells (Table 4). Neither of these two activities was altered after 3-day exposure to $1X10^{-9}M$ T_3.

DISCUSSION :

We have previously reported that GH_3 cells, a clonal line derived from a rat anterior pituitary tumor (28), contain the TRH-degrading enzymes pyroglutamyl peptidase I and prolyl endopeptidase (15). We have futher shown that exposure of these cells to 5-oxoprolinal, a pyroglutamyl peptidase I inhibitor, produces a marked and slelective increase in pyroglutamyl peptidase I activity (17). In this report we demonstrated that pyroglutamyl peptidase I can be induced in GH_3 cells by low concentrations of T_3. The increased activity is dependent on both the concentration of T_3 and the time of exposure to this hormone. An EC_{50} for T_3 of $5X10^{-10}$ M was found, although the true EC_{50} may be somewhat lower, since the serum used was not treated to remove endogenous T_3. The correction may not be large, since we found the response to T_3 in serum-containing medium to be similar to the response in serum-free medium. It has been estimated that the free T_3 concentration in serum is approximately 10^{-11} M (29).

Kinetic studies revealed that the increased activity was not due to an increase in the affinity of the enzyme for pGlu-NA. The increase in Vmax is indicative of an increase in the number of active sites . That this increased enzymatic activity is due to enzyme induction is seen by the ability of cycloheximide to totally block the T_3 stimulation without producing any cytotoxic effects.

Although the specific activity of prolyl endopeptidase in GH_3 cells is considerably greater than the specific activity of pyroglutamyl peptidase I, TRH is a very poor substrate for prolyl endopeptidase, The K_m of TRH for prolyl endopeptidase in GH_3 cells is 5.3 mM. Regulation is, therefore, exerted at the enzyme with low K_m and V_{max} rather than at the enzyme with high K_m and V_{max}. PDMK, a potent irreversible inhibitor of

pyroglutamyl peptidase I (19), was used to prove that the induced pGlu-NA-hydrolyzing activity was indeed due to pyroglutamyl peptidase I and not to another enzyme. It is known, for example, that rat pituitary also contains pyroglutamyl peptidase II , a membrane-bound enzyme which cleaves the pGlu-His bond of TRH with a high specificity. We found that PDMK inhibited more than 90% of the T_3-stimulated activity, demonstrating that this increased activity is indeed due to pyroglutamyl peptidase I.

The mouse pituitary cell line AtT20 has been shown to synthesize and release ACTH, but not other hormones of the anterior pituitary lobe. It is of interest that the levels of pyroglutamyl peptidase I and prolyl endopeptidase are similar in the AtT20 and GH_3 cell lines. Exposure of the AtT20 cells to T_3 did not lead to a stimulation of pyroglutamyl peptidase I activity, suggesting that this effect may be restricted to TRH target cells or T_3-dependent cells.

Regulation of the serum degradation of TRH by thyroid hormones has previously been demonstrated (12-14). Thus, the rate of degradation of TRH by serum from T_3-treated rats is significantly greater than the control value, whereas the serum of rats made hypothyroid by treatment with propylthiouracil degrades TRH at a rate significantly slower than the control value. The nature of the enzyme(s) mediating this effect in serum have not been established, but pyroglutamyl peptidase II plays a predominant role in the degradation of TRH by serum(31).

It is well established that an increase in the level of T_3 decreases TSH secretion, whereas a deficiency of T_3 leads to increased TSH secretion(32). The molecular mechanisms underlying the action of T_3 on the release and synthesis of TSH are still under active investigation. It has been shown that T_3 decreases TRH receptor concentration in pituitary tumor cells, leading to a decreased cellular responsiveness to TRH (8-11). Although Spira and Gordon (32) discuss evidence that T_3 and TRH may

affect TSH secretion by independent mechanisms, our studies, which demonstrate that T_3 induces a TRH-degrading enzyme, futher strengthen the link between T3 and TRH.

The relevance of these findings to the control of TSH secretion by T_3 in vivo remains to be determined. These results suggest a role for internalized TRH in this process. Thus, increased pyroglutamyl peptidase I should decrease intracellular TRH. Although the activation of this enzyme is relatively transient, with levels returning to baseline after 5 days of exposure to T_3, an effect on TSH may be translated over a more prolonged time frame.These studies provide a clear demonstration that the activity of a neuropeptide-degrading enzyme can be hormonally regulated. They further indicate that pyroglutamyl peptidase I controls the inactivation of TRH in GH_3 cells and possibly in the pituitary ae well.

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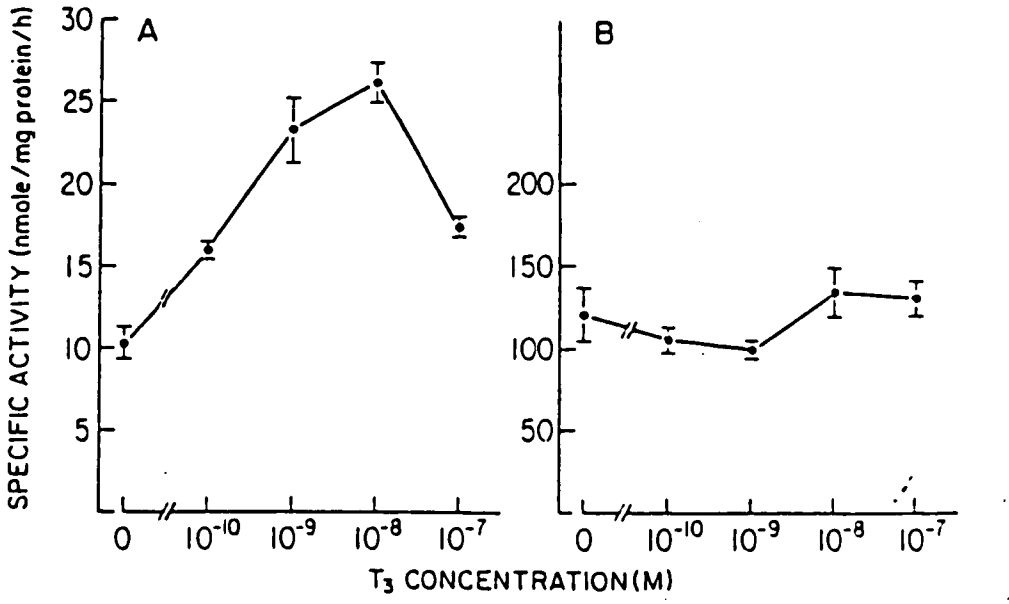

Fig. 1. Effect of varying concentrations of T_3 on the specifc activities of pyroglutamyl peptidase I (A) and prolyl endopeptidase (B) in GH_3 cells. Cells were exposed to T_3 for 3 days. Emzymatic activities were determined as described in *materials and Methods*. Each point represents the mean + SEM of three determinations.

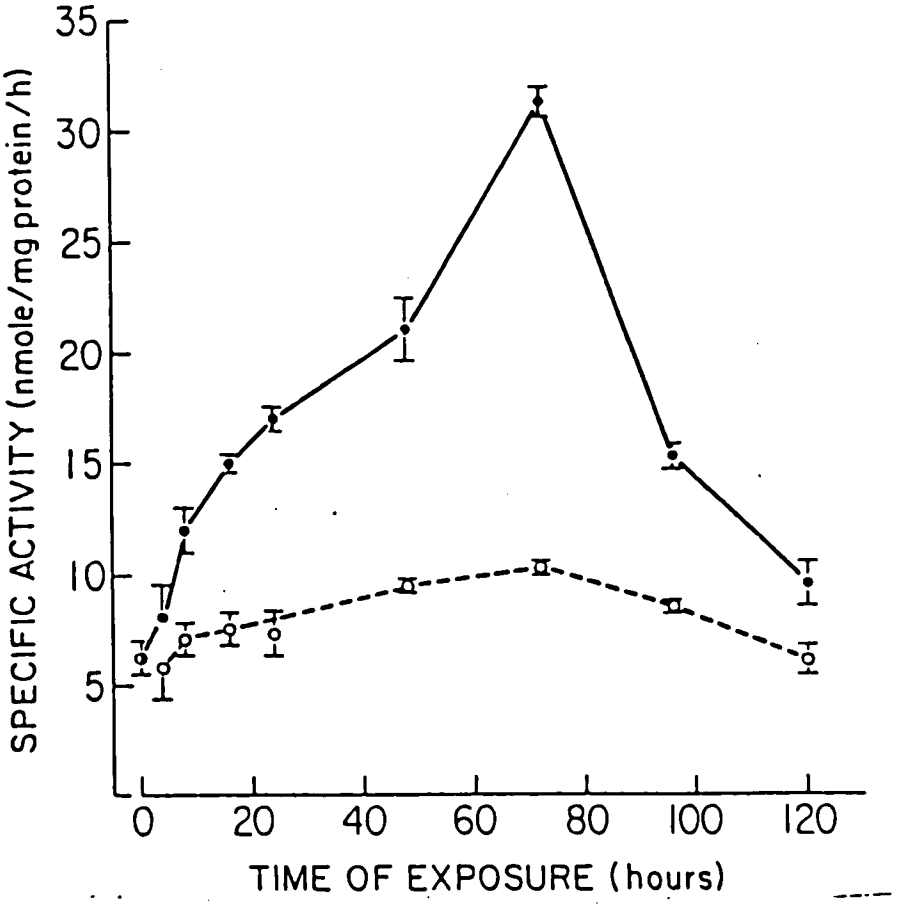

FIG.2. Effect of time of continuous exposure of GH_3 cells to T_3 ($1X10^{-9}M$) on the specific activity of pyroglutamyl peptidase I. Cells were harvested and assayed for pyroglutamyl peptidase I at the time periods designated .Solid line, Cells exposed to 10^{-9} M T_3; dashed line, cells not exposed to T_3.

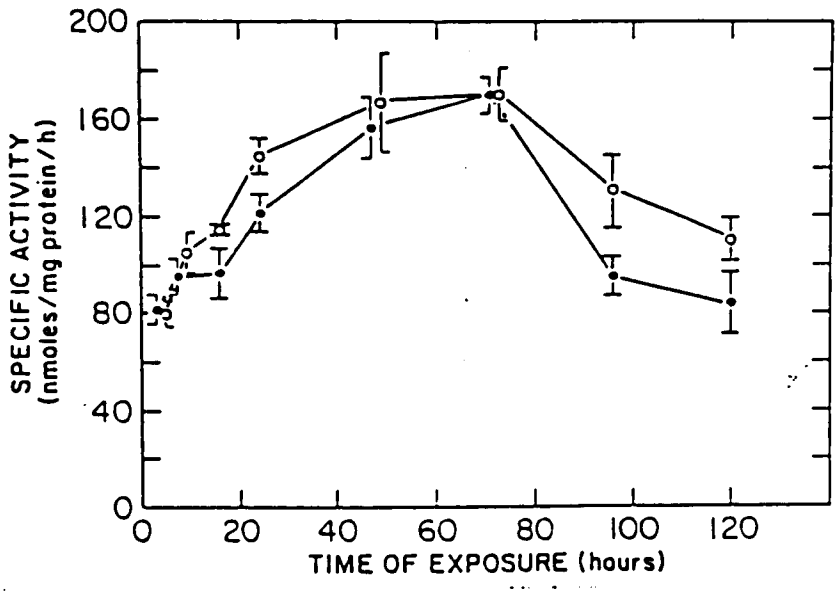

FIG. 3. Effect of time of continuous exposure of GH_3 cells to T_3 ($1X10^{-9}M$) on the specific activity of prolyl endopeptidase . Cells were harvested and assayed for prolyl endopeptidase at the time periods designated. O--O, Cells exposed to 10^{-9} M T_3; O--O, cells not exposed to T_3.

Table 1. Effect of time of continuous exposure to T_3 on specific activity of pyroglutamyl peptidase I:

Time of exposure to T_3 (h)	Specific Activity (nmol/mg protein.h)
o	13.5 +/- 1.1
1	17.1 +/- 0.4
8	22.0 +/- 0.1
24	31.8 +/- 2.2
48	35.4 +/- 3.1

GH_3 cells were exposed to $1X10^{-9}$ M T_3 for the time periods indicated. T_3 was then removed, and incubation was continued for a total time of 72 h. Pyroglutamyl peptidase I was measured as described in *Materials and Methods.*

Table 2. Effect of T_3 on the kinetic parameters of pGlu-NA hydrolysis by GH_3 cells

	K_m (mM)	V_{max} (nmol/mg protein.h)
Control	0.23 +/- 0.03 (4)	19.7 +/- 3.0 (4)
T_3 ($1X10^{-9}$M)	0.25 +/- 0.08 (4)	51.7 +/- 16 (4)

Cell homogenates were incubated with varying concentrations of pGlu-NA (0.05-3.2 mM), and the release of 2-naphthylamine was measured fluorimetrically, as described in *Materials and Methods.* Kinetic data were obtained by a computerized analysis of the direct linear plot (23).

Table 3. Effect of cycloheximide on T_3-stimulated pyroglutamyl peptidase I in GH_3 cells

Pyroglutamyl peptidase I activity (nmol/mg protein.h)			
Control:	T_3($1X10^{-9}$ M):	Cycloheximide (200ng/ml):	T_3 + Cycloheximide:
10.9+/-1.6	27.2 +/-1.1	5.5 +/-1.1	5.1+/-0.5

Flasks were incubated in the presence or absence of T_3 and cycloheximide for 24 h. Enzymatic activity was determined in whole homogenates. Values reported are the mean +/- SEM for three flasks each.

Table 4. Effect of T_3 on the specific activities of pyroglutamyl peptidase I and prolyl endopeptidase in AtT20 cells

Enzyme	Specific activity (nmol/mg protein.h)	
	control	T_3 ($1X10^{-9}$ M)
Pyroglutamyl peptidase I:	9.9 +/- 0.6	10.3 +/- 0.2
Prolyl endopeptidase :	186.5 +/- 30.4	221.7 +/- 10.4

Cells were exposed to $1X10^{-9}$ M T_3 for 3 days. Enzymatic activities were measured in whole cell homogenates, as described in *Materials and Methods*. Each value represents the mean +/- SEM of determinations from three flasks.

CHAPTER 2

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REGULATION OF THYROTROPIN RELEASING HORMONE DEGRADING ENZYMES IN RAT BRAIN AND PITUITARY BY L-3,5,3'-TRIIODOTHYRONINE

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SUMMARY :

The effect of treatment with L-3,5,3'-triiodothyronine (T_3) on the levels of pyroglutamyl peptidase I and pyroglutamyl peptidase II in rat brain regions, pituitary, and serum was studied. Pyroglutamyl peptidase I cleaves pyroglutamyl peptides such as thyrotropin releasing hormone (TRH), luteinizing hormone releasing hormone, neurotensin , and bombesin, whereas pyroglutamyl peptidase II appears to be specific for TRH. Acute administration of T_3 did not affect pyroglutamyl peptidase I in any of the regions studied, whereas pyroglutamyl peptidase II was significantly elevated in frontal cortex and pituitary. Treatment with T_3 for 10 or 14 days significantly elevated pyroglutamyl peptidase I in pituitary, hoypothalamus, olfactory bulb, hippocampus, and thalamus. Chronic T_3 treatment elevated pyroglutamyl peptidase II in frontal cortex and in serum. These studies demonstrate regulation of neuropeptide degrading enzymes by thyroid hormone in vivo. This regulation may play a role in the negative feedback control of thyroid status by T_3

INTRODUCTION :

The degradation of thyrotropin releasing hormone (TRH, pGlu-His-Pro-NH_2) is catalyzed by enzymes that cleave either the pGlu-His or Pro-NH_2 bonds (1,2). The latter reaction is catalyzed by prolyl endopeptidase (EC 3.4.21.26), which also cleaves peptide bonds at the carboxyl side of proline residues in a variety of peptides such as substance P, neurotensin, luteinizing hormone releasing hormone (LHRH), and angiotensin II (3). Two distinct pyroglutamyl peptidases, a cysteine protease and a metalloprotease, can cleave the pGlu-His bond. The former enzyme, pyroglutamyl peptidase I (EC 3.4.19.3) cleaves all pGlu-amino acid bonds except pGlu-Pro (4). Substrates of pyroglutamyl peptidase I in addition to TRH include LHRH, neurotensin, and bombesin, but not eledoisin which has a pGlu-Pro bond (5). Pyroglutamyl peptidase II (EC 3.4.19.-) is membrane bound and has been purified from guinea pig brain (6) and rabbit brain (7). This metalloenzyme of high molecular weight (230,000) was first detected in serum (8,9). It appears to be a specific TRH degrading enzyme and does not cleave the pGlu-His bond of LHRH (6,10). It has thus far been shown to act only on pGlu-His tripeptides (7).

Knights et al.(11) have suggested that polypeptide hormone levels may be controlled not only by their rate of synthesis, but also by their rate of enzymatic degradation. If control is expressed at the level of enzymatic inactivation, it would be reasonable to assume that the degradative enzymes are subject to regulation. Enzymatic inactivation of TRH by rat serum is regulated by thyroid hormone status. In rat serum TRH degrading activity is significantly increased in hyperthyroidism and decreased in hypothyroidism (12.13). Bauer has recently reported that acute treatment with L-3,5,3'-triiodothyronine (T_3) markedly stimulates pyroglutamyl peptidase II in rat adenohypophysis(14). We observed that exposure of rat pituitary GH_3 cells to 5-oxoprolinal, a specific inhibitor of pyroglutamyl peptidase I (15), led to a marked increase in the specific activity of this enzyme (16). We also found that pyroglutamyl peptidase I specific activity in GH_3 cells was increased by low concentrations of T_3 and that

this effect was blocked by cycloheximide (17). We now report on the effect of acute and chronic treatment with T_3 on the specific activity of the two pyroglutamyl peptidases in rat brain regions, pituitary, and serum.

MATERIALS AND METHODS :

pGlu-beta-naphthylamide (pGlu-NA) and T_3 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). pGlu-His-Pro-NA was synthesized by conventional techniques (S.Wilk and E.K.Wilk, submitted). Z-Pro-Prolinal and pyroglutamyl diazomethyl ketone were synthesized as previously described by Wilk and Orlowski(18) and Wilk et al. (19). Dipeptidyl peptidase IV (EC 3.4.14.5) was purified from rabbit kidney to apparent homogeneity by a modification of the procedure of Yoshimoto and Walter (20).

Animal experiments :

Male Sprague-Dawley rats weighing 225-250 g were used in all experiments. They were fed a commercial Purina laboratory chow diet. For acute studies, rats received a single subcutaneous injection of T_3 (100 ug). For chronic studies, rats received once daily subcutaneous injections of T_3 (100 ug) for a period of 10 or 14 days. Control rats received subcutaneous injections of vehicle (0.01 M NaOH). All animals were killed by decapitation 24 h following the last injection. Blood was collected, allowed to clot,and serum obtained after centrifugation for 10 min at 2,000g. Brains were rapidly removed; placed on ice; and hypothalamus, frontal cortex, hippocampus, striatum, olfactory bulb, amygdala, cerebellum, and brain stem dissected. The pituitary gland and spinal cord (cervical region) were also removed. Tissues were homogenized in five volumes of ice-cold 0.05M Tris-HCl buffer, pH=7.5, in a cooled Potter-Elvehjem homogenizer fitted with a Teflon pestle. Homogenates were centrifuged at 12,000g for 20 min. Supernatant fractions were removed and placed on ice. Pellets were washed twice by resuspension in the Tris-HCl buffer and recentrifuged. The washed pellets were resuspended in 250 ul of cold Tris-HCl buffer. The supernatant fraction was assayed for pyroglutamyl peptidase I and the particulate fraction assayed for pyroglutamyl peptidase II. Enzymatic activities obtained from T_3 and vehicle-treated animals were immediately determined in freshly prepared fractions.

Enzyme assays :

Enzymatic activities were measured spectrophotometrically by quantitating the release of NA from the appropriate chromogenic substrate by the method previously described in chapter 2. Protein was measured by the method of Lowry et al. Statistical significance was assessed by the Student's two-tailed t test.

RESULTS :

Acute studies :

As previously observed by Friedman et al (15) the distribution of pyroglutamyl peptidase I in rat brain regions is fairly uniform (Table 1). Highest activity was found in olfactory bulb, a region relatively rich in TRH (21). The activity of pyroglutamyl peptidase I in serum was very low. Acute treatment with T_3 did not alter pyroglutamyl peptidase I activity in pituitary, serum, or any of the brain regions studied.In agreement with our previous sutdies (22) pyroglutamyl peptidase II in brain displayed a more marked regional distribution. The specific activity of this enzyme in brain stem and spinal cord was three- to fourfold lower
than in frontal cortex, hippocampus, and olfactory bulb (Table 2 and 4). Acute treatment with T_3 produced a threefold increase in pituitary pyroglutamyl peptidase II activity, and a 70% increase in the activity of this enzyme in frontal cortex (Table).

Chronic studies :

The results of chronic treatment with T_3 on the activity of pyroglutamyl peptidase I are presented in Table 3. It can be seen that the activity of this enzyme is increased in hypothalamus, hippocampus, thalamus, and pituitary at both 10 and 14 days and in olfactory bulb at 14 days. This effect was most marked in hypothalamus and pituitary. It is notable that pyroglutamyl peptidase I activity was increased two- to threefold in these regions after treatment with T_3 for 10 or 14 days whereas no increase was seen after treatment for 1 day.The results of chronic treatment with T_3 on the specific activity of pyroglutamyl peptidase II are presented in table 4. Pyroglutamyl peptidase II activity was significantly elevated in frontal cortex and in serum after both 10 and 14 days. Although there was a tendency for activity in pituitary to be elevated at these times, this increase did not attain statistical significance. The activity of pyroglutamyl peptidase II in the brainstem of 14-day-treated animals was decreased compared to control.

DISCUSSION :

Thyroid hormones have been shown to regulate the activity of a number of enzymes. For example, livers from hyperthyroid rats have twice the pyruvate carboxylase activity of livers from normal rats (23), and the activity of malic enzyme in liver cells from chick embryos is increased 23-fold by T_3 (24). Enzymes involved in the metabolism of catecholamines and peptides are also affected by thyroid hormones. Thus Safari and Timiras (25) have shown that in two neuroblastoma cell lines cultured under hypothyroid condition, the activities of tyrosine hydroxylase and monoamine oxidase A are decreased. A dose-dependent induction of tyrosine hydroxylase by T_3 was also observed. Davis and Pieringer (26) studied cultures of dissociated brain cells from fetal mice. Dipeptidyl peptidase I was elevated in cells grown in hypothyroid calf serum and decreased when T_3 was added to the serum. Angiotensin converting enzyme was decreased when T_3 (50 nM) was added to this serum.

The ability of thyroid hormones to control the degradation of TRH by serum was first noted by Redding and Schally (27). Subsequent studies by White et al (12) and by Bauer (13) have documented a decreased degradation of TRH by serum from hypothyroid rat, and an increased degradation of TRH by serum from hyperthyroid rats. The serum enzyme regulated by thyroid hormones was shown by Emerson and Wu (28). to be the TRH-specific pyroglutamyl peptidase (pyroglutamyl peptidase II). Bauer (14) has recently demonstrated a rapid induction of a membrane-bound TRH degrading enzyme (pyroglutamyl peptidase II) in rat adenohypophysis. A similar finding was also recently reported by Ponce et al.(29). We have shown that low concentrations of T_3 induce pyroglutamyl peptidase I in the TRH-target GH_3 cell (17). These studies collectively point to control of TRH degradation by thyroid hormone as one of the factors contributing to the negative feedback regulation of thyroid status by thyroid hormones.

In addition to hypothalamus, rat brain regions were selected to include those relatively rich in TRH (olfactory bulb, brainstem, thalamus; Kreider et al.(21)), in TRH receptors (amygdala, hipocampus; Sharif and Burt (30)), and in pyroglutamyl peptidase II (frontal cortex; (22)). In this study we have found elevations in the activity of pyroglutamyl peptidase I in a number of brain regions and in the pituitary following chronic but not acute T_3 treatment (Table 1 and 3). Changes in the activity of pyroglutamyl peptidase I are not necessarily related to TRH since other pyroglutamyl peptides such as LHRH and neurotensin are substrates of this enzyme. However it is reasonable to postulate that the increased levels of the enzyme in pituitary and hypothalamus may contribute to the negative feedback regulation of thyroid status by T_3. This is supported by our in vitro studies on the TRH-target GH_3 cells(17). A recent report by Segerson et al. (31) indicates that thyroid hormone plays a role in controlling hypothalamic TRH. Their studies demonstrated an increase in proTRH mRNA and immunoreactive TRH in the paraventricular nucleus of the hypothyroid rat. Futher studies will be required to interpret the biological significance of the effect of T_3 on pyroglutamyl peptidase I in extrahypothalamic areas. It is of interest to note that changes in the activity of this enzyme occur in some areas relatively rich in TRH such as the thalamus and olfactory bulb (21).

Acute treatment with T_3 significantly elevates pyroglutamyl peptidase II in pituitary (Table 2). The specificity of this membrane-bound enzyme is such that changes in its activity can be reasonably interpreted in terms of TRH. The increased activity in pituitary following acute treatment has already been documented by Bauer (14). These findings indicate that the biological activity of TRH at the pituitary is terminated by pyroglutamyl peptidase II and that the increased enzymatic activity contributes to the negative feedback regulation of thyroid status by T_3. This increase is no longer seen in the chronically treated animals where other mechanisms such as induction of pyroglutamyl peptidase I may come into play. The increased activity of pyroglutamyl peptidase II in rat serum following chronic T_3 treatment (Table 4) is a well documented phenomenon (12,13).

We have observed further that both acute and chronic T_3 treatment

elevates pyorglutamyl peptidase II in frontal cortex (Table 2 and 4). Neither Bauer (14) nor Emerson and Wu (28) could detect a change in whole brain pyroglutamyl peptidase II by thyroid hormones. It is likely that the significant increase that we observed , which is restricted to the frontal cortex, is obscured when whole brain is studied. The biological significance of this increase and the decrease in pyroglutamyl peptidase II in brain stem after 14 days of treatment with T_3 is unclear. Although the anatomical distribution of TRH and its receptors in brain have been defined, there is currently a large gap between this knowledge and an understanding of the function of TRH in extrahypothalamic areas. These studies point to a need to explore futher the relationship between TRH function and thyroid hormones in these areas.

In summary, we have demonstrated that TRH-degrading enzymes can be regulated in vivo. These studies support the concept that pyroglutamyl peptidases play a role in termination of the biological activity of TRH.

Region	Enzymatic activity (U/mg of protein)[a] ± SEM	
	Control[b]	Treated[b]
Hypothalamus	7.6 ± 0.5	8.4 ± 0.5
Frontal cortex	5.2 ± 0.3	4.2 ± 0.4
Hippocampus	5.6 ± 0.2	5.7 ± 0.4
Striatum	5.2 ± 0.3	4.9 ± 0.2
Thalamus	5.6 ± 0.3	6.4 ± 0.6
Olfactory bulb	10.8 ± 0.5	10.8 ± 0.6
Cerebellum	5.8 ± 0.3	6.5 ± 0.4
Brainstem	5.7 ± 0.2	6.2 ± 0.5
Amygdala	5.1 ± 0.3	5.4 ± 0.3
Pituitary	7.8 ± 0.3	8.7 ± 0.6
Spinal cord	5.3 ± 0.5	6.0 ± 0.4
Serum	0.43 ± 0.04	0.47 ± 0.03

Table 1. Effect of treatment with T_3 for 24 h on the activity of pyroglutamyl peptidase I in rat brain regions : Male Sprague-Dawley rats weighing 225-250g were treated with 100ug of T_3 subcutaneously. Animals were decapitated 24 h after the injection. [a] One unit is defined as the amount of enzyme catalyzing the release of 1 nmole of NA/h from pGlu-NA. [b] Mean +/- SEM for eight animals in each group.

Region	Enzymatic activity (U/mg of protein)[a] ± SEM	
	Control[b]	Treated[b]
Hypothalamus	1.3 ± 0.1	1.2 ± 0.1
Frontal cortex	2.0 ± 0.1	3.4 ± 0.2[c]
Hippocampus	2.2 ± 0.1	2.4 ± 0.1
Striatum	1.0 ± 0.1	0.9 ± 0.1
Thalamus	1.2 ± 0.1	1.1 ± 0.1
Olfactory bulb	2.1 ± 0.1	2.5 ± 0.3
Cerebellum	0.8 ± 0.1	0.9 ± 0.1
Brainstem	0.7 ± 0.1	0.7 ± 0.1
Amygdala	1.9 ± 0.1	1.9 ± 0.1
Pituitary	0.8 ± 0.1	2.6 ± 0.2[c]
Spinal cord	0.5 ± 0.1	0.5 ± 0.1
Serum	1.0 ± 0.1	1.1 ± 0.1

Table 2. Effect of treatment with T_3 for 24 h on the activity of pyroglutamyl peptidase II in rat brain regions: Male Sprague-Dawley rats were treated with T_3 as described in the footnote to Table 1. [a] One unit is defined as the amount of enzyme catalyzing the degradation of 1 nmol of pGlu-His-Pro-NA/h. [b] Mean +/- SEM for eight animals in each group. [c] $P<0.01$.

Region	Enzymatic activity (U/mg protein)[a] ± SEM			
	10 days		14 days	
	Control	Treated	Control	Treated
Hypothalamus	7.9 ± 0.3 (4)	14.9 ± 0.5 (4)[b]	5.7 ± 0.3 (8)	14.5 ± 0.8 (9)[b]
Frontal cortex	5.0 ± 0.4 (4)	4.9 ± 0.7 (4)	5.4 ± 0.4 (8)	5.5 ± 0.4 (9)
Hippocampus	5.5 ± 0.3 (4)	8.1 ± 0.9 (4)[d]	5.7 ± 0.5 (8)	7.7 ± 0.6 (9)[d]
Striatum	5.2 ± 0.9 (4)	5.7 ± 0.5 (4)	5.5 ± 0.7 (8)	5.8 ± 0.4 (9)
Thalamus	4.8 ± 0.4 (4)	10.9 ± 1.1 (4)[c]	6.6 ± 0.4 (8)	10.5 ± 0.9 (9)[c]
Olfactory bulb	11.1 ± 1.8 (4)	16.4 ± 2.7 (4)	8.0 ± 0.9 (8)	16.2 ± 1.7 (9)[b]
Cerebellum	5.9 ± 0.3 (4)	7.9 ± 2.2 (4)	6.4 ± 0.5 (8)	7.3 ± 0.7 (9)
Brainstem	5.9 ± 0.4 (4)	6.5 ± 0.6 (4)	6.6 ± 0.8 (8)	7.4 ± 0.7 (9)
Amygdala	4.2 ± 0.5 (4)	6.1 ± 1.0 (4)	6.0 ± 0.7 (8)	6.5 ± 0.6 (9)
Pituitary	7.0 ± 1.1 (4)	20.4 ± 2.5 (4)[c]	7.5 ± 1.1 (8)	21.2 ± 2.1 (9)[b]
Spinal cord	5.3 (2)	6.4 (2)	8.7 ± 1.5 (3)	8.4 ± 0.6 (7)
Serum	0.57 (2)	0.56 (2)	0.32 ± 0.05 (4)	0.34 ± 0.03 (9)

Table 3. Effect of treatment with T_3 for 10 or 14 days on the activity of pyroglutamyl peptidase I in rat brain regions: Male Sprague-Dawley rats weighing 225-250g were treated daily with 100 ug of T_3 subcutaneously. Animals were decapitated 24 h after the last injection. Number of animals in each group is given in parentheses. [a] One unit is defined as the amount of enzyme catalyzing the release of 1 nmol of NA/h from pGlu-NA. [b]$P<0.001$;[c]$P<0.01$;[d]$P<0.05$.

Region	Enzymatic activity (U/mg protein)[a] ± SEM			
	10 days		14 days	
	Control	Treated	Control	Treated
Hypothalamus	1.2 ± 0.2 (4)	1.2 ± 0.3 (4)	1.6 ± 0.1 (8)	1.3 ± 0.1 (9)
Frontal cortex	1.9 ± 0.4 (4)	4.5 ± 0.5 (4)[b]	2.1 ± 0.2 (8)	3.8 ± 0.7 (9)[c]
Hippocampus	2.4 ± 0.8 (4)	2.5 ± 0.7 (4)	2.8 ± 0.4 (8)	2.0 ± 0.4 (9)
Striatum	0.9 ± 0.1 (4)	0.9 ± 0.2 (4)	1.2 ± 0.1 (8)	1.0 ± 0.3 (9)
Thalamus	1.0 ± 0.1 (4)	1.0 ± 0.2 (4)	1.7 ± 0.3 (8)	1.4 ± 0.1 (9)
Olfactory bulb	2.1 ± 0.3 (4)	2.3 ± 0.4 (4)	2.3 ± 0.2 (8)	2.2 ± 0.3 (9)
Cerebellum	1.1 ± 0.2 (4)	1.3 ± 0.3 (4)	2.0 ± 0.6 (8)	1.3 ± 0.3 (9)
Brainstem	0.5 ± 0.1 (4)	0.9 ± 0.2 (4)	0.7 ± 0.1 (8)	0.4 ± 0.1 (9)[b]
Amygdala	1.7 ± 0.2 (4)	2.2 ± 0.7 (4)	2.2 ± 0.3 (8)	2.0 ± 0.2 (9)
Pituitary	0.9 ± 0.3 (4)	1.3 ± 0.2 (4)	0.5 ± 0.1 (8)	1.0 ± 0.3 (9)
Spinal cord	0.45 (2)	0.5 (2)	0.4 ± 0.1 (4)	0.5 ± 0.1 (9)
Serum	1.1 ± 0.7 (3)	2.7 ± 1.8 (3)	0.5 ± 0.03 (5)	1.3 ± 0.14 (9)[b]

Table 4. Effect of treatment with T_3 for 10 or 14 days on the activity of pyroglutamyl peptidase II in rat brain regions: Male Sprague-Dawley rats were treated with T_3 as described in the footnote to table 1. Number of animals in each group is given in parentheses. [a]One unit is defined as the amount of enzyme catalyzing the degradation of 1 nmol of pGlu-His-Pro-NA/h. [b]$P<0.01$;[c]$P<0.05$.

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Chapter 3

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Occurrence of Pyroglutamyl Peptidase II, A Specific TRH Degrading Enzyme in Rabbit Retinal Membranes and in Human Retinoblastoma Cells

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Summary:

Pyroglutamyl peptidase II, a highly specific thyrotropin releasing hormone (TRH)-degrading enzyme is found in highest concentration in brain where it is localized to synaptic membranes. Retina contains relatively high concentrations of both immunoreactive TRH and TRH receptors. We report that the specific activity of pyroglutamyl peptidase II in rabbit retinal membranes exceeds that of all non-CNS tissues thus far studied. Nine clonal cell lines were screened for this enzymatic activity. The specific activity of pyroglutamyl peptidase II in Y79 retinoblastoma cells was greater than the highest activity found in other cell lines by approximately one order of magnitude. These studies further support a functional relationship between pyroglutamyl peptidase II and TRH and identify a cell line suitable for studies on the regulation of this enzyme.

INTRODUCTION :

Pyroglutamyl peptidase II (EC 3.4.19.-) is a highly specific TRH degrading enzyme. It was first detected in brain by Browne et al., (1), although eariler studies documented a similar activity in serum (2,3). Within brain the enzyme is found on synaptosomal membranes (4,5). Pyroglutamyl peptidase II is highly specific for the TRH molecule. It cleaves the pGlu-His bond of TRH but does not cleave the pGlu-His bond of luteinizing hormone releasing hormone (6,7). In earlier studies we found the highest activity of this enzyme in brain, with some activity in lung and serum, and negligible activity in liver, spleen, heart, kidney and skeletal muscle (8). Pyroglutamyl peptidase II, because of its localization and unique specificity was proposed as the first characterized neuropeptide-specific peptidase (9). If this is true, pyroglutamyl peptidase II should be closely associated with structures containing TRH and/or TRH receptors.

Retina is known to contain relatively high amount of immunoreactive TRH (10,11) and TRH receptors (12). In the retina of the rat, TRH receptor density exceeds that of pituitary and of all brain regions tested (13). Retinal membranes were therefore considered as a possible rich source of the enzyme. Studies on the regulation of pyroglutamyl peptidase II would be facilitated by identifying a cell line that expressed this activity. Nine clonal cell lines were screened for pyroglutamyl peptidase II and its activity compared to that of the less specific enzyme pyroglutamyl peptidase I (EC 3,4,19,3).

Materials and Methods :

Frozen rabbit brains (young, unstripped) were obtained from Pel Freez, Rogers, AK. Freshly dissected New Zealand white rabbit retinas were kindly suppiled by Dr. Thomas Mittag and Anne Tormay of the department of Ophthalmology and Pharmacology at the Mount Sinai School of Medicine. Pyroglutamyl diazomethyl ketone (PDMK) and Z-Pro-Prolinal were synthesized as previously described (14,15). The chromogenic substrate pGlu-His-Pro-naphthylamide (NA) was synthesized by conventional techniques (S.Wilk and E.K.Wilk, submitted). pGlu-NA was obtained from U.S. Biochemicals, Cleveland, OH. Dipeptidyl peptidase IV (EC 3,4.15.5) was purified to homogeneity from rabbit kidney by a modification of the procedure of Yoshimoto and Walter (16). All of the medium components were obtained from Gibco, Grand Island, NY except horse serum which was purchased from Hazelton Research Products, Lenexa, KS. GH_3 cells were kindly donated by Dr. Marvin Gershengorn of the Cornell University School of Medicine. AtT-20 and PC-12 cells were kindly donated by Dr. James Roberts of the Mount Sinai School of Medicine and NG-108 cells kindly donated by Dr. Christopher Molineaux of the Mount Sinai School of Medicine. All other cell lines were purchased from American Type Cell Collection (ATCC), Rockville, MD.

(1) Tissue Preparation :
Tissue samples were homogenized in 5 volumes ice cold 0.05 M Tris-HCl buffer, pH 7.5 in either a Potter Elvejhem homogenizer (retina) or a Waring blendor (brain). Aliquots were removed for measurement of enzymatic activity and homogenates were centrifuged for 30 min at 16,000g. The supernatant was discarded and the pellet washed three times by resuspending in the potassium phosphate buffer and centrifuging for 30 min at 16,000g each time. The washed pellet was resuspended in the original buffer volume and the suspension used for measurement of pyroglutamyl peptidase II activity.

(2) Cell cultures :
Human Y-79 retinoblastoma cells: These cells were grown in suspension as small clusters of 5-10 cells. They were cultured in RPMI medium containing 15% heat inactived fetal bovine serum, penicillin (100U/ml), streptomycin (100mg/ml) and glutamine (295ug/ml). Cells were grown in Corning 75 cm^2 tissue culture flasks in an incubator at 37 ^{0} with 5% CO_2 in humidified air as the gas phase. The culture medium was changed twice weekly. C6 glioma cells, NB41A3 (mouse neuroblastoma), CPAE(bovine pulmonary artery endothelium), PC-12 (mouse neuroblastoma), were cultured following conditions outlined by ATCC. GH_3 and AtT-20 cells were cultured as previously described (17). NG-108 cells (mouse neuroblastoma-glioma hybrid) were cultured as described for GH_3 cells (17). Cells were harvested by centrifugation for 10 min at 350g. The cell pellet was washed with 5ml phosphate-buffered saline and recentrifuged. The washed cell pellet was homogneized in 250 ul Tris-HCl (0.05M, pH 7.5) in a Potter-Elvejhem homogenizer. The homogenate was centrifuged for 20 min at 16,000g, the supernatant decanted and saved and the resulting membrane fraction washed with the Tris-HCl buffer. Following recentrifugation for 20 min at 16,000g the washed membrane fraction was resuspended in 250 ul of the Tris-HCl buffer.

3) Determination of pyroglutamyl peptidase I and II activities:
Enzyme activity was determined in a coupled assay with the substrate pGlu-His-Pro-NA in the presence of an excess of dipeptidyl peptidase IV as previously described (8). The components of the incubation mixture were : 100ul membrane suspension, 10ul Z-Pro-Prolinal (10^{-5}M final concentration), 10ul dipeptidyl peptidase IV (2unit), 10ul pGlu-His-Pro-NA (10mM in dimethyl sulfoxide) and 100ul Tris-HCl (0.05M;pH 7.5). Brain membrane and retinal membrane suspensions were incubated for 60 min at 37^{0}C. Membrane fractions from cultured cells were incubated at this temperature for 120 min. The reaction was terminated by addition of 250 ul 10% trichloroacetic acid and liberated naphthylamine determined colormetrically as described (8). Pyroglutamyl peptidase I was determined in whole retinal homogenates and in the supernatant fraction of cell homogenates as previously described (8). The incubation mixture (final volume 250 ul) contained 10ul 10mM pGlu-NA (in dimethyl sulfoxide), 20 ul dithiothreitol (20mM), 20ul EDTA (20 mM,pH 7.2), 50ul cell supernatant

or 100ul retinal homogenate and Tris-HCl (0.05M,pH 7.5). Samples were incubated for 1 h at 37°C and the reaction terminated by addition of 250ul 10% trichloroacetic acid. Protein was determined by the method of Lowry et al.,(18).

RESULTS:

The specific activity of pyroglutamyl peptidase II in rabbit brain homogenates was 6.1 nmoles mg protein/h. This specific activity was increased in the washed membrane fraction.Relatively high activity was also found in retinal membranes, and this activity also increased in the washed retinal membrane preparation (Table 1). To determine that the retinal activity was indeed due to pyroglutamyl peptidase II, membrane fractions from retina were incubated either in the absence of dipeptidyl peptidase IV or in the presence of 1 mM o-phenanthroline. Under these conditions there was no detectable release of naphthylamine from the substrate. The specific activity of pyroglutamyl peptidase I in retinal homogenates was 8.0 +/- 0.3 S.E. (N=4).

The values for the specific activity of pyroglutamyl peptidase II in the membranes of a number of clonal cell lines is summarized in Table 2. It is of interest that the specific activity of pyroglutamyl peptidase II in the Y-79 retinoblatoma cell exceeded the highest activity found in the other cell lines by approximately one order of magnitude. Activity in the C6, AtT-20, NB41A3 and NG108 cell lines was below the limit of sensitivity of the assay. To determine that the activity detected in the Y-79 retinoblastoma cell line was indeed due to pyroglutamyl peptidase II, incubations were run either in the absence of dipeptidyl peptidase IV or in the presence of o-phenanthroline. Under these conditions, no liberation of naphthylamine was observed.

The specific activity of pyroglutamyl peptidase I in the supernatant fraction of these same cell lines is shown in Table 3. In contrast to pyroglutamyl peptidase II, pyroglutamyl peptidase I is more uniformly distributed across the various cell lines. The specific activity of pyroglutamyl peptidase I is approximately ten fold higher than the specific activity of pyroglutamyl peptidase II in the Y-79 retinoblastoma cell line.

Discussion :

The chromogenic substrate pGlu-His-Pro-NA can be cleaved at the pGlu-His bond by both pyroglutamyl peptidase I and pyroglutamyl peptidase II. Cleavage at the Pro-NA bond is catalyzed by prolyl endopeptidase . Incubation mixtures for the measurement of pyroglutamyl peptidase II contained active site directed inhibitors of pyroglutamyl peptidase I and prolyl endopeptidase. Under these incubation conditions, direct release of naphthylamine from the substrate by the membrane suspension was not observed. Naphthylamine release was however dependent upon the presence of dipeptidyl peptidase IV in the assay mixture. This peptidase releases naphthylamine from His-Pro-NA therefore confirming initial hydrolysis of the pGlu-His bond. This activity was totally blocked by 1 mM o-phenanthroline consistent with pGlu-His clevage by the metalloprotease pyroglutamyl peptidase II.

The specific activity of pyroglutamyl peptidase II in retina is higher than in any other non-CNS tissue studied. In view of the presence of relatively high amounts of immunoreactive TRH and of TRH receptors in this structure, these finding support a functional relationship between TRH and pyroglutamyl peptidase II.

It has been reported that the concentration of immunoreactive TRH in rat retina is influenced by environmental lighting (10). Thus the highest concentration of immunoreactive TRH occurred during the daylight hours, with a gradual decrease after lights are turned off at night. The mechanism whereby the neuronal action of TRH is terminated is not known but may involve degradation catalyzed by the highly specific pyroglutamyl peptidase II (9). It would be of interest to determine whether the activity of this enzyme in retina is also altered during the light-dark cycle.

One of the features of pyroglutamyl peptidase II is its rather restrictive

tissue distribution in comparison to other peptidases (9). Pyroglutamyl peptidase I has a much more uniform tissue distribution (19) and is fairly uniformly distributed across the cell lines studied (Table 3). By contrast, the specific activity of pyroglutamyl peptidase II in the Y-79 retinoblastoma cell is much greater than any of the other cell lines studied (Table 2). The Y-79 retinoblastoma cell appears to be ideally suited for studies on the regulation of pyroglutamyl peptidase II. This cell line exhibits the interesting property of differentiating into a neuronal cell by culturing in a serum-free medium or a glial cell by addition of dibutyryl cAMP (20). The specific activity of pyroglutamyl peptidase II in the retinoblastoma cells is much lower than that of pyroglutamyl peptidase I although in retina the specific activities of these two enzymes are similar. One can speculate that neuronal differentiation of the Y-79 retinoblastoma cell may lead to a large increase in pyroglutamyl peptidase II activity.

Table 1 Specific Activity of Pyroglutamyl Peptidase II in Homogenates and Membrane Fractions of Rabbit Brain and Retina.

Tissue Fraction	Specific Activity
1) Brain homogenate	6.1 +/- 0.4 (6)
2) Brain membranes	9.0 +/- 0.9 (12)
3) Retinal homogenate	2.9 +/- 0.3 (7)
4) Retinal membranes	5.1 +/- 0.5 (7)

Specific activity is expressed as nmole naphthylamine released per mg protein per h +/- S.E. Numbers in parentheses indicated number of samples assayed. Pyroglutamyl peptidase II was determined as previously described (8).

Table 2 Specific Activity of Pyroglutamyl Peptidase II in Membrane Fractions of Clonal Cell Lines.

Cell Type	Specific Activity
1) Y-79 retinoblastoma	1.98 +/- 0.14 (6)
2) CPAE	0.23 +/- 0.03 (4)
3) PC-12	0.19 +/- 0.02 (4)
4) GH_3 cell	0.19 +/- 0.02 (4)
5) neuro-2A	0.18 +/- 0.02 (4)
6) C-6	< 0.06
7) AtT-20	< 0.06
8) $NB41A_3$	< 0.06
9) NG108	< 0.06

Specific activity is expressed as nmoles naphthylamine released per mg protein per h +/- S.E. Numbers in parentheses indicated number of determinations.

Table 3 Specific Activity of Pyroglutamyl Peptidase I in Supernatant Fractions of Clonal Cell Lines

Cell Type	Specific Activity
1) C-6 glioma	45.6 +/- 2.8 (6)
2) AtT-20	29.7 +/- 1.9 (6)
3) CPAE	22.1 +/- 1.8 (6)
4) Y-79 retinoblastoma	19.6 +/- 1.8 (6)
5) NG-108	18.5 +/- 0.9 (6)
6) PC-12	17.0 +/- 1.5 (6)
7) GH_3	14.6 +/- 1.2 (6)
8) NB41A3	12.7 +/- 1.2 (6)

Specific activity is expressed as nmole naphthylamine released per mg protein per h +/- S.E from pGlu-NA. Numbers in parentheses indicate number of determinations. Enzymatic activity determined as previously described (8).

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CHAPTER 4

ENDOCRINOLOGY **124** : 1654, 1989

Sodium Butyrate Induces Pyroglutamyl Peptidase I and Decreases Thyrotropin-Releasing Hormone Receptors in GH_3 Cells

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SUMMARY :

The effect of sodium butyrate treatment on TRH-degrading enzymes and TRH receptors in GH_3 cells was investigated. The specific activity of pyroglutamyl peptidase I (EC 3.4.19.3) was increased by exposure to sodium butyrate in a time- and concentration-dependent manner, whereas the specific activity of prolyl endopeptidase (EC 3.4.21.26) was unchanged. The maximal effect occurred at a concentration of 1 mM sodium butyrate and 16 h after exposure. The increase was reversible upon removal of sodium butyrate from the cell culture. Cycloheximide totally blocked the stimulation, indicating that the increase was due to new protein synthesis. Sodium butyrate had no effect on pyroglutamyl peptidase I activity in the AtT-20 cell line. [*methyl*-^{3}H] TRH binding to intact GH_3 cell was reduced to 70% of the control value when cells were exposed to 1 mM sodium butyrate for 8 h. A maximal decrease in binding to 40% of the control value occurred after 16 h of exposure. The K_d of [*methyl*-^{3}H] TRH binding was not changed. Sodium butyrate altered GH_3 cell morphology, but the morphological changes occurred after alterations of pyroglutamyl peptidase I activity. These results indicate that sodium butyrate can in some respects mimic the action of T_3 on GH_3 cells. Moreover, they provide further evidence that the activity of pyroglutamyl peptidase I, but not prolyl endopeptidase, is subject to regulation in the GH_3 cells.

INTRODUCTION :

Butyrate, a naturally occurring short chain fatty acid, produces a wide variety of effects on cells in culture (1,2). For example, it influences cellular proliferation and differentation (3,4) and alters the expression of specific genes (5-8). Sodium butyrate can induce beta-adrenergic receptors in Hela cells (9,10) and rat aortic smooth muscle cells (11). Sodium butyrate also increases the thyroid hormone nuclear receptor level in glial C_6 cells by decreasing receptor degradation (12,13). In contrast, nuclear thyroid hormone receptors in GH_1 cells are reduced by sodium butyrate. This effect is apparently related to a hyperacetylation of core histone in the presence of this fatty acid (14). Sodium butyrate can affect several neurotransmitter-metabolizing enzymes ; for example, it increases tyrsosine hydroxylase (2,15) and acetylcholinesterase activity (16) in mouse neuroblastoma cells.

GH_3 cells, a clonal cell line derived from a rat anterior pituitary tumor, synthesize and secrete GH and PRL (17). Sodium butyrate and sodium valerate increase the production of both GH and PRL 2- to 4-fold in the GH_3 cell. This effect is reversible and accompanied by morphological changes (18). Treatment of GH_3 cells with 1 mM sodium butyrate also reduces specific [^{3}H] TRH binding (19). We have previously reported that this cell line contains the TRH-degrading enzymes pyroglutamyl peptidase I (EC 3.4.19.3) and prolyl endopeptidase (EC 3.4.21.26) (20). Of these two activities, only pyroglutamyl peptidase I appears to be subject to regulation. Culturing in the presence of 5-oxoprolinal, a reversible inhibitor of this enzyme (21), or in the presence of L-T_3 (22) increases the activity of pyroglutamyl peptidase I, but not prolyl endopeptidase. We report here that sodium butyrate can selectively increase pyroglutamyl peptidase I activity in GH_3 cells in a time- and concentration-dependent manner. Moreover, this short chain fatty acid markedly decreases specific [*methyll*-^{3}H] TRH binding to intact cells without changing its apparent binding affinity. Although exposure to sodium butyrate produces dramatic changes in cell morphology, the biochemical changes observed precede the morphological effects.

MATERIALS AND METHODS :

Sodium butyrate, T_3, 12-0-tetradecanoyl phorbol-13-acetate, TRH, cycloheximide, and L-pyroglutamyl beta-naphthylamide (pGlu-NA) were obtained from Sigma Chemical Co. (St.Louis, MO). Epidermal growth factor was obtained from Biochemical Technologies, Inc. (Stoughton, MA). [^{3}H] Me-TRH (57.8Ci/mmol) and Aquasol-2 were purchased from NEN Research Products, (Boston, MA). All cell culture media, fetal bovine serum, and horse serum were purchased from Gibco (Grand Island, NY). N-Benzyloxycarbonyl-Gly-Pro-sulfamethoxazole (Z-Gly-Pro-SM) and 5-oxoprolinal were synthesized as described previously (23,24). The GH_3 cells were grown under previously described conditions (22). Briefly, these consisted of an atmosphere of 5% CO2, a temperature of 37C, and Ham's F-10 medium supplemented with 12.5% horse serum, 2.5% fetal bovine serum, 10 mM sodium bicarbonate, and HEPES buffer, pH 7.2. A penicillin-streptomycin solution (50U/ml; 50mg/ml; Gibco) was added to the medium.Stock cultures were grown in Falcon T-flasks (75 cm^2; Falcon Plastics, Oxnard,CA); cells for experiments were replated in 25 cm^2 tissue culture flasks. The medium was changed every 3 days. One week after plating, various concentrations of freshly prepared sodium butyrate were added to the flask. After 24 h of incubation at 37C, the cells were harvested. For time-course studies, 1mM freshly prepared sodium butyrate was added to the flask for times ranging from 4 h to 3 days. At varying time intervals, the cells were removed from the flask with Versene solution, pelleted, and then washed with PBS. The final pellets were placed on ice and homogenized in 250 ul 0.1 M Tris-HCl (pH=7.5) with a Potter-Elvejhem homogenizer. Activities of pyroglutamyl peptidase I and prolyl endopeptidase were measured as described previously (22). Pyroglutamyl peptidase I activity was determined with the substrate pGlu-NA, and prolyl endopeptidase activity was determined with the substrate Z-Gly-Pro-SM. The activities of both enzymes were measured by determination of release of the aromatic amine by the diazotization procedure of Bratton and Marshall, Jr. (25), as modified by Goldbarg and Rutenberg (26). Specific activity was expressed as nanomoles of amine released per mg protein/h. Protein concentration was measured by the method of Lowry *et all.* (27).

TRH receptor assay :

To measure the amount of [^{3}H] Me-TRH bound to intact GH_3 cells, various concentrations of [^{3}H] Me-TRH (final concentration, 0.05-15 nM) were prepared in the serum-free Ham's F-10 medium and added to the cells ($1.5X10^{-6}$ cells/tube). After incubation for 60 min at 25 C, the reaction mixture was diluted to 2 ml with ice-cold 0.9% NaCl solution and passed through a 25 mm Millipore HA filter (Millipore Corp., Bedford, MA) with gentle suction. The filters were washed three times with 2 ml cold 0.9% NaCl solution. For TRH receptor binding in the presence of sodium butyrate, GH_3 cells were incubated with sodium butyrate for 8,16, and 36 h, followed by washing twice with serum-free medium. Radioactive [^{3}H] Me-TRH was added, and the cells were incubated at 25 C for 1 h. Cells were filtered as described above. The radioactivity was determined by liquid scintillation spectrometry in 5 ml Aquasol-2 solution. Counting efficiency for ^{3}H was 62%. Nonspecific binding of [^{3}H] Me-TRH was determined in parallel assay tubes which contained a 1000-fold excess of unlabeled TRH. Nonspecifc binding comprised 3-5% of total binding. The specific binding of [^{3}H] Me-TRH was obtained by subtraction of nonspecific binding from the total binding. All measurements were carried out in triplicate. Values for the Kd and the total number of receptor sites (B_{max}) were determined by a computerized least square fit of the Scatchard plot.

RESULTS :

The effects of sodium butyrate on the enzymatic activity of pyroglutamyl peptidase I and prolyl endopeptidase in GH_3 cells were studied. After a lag of slightly less than 10 h, 1 mM sodium butyrate caused a 3-fold increase in pyroglutamyl peptidase I specific activity (FIG.1). Pyroglutamyl peptidase I specific activity was maximally increased by 16 h and remained elevated thereafter. The activation of pyroglutamyl peptidase I was not due to a direct effect of the fatty acid, since addition of sodium butyrate to assay mixtures had no effect on pyroglutamyl peptidase I. The stimulation of pyroglutamyl peptidase I was dependent upon the concentration of sodium butyrate used. As shown in Fig.2, 1 mM sodium butyrate elicited a maximal stimulation of pyroglutamyl peptidase I activity. There was a visible cytotoxic effect when the concentration of sodium butyrate was increased to 5 mM. There was no change in prolyl endopeptidase specific activity (data not shown).

The sodium butyrate-induced increase in pyroglutamyl peptidase I activity was completely reversible, as shown in Fig.3. After removal of sodium butyrate from the culture medium, the enzyme level declined rapidly and essentially returned to control levels within 2 days. Experiments were designed to determine whether the increased activity of pyroglutamyl peptidase I was due to *de novo* protein synthesis. GH_3 cells were treated with sodium butyrate (1mM), cycloheximide alone (200 ng/ml), or sodium butyrate plus cycloheximide. This concentration of cycloheximide did not produce cytotoxicity in the cells, as judged by trypan blue exclusion. As shown in Fig. 4, the basal activity of pyroglutamyl peptidase I was reduced by exposure to cycloheximide, presumably as a consequence of decreased protein synthesis. The sodium butyrate-induced increase in activity of pyroglutamyl peptidase I was totally blocked by cycloheximide. The results indicate that the increase in specific activity of pyroglutamyl peptidase I by sodium butyrate is due to new enzyme synthesis.

We previously reported that exposure of rat pituitary GH_3 cells to 5-

oxoprolinal (21) or low concentrations of T_3 (22) increases the specific activity of pyroglutamyl peptidase I. Table 1 shows the results of experiments in which GH_3 cells were incubated with sodium butyrate, 5-oxoprolinal, and T_3 separately and in combination. 5-oxoprolinal combined with either T_3 or sodium butyrate has an additive effect on pyroglutamyl peptidase I specific activity. By contrast, cells treated with sodium butyrate and T_3 did not display an increase in pyroglutamyl peptidase I activity above that caused by either compound alone.

We also evaluated the effect of sodium butyrate on pyroglutamyl peptidase I activity in another mouse pituitary tumor cell line (AtT-20). The basal level of pyroglutamyl peptidase I activity in AtT-20 cells was 20.4 +/- 2.6 nmol/mg protein.h (n=6), a value virtually identical to the enzymatic activity in the AtT-20 cells treated with 1 mM sodium butyrate for 24 h (20.6 +/-3.1nmol/mg protein.h;n=6). Previous studies have shown that several compounds change the morphology of GH cells (18,28-30). The morphological changes in GH_3 cells are not related to the induction of pyroglutamyl peptidase I. Of the compounds known to alter cell morphology, only sodium butyrate increased pyroglutamyl peptidase I specific activity (Fig. 5).

Saturation binding of [^{3}H]Me-TRH to intact GH_3 cells was conducted in the absence and presence of sodium butyrate (Fig. 6). The B_{max} of the control cells (0.89 +/- 0.03 pmol/mg protein) was reduced to 0.62 +/- 0.04 pmol/mg protein when cells were treated with 1 mM sodium butyrate for 8 h and to 0.39 +/- 0.02 pmol/mg protein when cells were treated with sodium butyrate for 16 h. There was no further decrease in the B_{max} of [^{3}H]Me-TRH binding upon longer exposure to sodium butyrate (Fig. 7). The apparent dissociation constant was not significantly altered. The Kd of [^{3}H]Me-TRH binding to the control cells was 1.41 +/- 0.11 nM (n=5) compared to 1.85 +/- 0.15 nM (n=5; $P>0.05$) for the cells treated with sodium butyrate. We have studied the time-dependent changes in GH_3 cell morphology produced by exposure to sodium butyrate. GH_3 cell are characteristically spherical in shape and loosely adherent to the culture dish. Treatment of GH_3 cells with 1 mM sodium butyrate caused the cells to flatten, have more angular borders, adhere more firmly to the

substrate, and enlarge. Some cells appeared fused. These effects were time dependent. No changes in morphology were apparent for the first 8 h. Some changes were noted at 24 h, but were most prominent at 48 h (Fig. 8). The time course of the decrease in number of [^{3}H]Me-TRH-binding sites in GH_3 cells did not correlate with the change in cell morphology (Fig. 8).

DISCUSSION :

Sodium butyrate exerts a wide variety of complex effects on cells in culture (2). In GH_3 cells, this compound has been reported to change cell morphology and increase GH and PRL production (18). We report that sodium butyrate induces the TRH-degrading enzyme pyroglutamyl peptidase I and extend the observations of Imai and Gershengorn (19) on the reduction in TRH receptors. We have previously reported that GH_3 cells contain the TRH-degrading enzymes, pyroglutamyl peptidase I and prolyl endopeptidase (20). The activity of pyroglutamyl peptidase I, but not prolyl endopeptidase, is regulated by 5-oxoprolinal (21) and T_3 (22). The effect of T_3 is also seen *in vivo*. We have recently shown that pyroglutamyl peptidase I in rat pituitary gland and hypothalamus is increased by chronic treatment with T_3 (31). This implies that the induction of pyroglutamyl peptidase I by T_3 may contribute to the negative feedback regulation of thyroid hormone status.

In this report we have shown that pyroglutamyl peptidase I is also increased by sodium butyrate. The maximal increase in pyroglutamyl peptidase I activity occurs 16 h after treatment with 1 mM sodium butyrate and is totally reversible upon removal of this fatty acid. It is also blocked by cycloheximide, indicating that the increase is due to new enzyme synthesis. This effect is very similar to that of T_3, as previously reported (22). We were interested in whether the mechanism of enzyme elevation by sodium butyrate differs from that of 5-oxoprolinal or T_3. The results in Table 1 show that 5-oxoprolinal has an additive effect in combination with either sodium butyrate or T_3 on the activity of pyroglutamyl peptidase I. We have previously found that the increase in pyroglutamyl peptidase I by 5-oxoprolinal could not be blocked by cycloheximide. Thus, 5-oxoprolinal increases pyroglutamyl peptidase I by a mechanism different from that of T_3 or sodium butyrate, perhaps by decreasing the degradation of this enzyme. However, addition of T_3 to sodium butyrate produced no further increase above that caused by either agent used separately. Samuels et al. (14) have shown previously that butyrate reduces thyroid hormone nuclear receptor levels in GH cells and

that reduction of thyroid hormone receptor may be secondary to a process that involves inhibition of histone deacetylase activity. This probably explains the finding that sodium butyrate and T_3 do not have an additive effect on pyorglutamyl peptidase I. By decreasing thyroid hormone receptors in GH_3 cells, sodium butyrate will diminish the effect of T_3.

Others have shown that the morphology of GH_3 cells changes from a spherical appearance to an elongated flattened shape after treatment with epidermal growth factors and TRH (28,29), phorbol ester (30), and sodium butyrate (18). After incubation of GH_3 cells with these compounds for 36 h, only sodium butyrate increased pyroglutamyl peptidase I activity. This suggests that the induction of enzyme activity is independent of alterations in cell morphology.

The TRH receptor in the GH_3 cell has been well characterized (32-34). The number of TRH receptors in GH_3 cells is regulated by TRH (35) and T_3 (36,37). We have found a marked decrease in [^{3}H]Me-TRH binding after exposure of GH_3 cells to sodium butyrate. Similarly, Imai and Gershengorn (19) reported approximately a 55% decrease in [^{3}H]TRH binding after 16-h exposure to 1 mM sodium butyrate. It was, therefore, of interest,in order to rule out nonspecific effects, to determine whether this reduction in binding was independent of changes in cell morphology. [^{3}H]Me-TRH binding in the presence of sodium butyrate decreased to 70% of the control value after 8 h. By 16 h binding decreased to 70% of the control value. No further decrease in radioligand binding occurred after longer treatment with sodium butyrate. The reduction in binding occurs before noticeable changes in cell morphology, which are seen only after 24-36 h of incubation with sodium butyrate. From the Scatchard plot analysis, the apparent affinity of [^{3}H]Me-TRH in untreated cells was similar to the affinity of this ligand to the cell treated with sodium butyrate.

It is of interest that in some respects sodium butyrate can mimic the action of T_3 on GH_3 cells. Thus, both compounds 1) induce pyroglutamyl peptidase I in a time- and dose-dependent manner (hence, this effect can be blocked by cycloheximide) (22), 2) decrease TRH receptor number

without changing the affinity of TRH for its receptor (37), 3) induce GH synthesis (18,37), and 4) alter cell morphology from a spherical to a more flattened shape (38). The possibility of coordinate regulation of TRH receptors and TRH-degrading activity so as to decrease the sensitivity of the cell to TRH is worthy of consideration. Futher experimentation would be required to establish whether this is indeed the case. At any rate these studies reveal a third mechanism of regulation of pyroglutamyl peptidase I and support a role for this enzyme in the control of the biological activity of TRH.

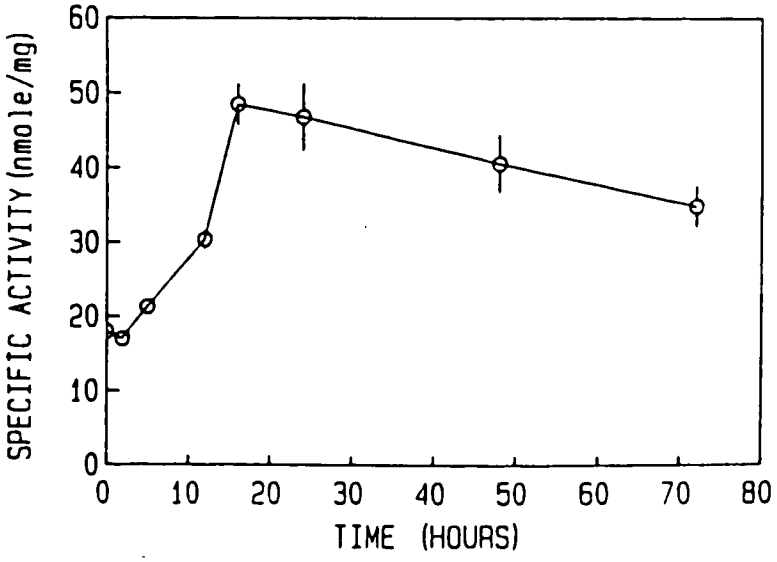

Fig. 1. Pyroglutamyl peptidase I activity in GH_3 cells as a function of time of exposure to sodium butyrate. Cells were plated as described in *Materials and Methods* and treated for the indicated period of time with 1 mM sodium butyrate. Each point represents the mean +/- SEM of five determinations. Specific activity is expressed as nanomoles of 2-napthylamine released from pGlu-NA per mg protein/h.

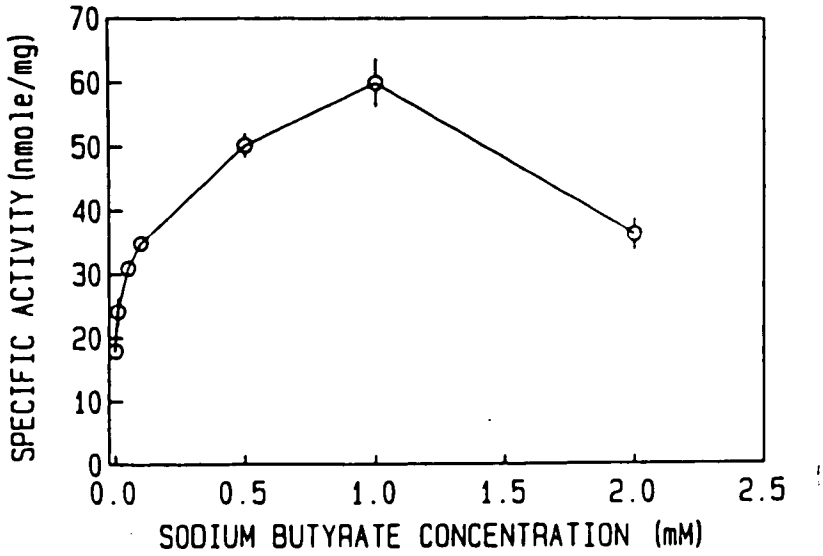

Fig. 2. Dose-response effect of sodium butyrate on the specific activity of pyroglutamyl peptidase I in GH_3 cells. Specific activity is expressed as described in Fig. 1. Cells were plated as described and treated for 24 h with the indicated concentration of sodium butyrate. Each point represents the mean +/- SEM of five determinations.

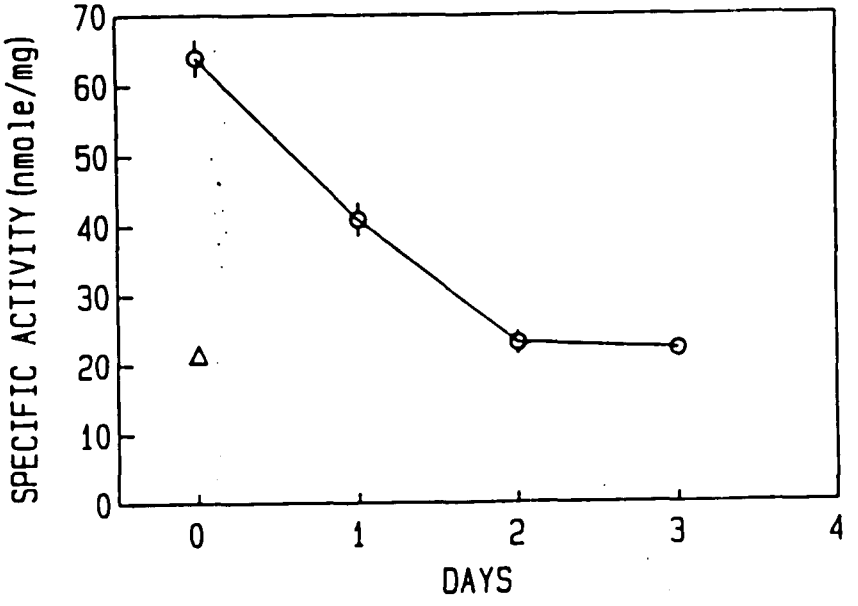

Fig. 3. Reversibility of the effect of sodium butyrate on the specific activity of pyroglutamyl peptidase I in GH_3 cells. Specific activity is expressed as described in Fig. 1. Cells were treated for 24 h with 1 mM sodium butyrate and after this time were grown in a sodium butyrate-free medium. Cells were harvested 0,1,2,and 3 days after medium replacement. A, The basal activity of pyroglutamyl peptidase I in cells never exposed to sodium butyrate. Each point represents the mean +/- SEM of five determinations.

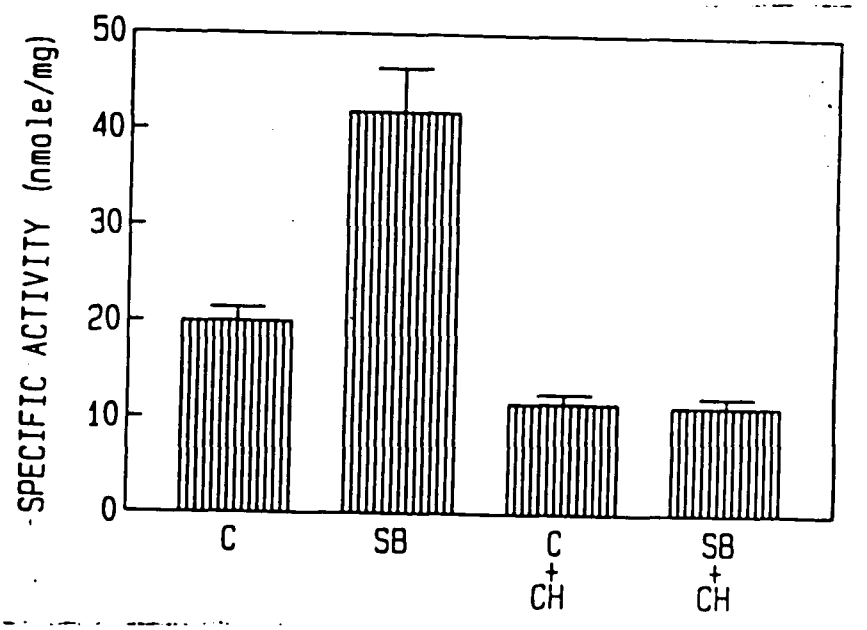

Fig. 4. Effect of cycloheximide on the sodium butyrate-induced increase in pyroglutamyl peptidase I specific activity in GH_3 cells. Specific activity is expressed as described in Fig. 1. Flasks were incubated in the presence and absence of sodium butyrate (1 mM) and cycloheximide (200 ng/ml) for 24 h. Each bar represents the mean +/- SEM of determinations from five flasks. C, Control (no sodium butyrate); SB, sodium butyrate treated; CH, Cycloheximide treated.

Additions	Pyroglutamyl peptidase I specific activity (nmol/mg protein·h)
None	16.0 ± 0.9
Sodium butyrate	32.5 ± 2.2
T_3	22.5 ± 1.1
5-Oxoprolinal	32.7 ± 2.2
Sodium butyrate + T_3	31.6 ± 2.3
Sodium butyrate + 5-oxoprolinal	48.5 ± 0.8
T_3 + 5-oxoprolinal	48.5 ± 0.8

Table 1. Effects of sodium butyrate, T_3, and 5-oxoprolinal on the specific activity of pyroglutamyl peptidase I in GH_3 cells: Cells were exposed to sodium butyrate (1mM), T_3 ($1X10^{-8}M$), and 5-oxoprolinal ($1X10^{-5}M$) either singly or in combination for 24 h. Activity of pyroglutamyl peptidase I was measured in whole cell homogenates as described in Materials and Methods. Each value represents the mean +/- SEM of five determinations from five flasks.

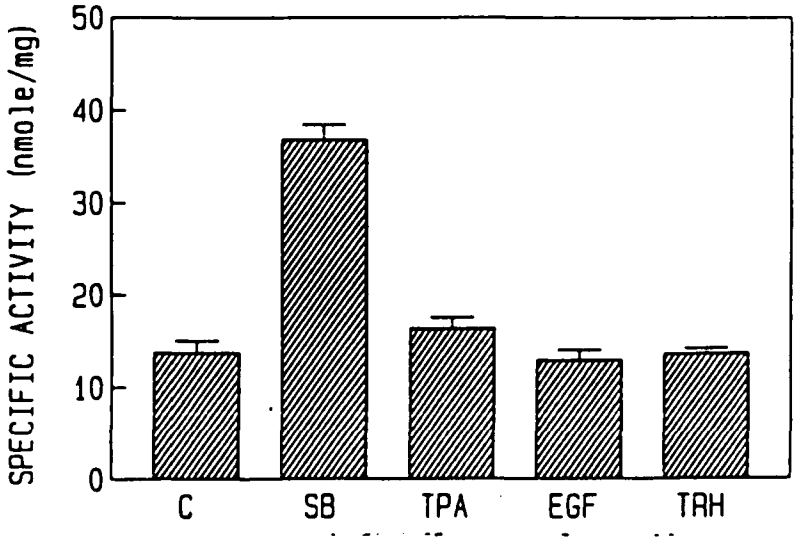

Fig. 5. Effect of agents demonstrated to alter GH_3 cell morphology on the specific activity of pyroglutamyl peptidase I in GH_3 cells. Specific activity is expressed as described in Fig. 1. GH_3 cells (C,control) were treated for 36 h with sodium butyrate (SB;1mM), 12,0-tetradecanoyl phorbol-13-acetate (TPA;$1X10^{-7}$M), epidermal growth factor (EGF; 50ng/ml), or TRH ($1.4X10^{-7}$M). Cells were then harvested, and the specific activity of pyroglutamyl peptidase I was determined as described in Materials and Methods. Each bar represents the mean +/- SEM of determinations from five flasks.

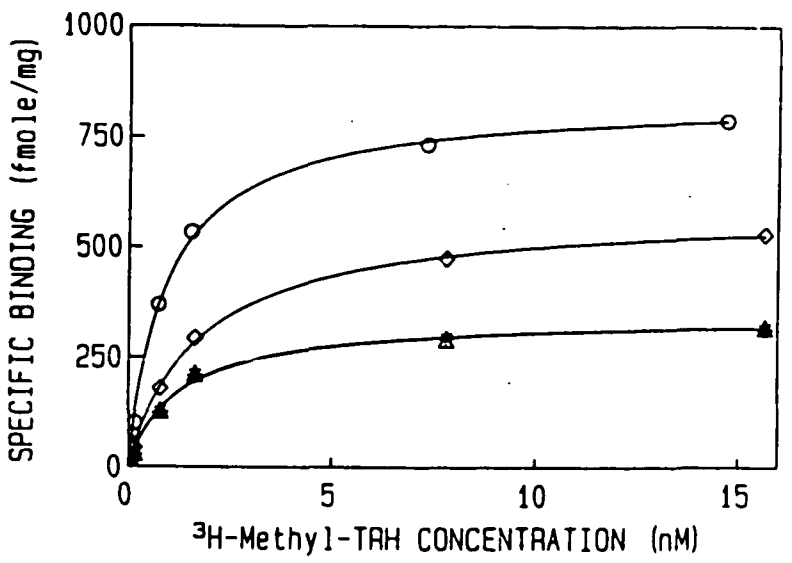

Fig. 6. Effect of sodium butyrate on the saturable binding of [^{3}H] Me-TRH to intact GH_3 cells. Specific binding, defined as total binding minus binding in the presence of a 1000-fold molar excess of TRH and expressed as femtomoles per mg protein, is displayed. Incubations were conducted at 25 C for 1 h in a total volume of 0.25 ml. Each point represents the mean of triplicate determinations. O,control; ,1mM sodium butyrate 8h; *,1mM sodium butyrate 16 h; , 1mM sodium butyrate 36 h.

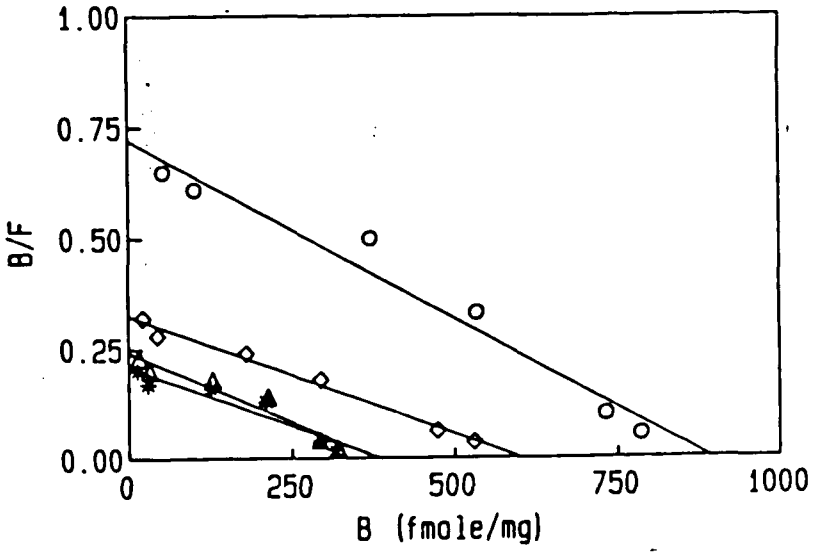

Fig. 7. Scatchard plot of the data represented in Fig. 6. The B_{max} of control cells was 890 fmol/mg protein. The B_{max} values of cells exposed to 1mM sodium butyrate for varying periods of time were : 8h, 620 fmol/mg protein; 16h, 390 fmol/mg protein; 36h, 400 fmol/mg protein.

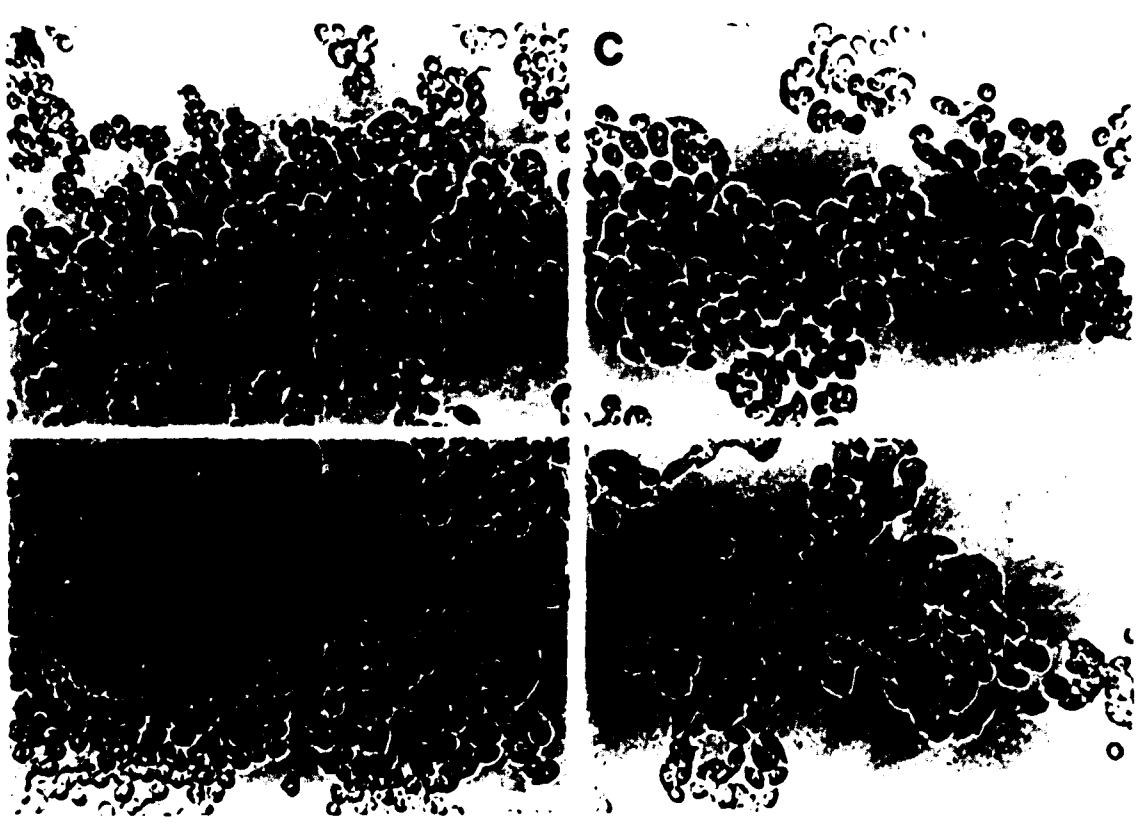

Fig. 8. Change in GH_3 cell morphology as a function of time of exposure to 1 mM sodium butyrate. A, zero hours; B, 8h; C, 24h; D, 48h.

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CHAPTER 5

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INACTIVATION AND PHOSPHORYLATION OF PYROGLUTAMYL PEPTIDASE II BY PHORBOL ESTER ACTIVATED PROTEIN KINASE C IN Y-79 HUMAN RETINOBLASTOMA CELLS

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SUMMARY :

Pyroglutamyl peptidase II (EC 3.4.19.-), a membrane bound metalloproteinase, is a highly specific thyrotropin releasing hormone (TRH) degrading enzyme. Exposure of Y-79 human retinoblastoma cells to 12-0-tetradecanoyl phorbol 13-acetate (TPA) decreased the activity of this enzyme in a time and concentration dependent manner ($IC_{50} = 5 \times 10^{-9}$ M). After 15 min of TPA treatment , only 10% of pyroglutamyl peptidase II activity remained. TPA treatment did not affect the activity of the cytosolic enzyme pyroglutamyl peptidase I (EC 3.4.19.3.) or the membrane-bound enzyme dipeptidyl peptidase IV (EC 3.4.19.3). Pretreatment of the cells with the protein kinase C inhibitors, H-7 or sphingosine, prevented the inactivation of pyroglutamyl peptidase II by TPA. The time course of the TPA-mediated effect paralleled the time course of translocation and activation of protein kinase C in this cell line. Immunoblot analysis demonstrated that inactivation of pyroglutamyl peptidase II was not due to dissociation and/or internalization of this enzyme molecule. Incubation of TPA-activated Y-79 cell membranes with γ-^{32}P-ATP followed by immunoprecipitation revealed phosphorylation of a 48 kDa subunit of pyroglutamyl peptidase II. Protein kinase C-mediated inactivation of pyroglutamyl peptidase II may provide a mechanism for potentiation of the action of TRH at its target sites.

INTRODUCTION:

Thyrotropin releasing hormone (TRH, <Glu-His-Pro-NH2) is cleaved at the <Glu-His bond by two pyroglutamyl peptidases designated as I and II (1). Pyroglutamyl peptidase I (EC 3.4.19.3), a widely distributed cytosolic cysteine proteinase cleaves all <Glu-amino acid bonds except the <Glu-Pro bond (2). Pyroglutamyl peptidase II (EC 3.4.19.-), a membrane-bound metalloproteinase(3), is found predominantly in brain (4) where it is present as an ectoenzyme (5) on synaptosomal membranes (6,7). It has a very restricted specificity cleaving the <Glu-His bond of TRH but not the <Glu-His bond of gonadotropin hormone releasing hormone (3,8). The activity of pyroglutamyl peptidase II in pituitary and frontal cortex is increased by thyroid hormone treatment in a mechanism apparently due to induction of protein synthesis (9). CPHNA, an inhibitor of pyroglutamyl peptidase II enhances the basal and K+ stimulated recovery of TRH from brain slices(10). These properties suggest that pyroglutamyl peptidase II may act to terminate the biological activity of TRH.

It is known that the activation of TRH receptors in GH_3 cells leads to the hydrolysis of phosphatidyl inositol, generating the dual signals inositol triphosphate and diacylglycerol (11). Diacylglycerol is an endogenous activator of the Ca^{+2} and phospholipid-dependent protein kinase (protein kinase C). In the GH_3 cell, TRH stimulates translocation of protein kinase C from cytoplasm to membrane (12) leading to protein kinase C-mediated phosphorylation of protein substrates (13). The function of protein phosphorylation following exposure of GH_3 cells to TRH is presently unknown.

We considered the possibility that pyroglutamyl peptidase II may be subject to short term regulation and that protein kinase C-mediated phosphorylation would be a likely mechanism for this effect. We have investigated this possibility in the Y-79 retinoblastoma cell which contains relatively high concentrations of pyroglutamyl peptidase II (14).

We report that exposure of Y-79 cells to 12-0-tetradecanoyl phorbol 13-acetate (TPA) leads to a rapid and marked inactivation of pyroglutamyl peptidase II and that this effect is due to phosphorylation of pyroglutamyl peptidase II by protein kinase C.

MATERIALS AND METHODS :

Materials :
Cell culture media and sera were purchased from GIBCO (Grand Island, NY). r-[^{32}P]-ATP (25 Ci/mmole) was obtained from New England Nuclear (Boston, MA). [^{125}I]-protein A and monoclonal antobodies to protein kinase C were from Amersham Corp. (Arlington Heights, IL). Nonidet P-40 (NP-40) was obtained from Pierce, Inc. Rockford, IL. TPA, phorbol 12,13-dibutanoate, 4 alpha-TPA, H-7, sphingosine, phosphatidyl serine, diolein, histone (type III-S), affinity purified secondary antibodies, protein A-Sepharose and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Immobilon membranes were obtained from Millipore (Bedford, MA). Y-79 human retinoblastoma cells, GH_3 cells and AtT-20 cells were purchased from American Type Cell Collection (ATCC), Rockville, MD. <Glu-2-naphthylamide (<Glu-NA) was obtained from Bachem Bioscience Inc. (Philadelphia, PA). Dipeptidyl peptidase IV (EC.3.4.15.5) was purified to homogeneity from rabbit kidney (15). pGlu-His-Pro-NA, Z-Pro-Prolinal and pyroglutamyl diazomethyl ketone were synthesized as previously described (8,16,17). Gly-Pro-NA was synthesized by conventional procedure. Aquasol-2 was purchased from Dupont (Boston, MA). Pyroglutamyl peptidase II was purified from rabbit brain to apparent homogeneity as described (8).

Cell Cultures :
Human Y-79 retinoblastoma cells were grown in suspension as small clusters of 5-10 cells. These were cultured in RPMI medium containing 15% heat inactivated fetal bovine serum, penicillin (100u/ml), streptomycin (100ng/ml) and glutamine. Cells were grown in Corning 75 cm^2 tissue culture flasks in an incubator at 37^{o}C with 5% CO_2 in humidified air as the gas phase. The culture medium was changed twice weekly.

Measurement of activities of TRH degrading enzymes and dipeptidyl

peptidase IV :
The activities of all peptidases were determined with synthetic naphthylamide substrates. Substrate cleavage leads to release of free 2-naphthylamine which is measured by a diazotization procedure (18,19). The absorbance of the chromogen formed was read in a spectrophotometer at 580 nm. Specific activity was expressed in term of nmoles of naphthylamine released/mg protein/h. Protein was measured by the method of Lowry et al (20).

a) Pyroglutamyl peptidase I : This enzyme was determined with the substrate <Glu-NA. The incubation mixture (final volume 250 ul) contained 10 ul of a 10 mM substrate solution prepared in dimethylsulfoxide, 20 ul of 10 mM dithiothreitol (DTT), 20 ul of 20 mM EDTA (pH 7.2) , 50 ul of cell homogenate and 50 mM Tris-HCl buffer, pH 7.5. Tubes were incubated at 37°C for 1 h and the reaction was terminated by addition of 250 ul of 10% trichloroacetic acid.

b) Pyroglutamyl peptidase II : Enzymatic activity was determined with the substrate pGlu-His-Pro-NA in a coupled assay in the presence of excess dipeptidyl peptidase IV as described (4). The incubation mixture (final volume 250 ul) contained 10 ul of a 10 mM substrate solution in dimethylsulfoxide, 10 ul each of Z-Pro-Prolinal and pyroglutamyl diazomethyl ketone (10^{-5} M final concentration of each, added to inhibit prolyl endopeptidase and pyroglutamyl peptidase I respectively), 10 ul of dipeptidyl peptidase IV (2 ug protein), 50 ul cell homogenate and 50 mM Tris-HCl buffer, pH 7.5. Incubations were conducted for 1 h at 37°C and terminated by addition of 250 ul 10% trichloroacetic acid.

c) Dipeptidyl peptidase IV : This enzyme was measured with Gly-Pro-NA as substrate. The incubation mixture (final volume 250 ul) contained 10 ul of a 10 mM substrate solution in dimethylsulfoxide, 25 ul cell homogenate and 50 mM Tris-HCl buffer, pH 7.5. Tubes were incubated at 37°C for 30 min and the reaction was stopped by the addition of 250 ul of trichloroacetic acid.

Determination of protein kinase C activity in Y-79 cells :
Protein kinase C activity was measured by the method of Thomas et al

(21). Cells were treated with TPA (1.6X10-7M) for varying times and then quickly spun down in a microfuge. The cells were washed twice with cold phosphate buffered saline (PBS). The cells were resuspended in 250 ul of buffer A (20mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 25 ug/ml leupeptin), and homogenized at 4°C. The cytosol and particulate fractions were separated by centrifugation at 16,000 g for 15 min. The particulate fraction was washed once with buffer A and recentrifuged. Detergent-solubilized protein kinase C was obtained from the particulate fraction by adding 250 ul of buffer A containing 1% NP-40. The tubes were rotated slowly for 30 min at 4°C.Following centrifugation at 16,000 g for 15 min, the supernatant was retained. The original cytosol and the detergent-solubilized particulate fraction were partially purified on DE-52 columns (10 cm X 11 cm), equilibrated with buffer B (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA), and washed well with the same buffer. Protein kinase C was eluted with buffer B containing 0.1 M NaCl. Protein kinase C activity was assayed by measuring the incorporation of ^{32} P phosphate into histone type III-S. The assay mixture contained 20 mM Tris-HCl, pH 7.5, 0.75 mM $CaCl_2$, 10 mM magnesium acetate, 50 ug histone type III-S, 50 uM r-[^{32}P]-ATP, 12.5 ug leupeptin, 24 ug phosphatidyl serine, 1.6 ug diolein and 80 ul of enzyme preparation in a final volume of 250 ul. The tubes were incubated at 30 C for 3 min and the reaction terminated by the addition of 1 ml of cold 25% trichloroacetic acid. The precipitate was collected by filtration over a millipore filter (0.45 uM). The filters were washed five times with cold 10% trichloroacetic acid, dissolved in Aquasol-2 and radioactivity measured by liquid scintillation spectroscopy. Protein kinase C activity was expressed as picomoles of ^{32} P incorporated per min per ug of protein.

Immunoblot analysis of pyroglutamyl peptidase II and protein kinase C in Y-79 cells :

Y-79 cells were treated with TPA for various times. The reaction was stopped by rapid centrifugation and the cells washed twice with cold PBS. The cells were resuspended in 250 ul of buffer A, homogenized and centrifuged at 4°C for 15 min at 16,000g. The supernatant (cytosolic fraction) was collected and saved. Membrane proteins were extracted from the particulate fraction by adding 100 ul of buffer A containing 1% NP-40

and incubating for 1 h at 4°C. The cytosolic and solubilized membrane proteins were separated by electrophoresis on 10% SDS polyacrylamide gels. The proteins were electrophoretically transferred to Immobilon membranes. After electroblotting, the membranes were placed overnight in TBS buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl) containing 5% BSA to block nonspecific binding. For protein kinase C quantitation, the membranes were incubated overnight with a protein kinase C monoclonal antibody (1:100 dilution). Goat antimouse antibody (1 ug/ml) and rabbit antigoat antibody (1 ug/ml) were added sequentially and membranes were incubated for an additional 2 h. For pyroglutamyl peptidase II quantitation, the membranes were incubated overnight with a polyclonal antibody to pyroglutamyl peptidase II (1:500 dilution). Rabbit antiguinea pig antibody was then added and the membranes incubated for another hour. The immunoreactive bands were visualized by adding [^{125}I]-protein A (0.5 uCi/ml) in TBS containing 5% BSA for 1 h. The membranes were washed extensively several times with TTBS (TBS buffer containing 0.05% Tween-20) between each addition of antibodies and then air-dried. The dried membranes were subjected to autoradiography.

Phosphorylation and immunoprecipition of pyroglutamyl peptidase II :
Y-79 cells were incubated for appropriate times with TPA (1.6 X 10^{-7} M), and then washed twice with cold PBS and resuspended in buffer A. The cells were then homogenized and centrifuged at 16,000g at 4°C for 15 min. The crude membrane fractions were washed once with buffer A and resuspended in phosphorylation buffer (20 mM Tris-HCl, 10 mM $MgSO_4$, 2 mM PMSF, 50 mM NaF and 200 uCi of r-[^{32}P]-ATP), for 3 min at 30°C. Phosphorylation was stopped by adding cold methanol to precipitate the proteins. The pellets were resuspended in 200 ul of cold immunoprecipition buffer (10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.5% SDS, 50 mM NaF, 2 mM Na_3VO_4, 2 mM PMSF, and 250 ug/ml leupeptin pH 7.5) and then 100 ul of polyclonal antibody to pyroglutamyl peptidase II or preimmune serum diluted 200 fold in binding buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% BSA, 0.5% SDS) was added. After incubation at 4°C overnight, the immunocomplexes were formed by adding 100 ul of 10% protein A-Sepharose, incubating for 2 h and precipitating by centrifugation at 16,000g for 15 min at 4°C.The supernatant was carefully removed, the pellets washed several times

with immunoprecipition buffer and then washed twice in a solution of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40. The pellets were then resuspended in SDS sample buffer and boiled for 5 min. All samples were then subjected to SDS polyacrylamide gel electrophoresis on 10 % gels. The gels which contained the ^{32}P labelled proteins were dried and subjected to autoradiography.

RESULTS:

Inactivation of pyroglutamyl peptidase II by TPA :
Exposure of Y-79 retinoblastoma cells to $1.6X10^{-7}M$ TPA produced a rapid and marked inactivation of pyroglutamyl peptidase II (Fig.1). The activity of pyroglutamyl peptidase II was decreased to 10% of control after 15 min treatment, and this effect was sustained to 30 min. Enzymatic activity returned to 70% of control after longer treatment (data not shown). There was no significant change in the activity of pyroglutamyl peptidase I (Fig.1). The effect of TPA was dose-dependent in the range of $10^{-10}M$ to $10^{-6}M$ (Fig.2), with an IC 50 of approximately $5 X 10^{-9}M$. Significant inhibition of enzymatic activity was observed at a concentration of TPA as low as $10^{-10}M$. In order to rule out the possibility that inactivation of this membrane-bound enzyme was not due to a nonspecific membrane effect produced by TPA, we examined the effect of TPA on another membrane-bound peptidase, dipeptidyl peptidase IV (DPP IV). TPA tested at different concentrations and at different times of exposure did not significantly alter the activity of DPP IV (Fig. 3). To determine whether the TPA effect was mediated by protein kinase C we tested the effect of other phorbol esters and of protein kinase C inhibitors on the activity of pyroglutamyl peptidase II in the Y-79 cells.Phorbol-12,13-dibutyrate, a less potent phorbol ester, caused a 60% inhibition of pyroglutamyl peptidase II activity at a concentration of $1.6 X 10^{-7}M$, whereas 4-alpha-TPA, an inactive form of phorbol ester, did not change the activity of pyroglutamyl peptidase II (Table 1). Pretreating the cells with the protein kinase C inhibitors, H-7 or sphingosine, abolished the TPA mediated inactiviation of pyroglutamyl peptidase II. These two inhibitors by themselves did not affect pyroglutamyl peptidase II activity. Inhibition by TPA was not due to a direct effect on the pyroglutamyl peptidase II molecule. Purified rabbit brain pyroglutamyl peptidase II was not altered by TPA at a concentration as high as $1.6 X 10^{-7}M$.

TPA has been shown to directly activate protein kinase C in many systems. We determined whether TPA could also mediate the translocation and

activation of protein kinase C in Y-79 cells. Cytosol and membrane proteins were separated after treatment of the cells with TPA for various times. After partial purification of both fractions by DE-52 chromatography, protein kinase C activity was determined by measuring ^{32}P transfer to histone II-S. Exposure of Y-79 cells to 1.6 X 10^{-7}M TPA led to a rapid time-dependent decrease in PKC activity in the cytosolic fraction (Fig. 4A). A commerically available monoclonal antibody against purified bovine brain protein kinase C was used to determine the translocation of protein kinase C in Y-79 cells. Cytosol and membrane fractions were separated, the proteins resolved by SDS PAGE, transferred to Immobilon membranes and blotted with the monoclonal antibody. Protein kinase C was visualized by autoradiography following addition of [^{125}I]-protein A. There was a rapid disappearance of cytosolic protein kinase C (Fig.5A), whereas protein kinase C in the membrane fraction increased gradually reaching a maximum at 30 min (Fig.5B). The time course of immunoreactive protein kinase C translocation was parallel to that of the increase in protein kinase C activity in membranes.

Quantitative immunoblot analysis of pyroglutamyl peptidase II:
A polyclonal antibody to pyroglutamyl peptidase II was used to investigate whether the decreased activity following TPA treatment was caused by dissociation and/or internalization of pyroglutamyl peptidase II. A polyclonal antibody to the rabbit brain enzyme was raised in guinea pigs. Western blotting revealed staining of the native 230,000 kDa brain enzyme at a 1:1000 dilution of antiserum and the antiserum also stained the major subunit (48 kDa) of this enzyme on SDS-PAGE gels. The antiserum was found to also stain on SDS-PAGE gels a 48 kDa protein in the solubilized membrane fraction of Y-79 cells (Fig. 6 lane 1). This protein was not recognized by preimmune serum (lane 2). To further verify the antibody specificity, we examined two additional clonal cell lines which had been reported previously to have lower pyroglutamyl peptidase II activities than Y-79 cells (14). Cytosol and membrane proteins of the cell lines were separated and transferred to Immobilon membranes. The antibody recognized a protein with molecular weight of about 48 kDa in the membrane fraction of each cell line (Fig. 7A). There were no specific proteins in the cytosolic fraction recognized by this antibody. Some nonspecific proteins bands were detected and attributed to binding of

secondary antibody (Fig 7B). The immunoblot results showed that the amount of the 48 kDa band in Y-79 cells (lane 1) was more abundant than that of GH_3 cells (lane 2) and AtT-20 cells (lane 3). These results are consistent with our eariler studies demonstrating greater pyroglutamyl peptidase II activity in Y-79 cells compared to GH_3 or AtT-20 cells. The amount of enzyme present in the membrane fraction after TPA treatment was examined to rule out the possibility of dissociation and/or internalization of pyroglutamyl peptidase II. There was no quantitative difference in the amount of pyroglutamyl peptidase II in Y-79 cells after the cells were treated with TPA from 5 to 30 min (Fig. 8). The results indicate that the decreased enzymatic activity was not due to the dissociation or internalization of pyroglutamyl peptidase II.

Phosphorylation of pyroglutamyl peptidase II by TPA activated protein kinase C :

Since the time course of inactivation of pyroglutamyl peptidase II and the time course of translocation and activation of protein kinase C were similar, we considered the possibility that enzymatic inactivation was due to phosphorylation by protein kinase C. In order to decrease the high background and nonspecific dephosphorylation by cytosolic phosphatases, we separated membrane proteins from cytosol after the cells were treated with TPA, and phosphorylated crude membrane proteins with [^{32}P]-ATP in vitro. After phosphorylation, the polyclonal antibody to pyroglutamyl peptidase II was employed for immunoprecipitation. We observed phosphorylation of a 48 kDa protein consistent with our previous immunoblot results (Fig 7A). The preimmune serum did not immunoprecipitate this protein (lane 2). The phosphorylation of pyroglutamyl peptidase II was time-dependent. Phosphorylation of the 48 kDa subunit of this enzyme was increased with increasing time of exposure to TPA and reached a maximum after 30 min (Fig 9B). Preincubation of the cells with H-7 caused a reduction of TPA mediated phosphorylation of the 48 kDa protein (data not shown).

DISCUSSION:

TRH, synthesized and released from the hypothalamus, is rapidly degraded in plasma and target tissues (22). Three enzymes can catalyze the initial degradation of this tripeptide (1). Prolyl endopeptidase and pyroglutamyl peptidase I are cytosolic enzymes that degrade a number of peptides in addition to TRH. Pyroglutamyl peptidase II on the other hand is a membrane-bound enzyme and may be TRH specific. Specific inhibitors of prolyl endopeptidase and pyroglutamyl peptidase I failed to increase the recovery of TRH from K^+-depolarized brain slices (23) whereas an increased recovery was obtained in the presence of CPHNA, a specific inhibitor of pyroglutamyl peptidase II (10). Pyroglutamyl peptidase II is therefore an excellent candidate for controlling the level of TRH at its site of action. Several groups have reported that the activity of pyroglutamyl peptidase II in the rat anterior pituitary gland is increased acutely by in vivo administration of thyroid hormone (9,24,25). The induction of this enzyme by thyroid hormone may contribute to the negative feedback regulation of thyroid hormone levels.

It has been demonstrated that TRH acts to stimulate the hydrolysis of phosphatidyl inositol by a phospholipase C to yield inositol triphosphate and diacylglycerol in GH_3 cells (11). We sought to investigate whether TRH could regulate its own degradation through TRH-activated signal processes. The Y-79 retinoblastoma cells although containing relatively high amounts of pyroglutamnyl peptidase II, do not have measurable TRH receptors (unpublished results). Therefore TPA was used instead of TRH to directly stimulate protein kinase C. TPA caused a time (Fig. 1) and dose (Fig. 2) dependent decrease in pyroglutamyl peptidase II activity but did not alter pyroglutamyl peptidase I activity. The inactivation of enzymatic activity was rapid and only 10% of the enzyme activity remained after 15 min. Although TPA is a hydrophobic molecule, the effect was not due to a nonspecific disturbance of the cell membrane integrity since membrane bound DPP IV was unaltered by this treatment (Fig. 3A and B). 4-alpha-TPA, an inactive form of phorbol ester, did not cause inactivation of

pyroglutamyl peptidase II, and pretreatment of the cells with the protein kinase C inhibitors, H-7 or sphingosine, prevented the decrease of pyroglutamyl peptidase II activity by TPA(Table 1). These results demonstrate that the rapid inactivation of pyroglutamyl peptidase II is a specific effect, possibly mediated by TPA activated protein kinase C.

Protein kinase C, a family of Ca^{+2}/phospholipid dependent phosphotransferases, is present in different tissues and cells (26) . Phorbol ester (27) and several hormones, such as TRH (12) and gonadotropin releasing hormone (28), cause rapid translocation and activation of protein kinase C from cytosol to the plasma membrane. We have shown that the Y-79 cell contains protein kinase C activity in the cytosol (Fig. 4A) and membrane (Fig. 4B) fractions. We have further demonstrated translocation of protein kinase C from the cytosol to membrane in a temporal fashion similar to the time dependent inactivation of pyroglutamyl peptidase II.

In order to elucidate the mechanism involved in the rapid inactivation of pyroglutamyl peptidase II, polyclonal antibodies of this enzyme were used. We have previously purified pyroglutamyl peptidase II from rabbit brain (8). and raised polyclonal antibodies to this enzyme in the guinea pig. The specificity of the antibodies have been checked with purified pyroglutamyl peptidase II. Although not anticatalytic, a 1 :1000 dilution of the antiserum detects the native enzyme on a Western blot and recognizes a 48 kDa subunit on SDS-PAGE gels. We have shown that the antiserum also recognizes a 48 kDa protein in Y-79 membranes but not in cytosol (Fig. 6). Diminished amounts of the 48 kDa protein were found in two cell lines having markedly lower levels of pyroglutamyl peptidase II activity than the Y-79 cells (14).

The multifunctional nature of protein kinase C is expressed by its role in both positive and negative regulation of biological responses. A large body of evidence demonstrates that protein kinase C provides negative feedback control over various steps of cellular responses. For example, protein kinase C inhibits Ca^{+2} mobilization by blocking the receptor-mediated

hydrolysis of inositol phospholipid (29), decreases muscarinic acetylcholine receptors by rapid internalization and subsequent degradation (30), inhibits ligand-mediated tyrosine kinase activity of EGF and insulin receptors (31,32), and inhibits Na+-K+-ATPase activity in renal proximal tubules leading to a decrease in sodium ion transport (33). It was recently reported (34) that neutral endopeptidase 24.11, (EC 3.4.24.11) an integral membrane protein in human neurotrophils, is rapidly inactivated by phorbol ester-mediated internalization and proteolytic degradation. We determined whether the rapid decrease in pyroglutamyl peptidase II activity was caused by TPA mediated dissociation and/or internalization of this enzyme molecule from the plasma membrane. The results in Fig. 8 show that the rapid inactivation of this enzyme is not due to dissociation and/or internalization of pyroglutamyl peptidase II from the membrane. Inactivation of pyroglutamyl peptidase II is likely due to phosphorylation (Fig.9). Phosphorylation of the 48 kDa subunit was time dependent, evident after 5 min exposure to TPA, and reached a maximum at 30 min.

In summary, pyroglutamyl peptidase II, a specific TRH degrading enzyme, was rapidly inactivated by TPA, a protein kinase C activator, in Y-79 retinoblastoma cells. The inactivation of this enzyme was time and concentration dependent, not due to dissociation and/or internalization, but rather to phosphorylation. Further experiments will be required to determine whether TRH can rapidly down-regulate pyroglutamyl peptidase II by a similar mechanism to potentiate its cellular response. In this respect it is of interest to note that administration of TPA to mice has been shown to augment the hypoglycemic effect of TRH (35)

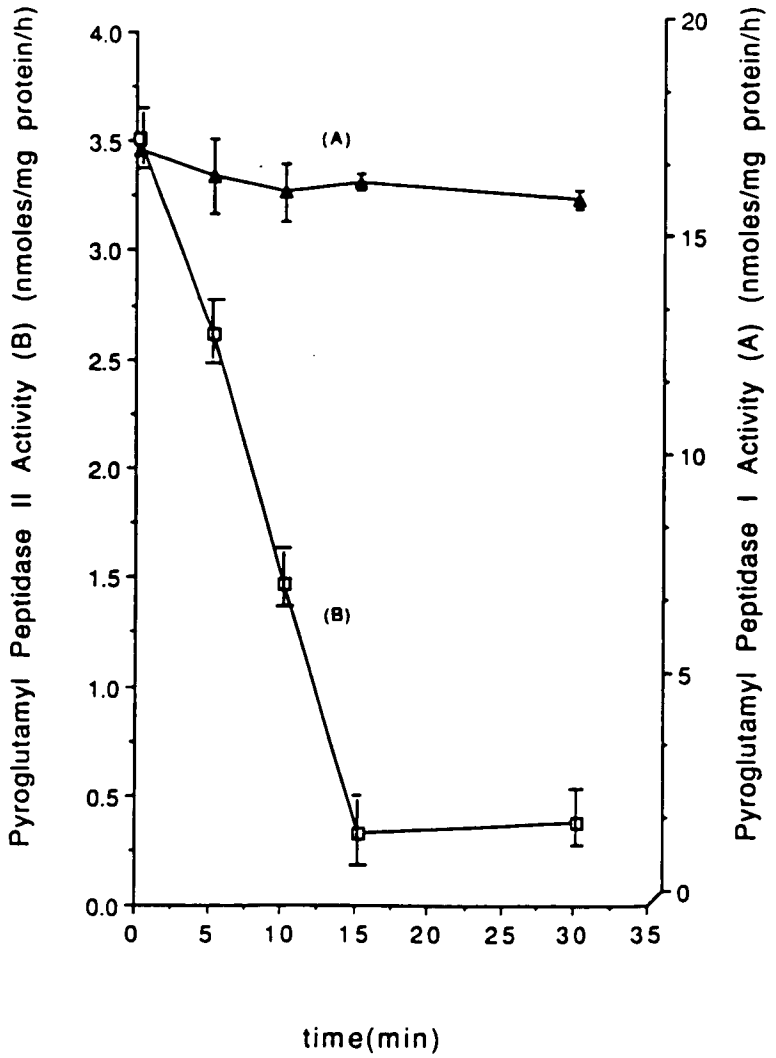

Fig. 1. Activities of pyroglutamyl peptidase I (A) and pyroglutamyl peptidase II (B) in Y-79 cells as a function of time of exposure to phorbol ester: Cells were treated with TPA ($1.6X10^{-7}M$) for various times. The cells were collected and the activities of pyroglutamyl peptidase I and II were measured separately as described in methods. Each point represents the mean +/- SEM of five determinations.

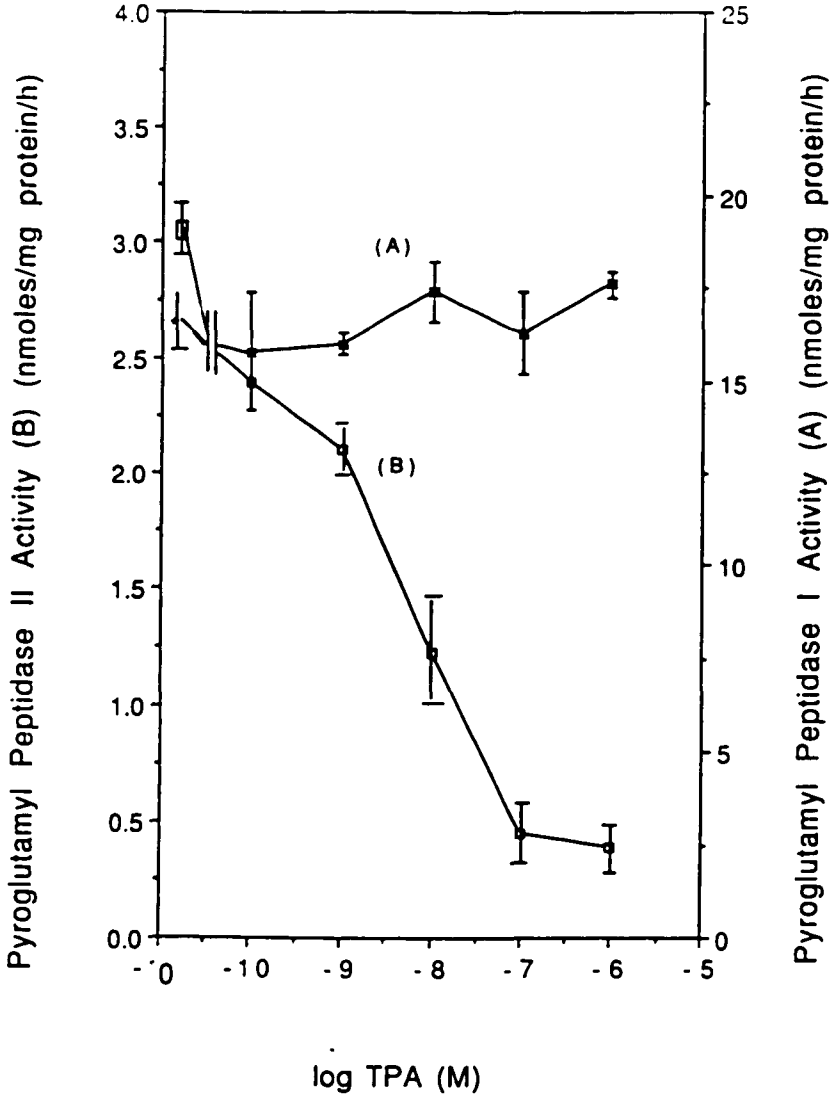

Fig. 2. Dose-response effects of TPA on the specific activities of pyroglutamyl peptidase I and II in Y-79 cells: Cells were treated with TPA (concentrations from 10^{-10}M to 10^{-6}M) for 15 min. Both enzymes were then measured as described in methods. Each point represents the mean +/- SEM of five determinations.

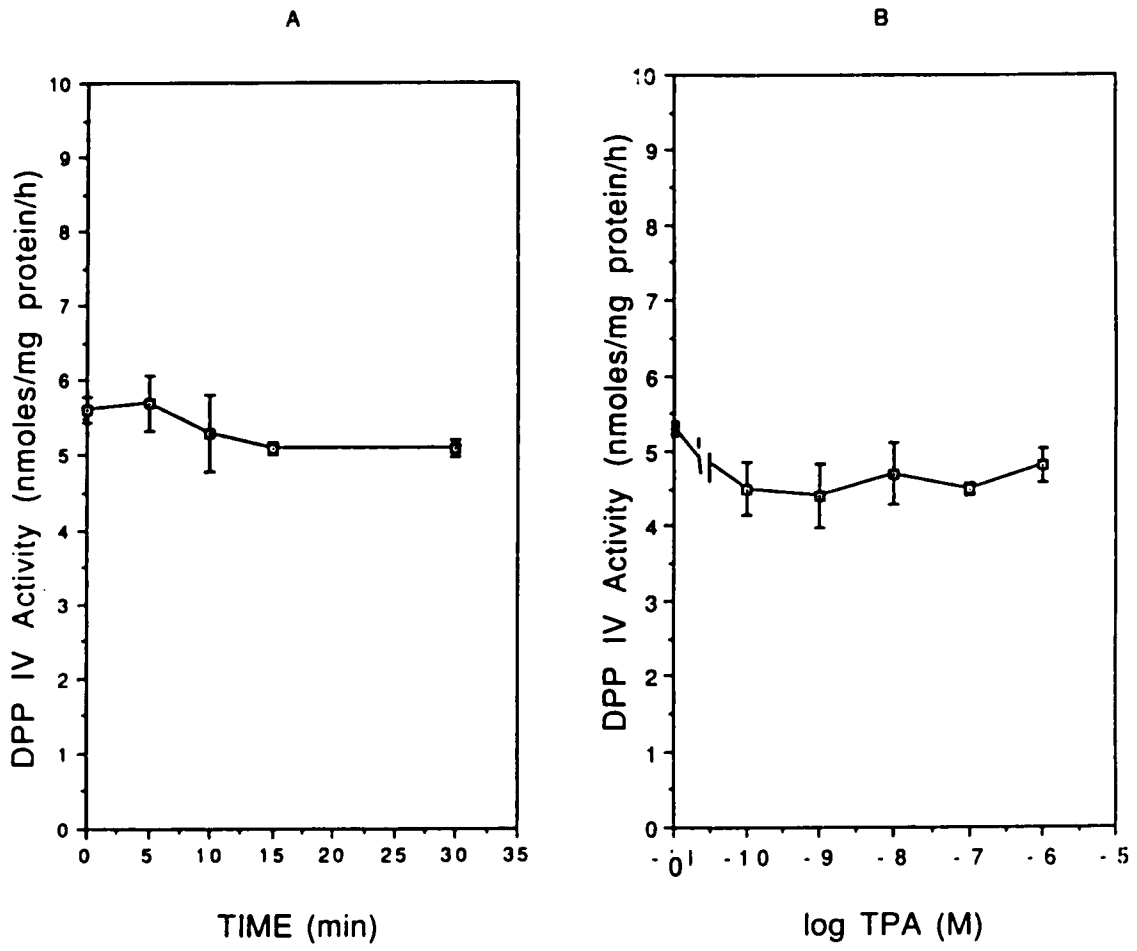

Fig. 3. Enzymatic activity of dipeptidyl peptidase IV (DPP IV) following TPA treatment in Y-79 cells: Cells were treated with either 1.6X10-7M TPA for different times (A) or various concentrations of TPA for 15 min (b). The membrane fractions were collected and DPP IV activity was measured as described in methods. Each point represents the mean +/- SEM of five determinations.

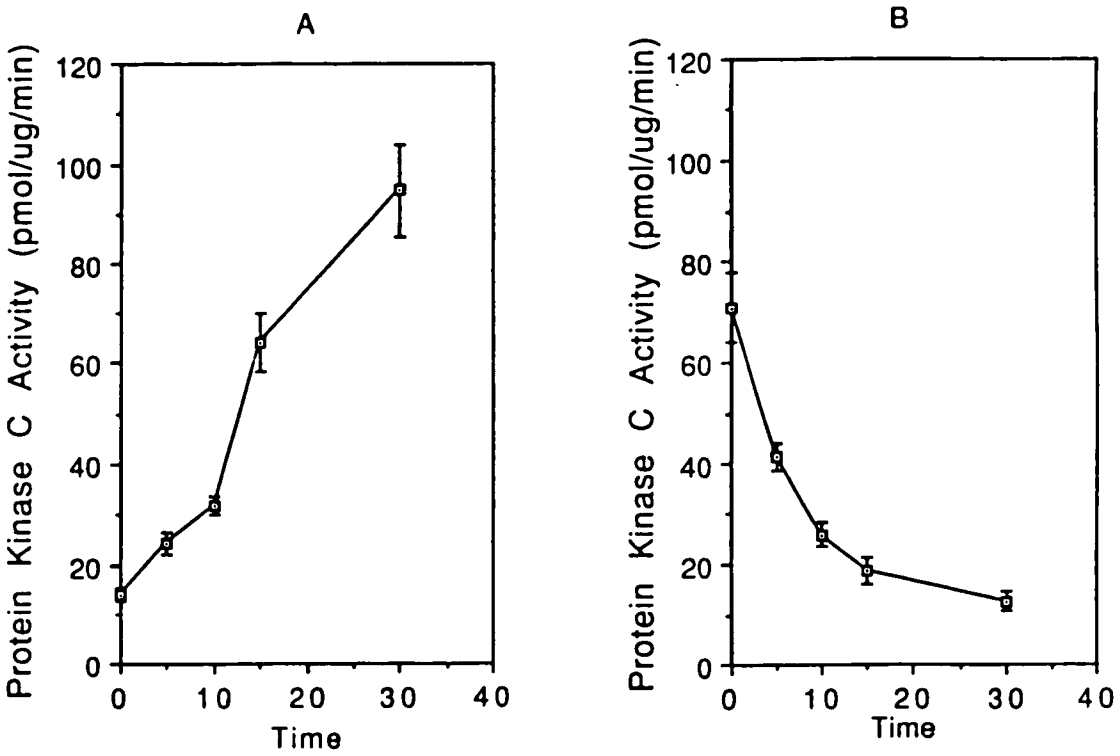

Fig. 4. Translocation of protein kinase C activity from cytosol to membrane fractions of Y-79 cells following exposure to TPA: Cells were treated with $1.6X10^{-7}$ M TPA for 5,10,15, and 30 min. Cytosol and membrane fractions were separated, partially purified by DE-52 chromatography, and protein kinase C activity was measured as described in methods. Each point represents the mean +/- SEM of three determinations.

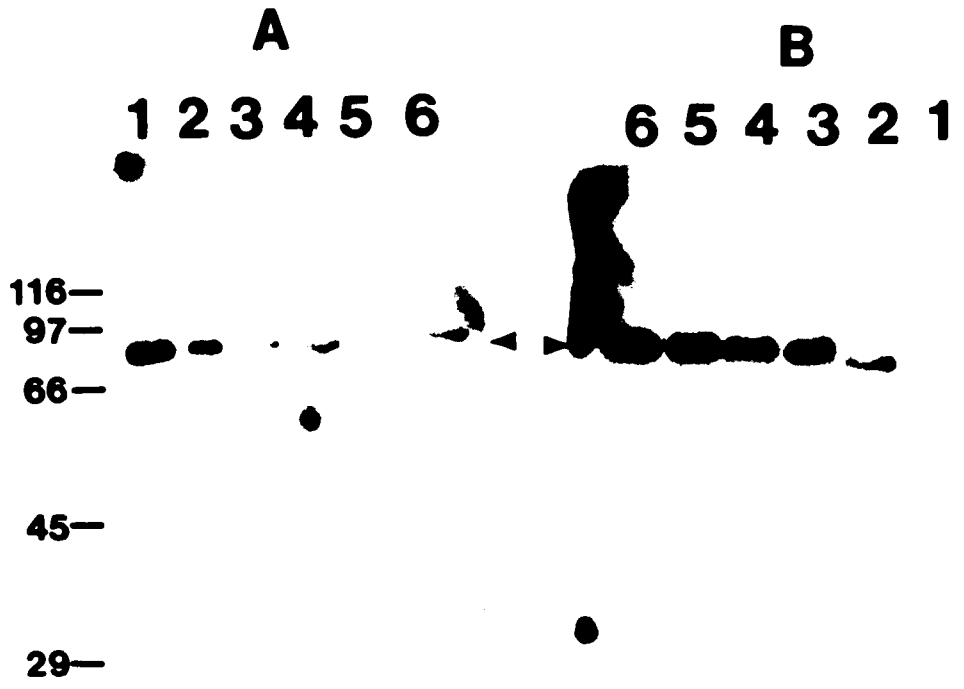

Fig. 5. Immunoblot measurement of protein kinase C translocation in Y-79 cells following exposure to TPA: After treatment of the cells with $1.6X10^{-7}M$ TPA for various times, Cytosol (A) and membrane (B) proteins were separated on 10% SDS-PAGE gels. The proteins were transferred to Immobilon membranes and monoclonal antibody to protein kinase C was added. Immunoreactive protein kinase C was visualized by autoradiography after adding [^{125}I]-protein A. Lane 1: control, lanes 2 to 6 : 2.5 min, 5 min, 10 min, 15 min, 30 min respectively.

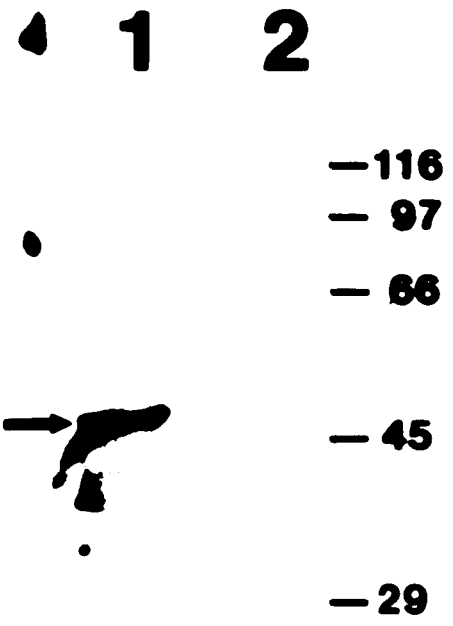

Fig. 6. Specificity of the polyclonal antibody to pyroglutamyl peptidase II: Solubilized particulate fraction proteins from Y-79 cells were separated on 10% SDS-PAGE gels and transferred to Immobilon membranes. Immune serum to pyroglutamyl peptidase II (lane 1) or preimmune serum (lane 2) were added and membranes incubated overnight. Immunoreactive proteins were visualized by autoradiography following addition of [^{125}I]-protein A.

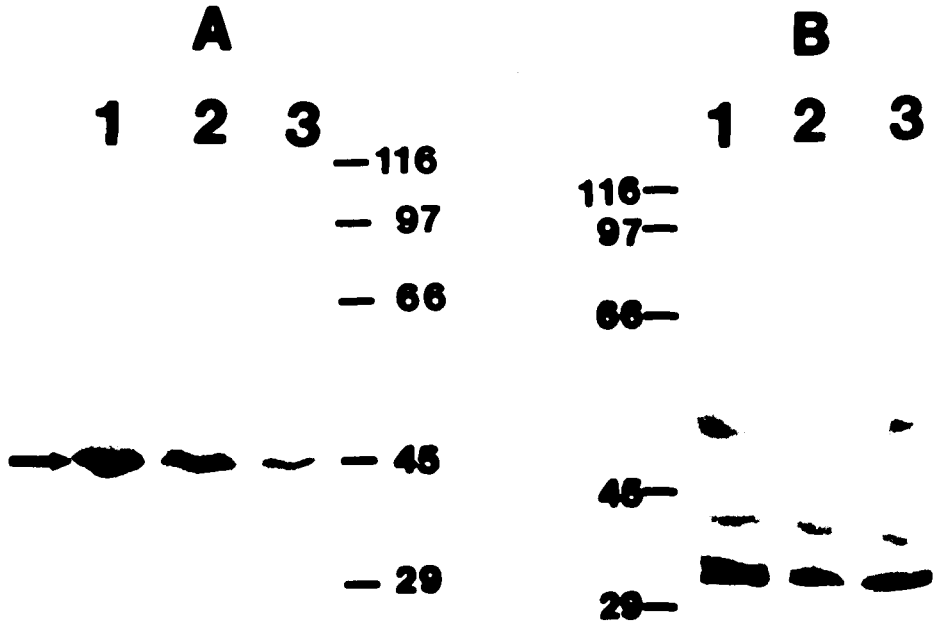

Fig. 7. Immunoblot detection of pyroglutamyl peptidase II in three different cell lines: Membrane (A) and cytosol (B) proteins of three different cell lines were separated on 10% SDS-PAGE gels and transferred to Immobilon membranes. Immune serum to pyroglutamyl peptidase II was added and membranes incubated overnight. Immunoreactive proteins were visualized by autoradiography following addition of [^{125}I]-protein A. Lane 1: Y-79 cells, Lane 2: GH_3 cells, Lane 3: AtT20 cells.

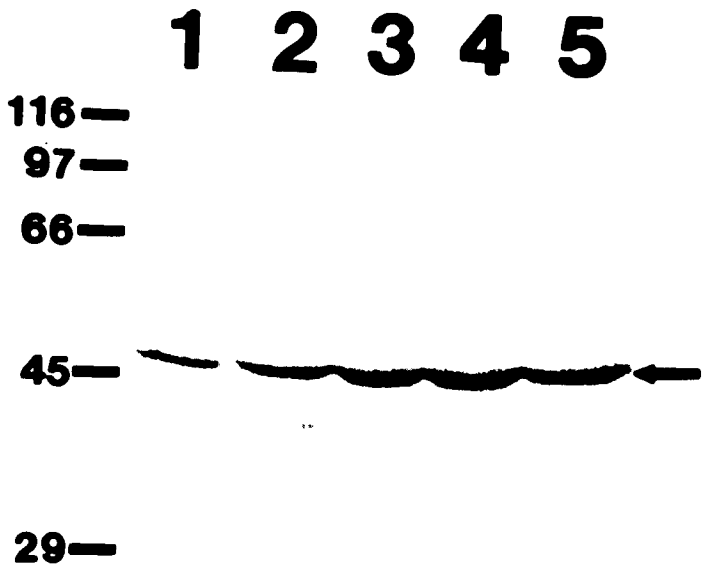

Fig. 8. Quantitative analysis of pyroglutamyl peptidase II in Y-79 cells as a function of time of exposure to TPA : After treatment of cells with $1.6X10^{-7}M$ TPA, the solubilized membrane proteins were subjected to electrophoresis. Proteins were then transferred to Immobilon membranes, immune serum added and membranes incubated overnight. Pyroglutamyl peptidase II was visualized by autoradiography following addition of [^{125}I]-protein A. Lane 1: control, Lane 2 to 5 : 5 min, 10 min, 15 min, 30 min. min.

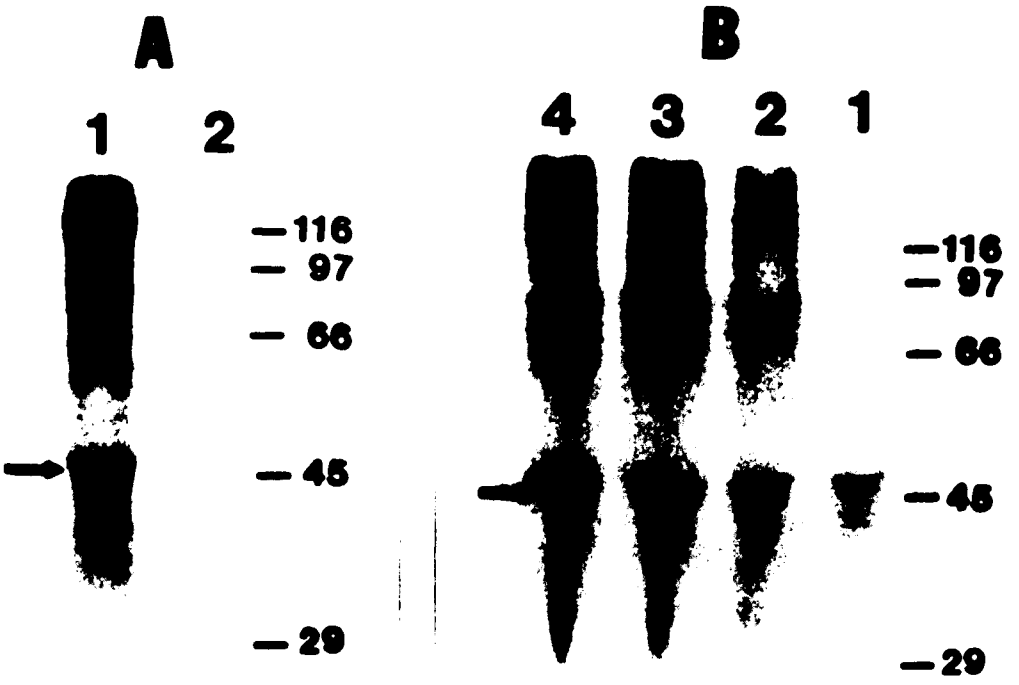

Fig. 9. Phosphorylation and immunoprecipitation of pyroglutamyl peptidase II in membrane fractions of Y-79 cells: Cells were treated with $1.6X10^{-7}M$ TPA for various times. The membrane proteins were separated from the cytosol, r-[^{32}P] ATP was added and samples were incubated at 30°C for 5 min. Immunocomplexes were precipitated by adding ploYclonal antiserum to pyroglutamyl peptidase II and protein A-Sepharose and were analyzed on 10% SDS-PAGE gels. (A) Cells treated with TPA for 15 min. Lane 1: immune serum, Lane 2: preimmune serum. (B) time course of phosphorylation. Lane 1 : control, Lane 2 to 4 : 5 min, 15 min, 30 min respectively.

Table 1:

The Effect of Phorbol Ester, Phorbol Ester Analogs and Protein Kinase C Inhibitors on the Enzymatic Activities of Pyroglutamyl peptidases I and II and Dipeptidyl Peptidase IV :

Enzymatic Activity (nmoles/mg protein/h) +/- SEM

Additions*	Pyroglutamyl Peptidase I.	Pyroglutamyl Peptidase II.	Dipeptidyl Peptidase IV.
1) Control	15.7 +/- 0.3 (3)	3.2 +/- 0.3 (3)	5.3 +/- 0.3 (3)
2) TPA	15.0 +/- 0.6 (3)	0.5 +/- 0.3 (3)[a]	5.8 +/-0.3(3)
3) Phorbol-12,13 -Dibutyrate	14.8 +/- 0.8 (3)	1.8 +/- 0.4 (3)[a]	5.1 +/- 0.2 (3)
4) 4-alpha-TPA	13.2 +/- 0.7 (3)	3.1 +/- 0.3 (3)	5.6 +/- 0.5 (3)
5) H-7	14.4 +/- 0.9 (3)	3.1 +/- 0.7 (3)	6.1 +/- 0.8 (3)
6) Sphingosine	16.7 +/- 1.5 (3)	2.9 +/- 0.6 (3)	5.9 +/- 0.7 (3)
7) TPA + H-7	14.9 +/- 1.4 (3)	3.0 +/- 0.4 (3)[b]	6.1 +/- 0.5 (3)
8) TPA + Sphingosine	14.6 +/- 1.5 (3)	3.0 +/- 0.4 (3)[b]	5.2 +/- 0.6 (3)

* TPA ($1.6X10^{-7}M$), Phorbol-12,13-dibutyrate ($1.6X10^{-7}M$), 4-alpha-TPA ($1.6X10^{-7}M$), H-7 ($1.0X10^{-5}M$), Sphingosine ($1.0X10^{-5}M$). Each reaction was carried out in duplicate. Numbers in parentheses indicate number of separate experiments. [a]$P<0.01$; compared to control [b]$P<0.05$ compared to TPA.

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Chapter 6

Submitted to Endocrinology

Inhibition of the Synthesis of Pyroglutamyl Peptidase II, A TRH Degrading Enzyme, by Phorbol Ester in Human Retinoblastoma Cells.

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SUMMARY :

Pyroglutamyl peptidase II (EC 3.4.19.-), a highly specific membrane-bound TRH degrading enzyme, in Y-79 cells is inactivated by TPA (12-0-tetradecanoyl phorbol-13-acetate) in a biphasic manner. A rapid decrease of pyroglutamyl peptidase II activity occurs within 30 min and then returns to 70% of control level after 60 min. We have previously shown that the rapid inactivation of pyroglutamyl peptidase II results from its phosphorylation by TPA activated pretein kinase C. A time- and concentration-dependent delayed inactivation of pyroglutamyl peptidase II is seen after longer exposure of TPA to cells (IC_{50} of approximately 10^{-8}M). The specificity of this inactivation was studied by measuring two other enzymes, pyroglutamyl peptidase I and dipeptidyl peptidase IV. Both enzyme activities were not altered by longer exposure to TPA. Pretreatment of the cells with H-7, a protein kinase C inhibitor, prevented the TPA mediated inactivation of pyroglutamyl peptidase II. Immunoblot analysis showed that pyroglutamyl peptidase II in the cell membrane decreased with increasing time of incubation with TPA. To further explore this phenomenon, Y-79 cells were labelled with ^{35}S-methionine and immunoprecipitated. There was a parallel time course of decreasing ^{35}S-methionine incorporated into pyroglutamyl peptidase II and a decreasing enzyme activity. These studies demonstrate that the late phase of inactivation of pyroglutamyl peptidase II is caused by TPA-mediated inhibition of de novo synthesis of this enzyme.

INTRODUCTION :

Thyrotropin-releasing hormone (TRH, pGlu-His-Pro-NH_2) is rapidly degraded in plasma and tissues(1). The cleavage of the pGlu-His bond is catalyzed by two pyroglutamyl peptidases (2), and both enzymes are subject to regulation. It has been previously reported that pyroglutamyl peptidase I, a widely distributed cytosolic cysteine proteinase which cleaves all pGlu-amino acid bonds except the pGlu-Pro bond, is regulated by thyroid hormone(3) and sodium butyrate(4) in GH_3 cells. Pyroglutamyl peptidase II, a highly specific membrane-bound TRH degrading enzyme, cleaves only pGlu-His tripeptides(5). The highest activity of this enzyme was found in brain(6) and retina(7). Several groups reported that pyroglutamyl peptidase II activity in rat pituitary gland and frontal cortex is elevated acutely after the administration of T_3 in vivo (8,9,10). Tumor-promoting phorbo esters, such as 12-0-tetradecanoyl-phorbol-13-acetate (TPA), directly activate the Ca^{+2} and phospholipid dependent protein kinase (protein kinase C). This in turn elicits a variety of biological actions in a manner very similar to the effect of hormones(11). Phorbol esters, which were shown to activate protein kinase C directly in GH_3 cells, produced TRH-like effects (12). Recently we found that exposure of human retinoblastoma Y-79 cells to phorbol ester (TPA) led to a rapid and marked decrease in pyroglutamyl peptidase II activity. The initial and maximal inactivation of this enzyme occurred after 15 min and returned to 70% of control level by 60 min. We futher demonstrated that inactivation of pyroglutamyl peptidase II was due to activated protein kinase C-mediated phosphorylation of a 48 kD_a subunit of this enzyme. In the studies reported here, prolonged exposure to TPA caused a second phase of inactivation of pyroglutamyl peptidase II. Experiments with ^{35}S-methionine labeling of proteins showed that this second phase of inactivation was due to an inhibition of de novo synthesis of this enzyme.

MATERIALS and METHODS :

TPA (12-0-tetradecanoyl-phorbol-13-acetate), Phorbol 12,13, dibutyrate,4-alpha-TPA , H-7 (1-(5-isoquinolinesulfonyl)-2-methyl piperazine dihydrochloride), affinity purified rabbit antiguinea pig antibody, and protein A-Sepharose were obtained from Sigma Chemical Co. (St.Louis, MO). Polyclonal antibodies to purified rabbit brain pyroglutamyl peptidase II were raised in guinea pigs. ^{125}I-protein A was obtained from Amersham Corp (Arlington Heights. IL). Immobilon membranes were obtained from Millipore (Bedford,MA). ^{35}S-methionine (specific activity 1156 Ci/mmole) was from ICN (Irvine CA). Cell culture media and sera were purchased from GIBCO (Grand Island. NY). pGlu-NA and pGlu-His-Pro-NA were obtained from Bachem Bioscience INC (Philadephia, PA). Gly-Pro-NA, Pyroglutamyl diazomethyl ketone (PDMK) and Z-Pro-Prolinal were synthesized by this laboratory. Dipeptidyl peptidase IV (EC.3.4.15.5) was purified to apparent homogeneity from rabbit kidney. Y-79 human retinoblastoma cells, purchased from American Type Cell Collection (ATCC), were cultured in RPMI medium containing 15% heat inactivated fetal bovine serum. They were grown in an incubator at 37^{0} C with 5% $C0_2$ in humidified air as the gas phase. The culture medium was changed twice weekly.

Measurement of Activities of Pyroglutamyl Peptidase I and II and Dipeptidyl Peptidase IV :

The activities of the three peptidases were measured as previously described (6,13). In all cases the release of 2-naphthylamine from substrates was quantitated by the diazotiaztion procedure of Bratton and Marshall(14) as modified by Goldbarg and Rutenberg (15). The absorbance of the chromogen was measured in a spectrophotometer at 580 nm. a) pyroglutamyl peptidase I was determined with the substrate pGlu-NA. The incubation mixture contained 10 mM pGlu-NA, 20 mM EDTA, 50 mM Tris-HCl buffer (pH=7.5) and 50 ul of cell homogenate in a final volume of 250 ul. Tubes were incubated at 37^{0} C for 60 min. b) pyroglutamyl peptidase II was determined with the substrate pGlu-His-Pro-NA in a

coupled assay with excess dipeptidyl peptidase IV as described (6). The incubation mixture contained Z-Pro-Prolinal and pyroglutamyl diazomethyl ketone (10-5 M final concentration of each, added to inhibit prolyl endopeptidase and pyroglutamyl peptidase I respectively), 50 mM Tris-HCl pH=7.5, dipeptidyl peptidase IV (2 ug protein), 10 ul 10mM pGlu-His-Pro-NA in dimethyl sulfoxide and 50 ul membrane homogenate in a final volume of 250 ul. Incubations were conducted for 60 min at 37^0 C. c) dipeptidyl peptidase IV was measured with Gly-Pro-NA as substrate. The incubation mixture contained 10 ul 10 mM substrate in dimethyl sulfoxide, 50 mM Tris-HCl pH=7.3 and 50 ul membrane homogenate in a final volume of 250 ul. Tubes were incubated at 37^0 C for 30 min. Specific activity was expressed as nanomoles of amine released per mg protein/h. Protein concentration was measured by the method of Lowry et al (16).

Immunoblot Analysis of Pyroglutamyl Peptidase II :

After treatment of the Y-79 cells with 1.6 X 10^{-7}M TPA for various times, the reaction was stopped by rapid centrifugation and the cells were washed twice with cold PBS. The cell pellets were resuspended in 250 ul of buffer A (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 25/ml leupeptin), homogenized and centrifuged at 4^0 C for 15 min at 16,000g. The supernatant was discarded, and membrane proteins extracted from particulate fraction by incubating for 60 min at 4^0 C with 100 ul of buffer A containing 1% NP-40. The solubilized membrane proteins were separated by electrophoresis on 10% SDS polyacrylamide gels. The proteins were then electrophoretically transferred to an Immobilon membrane. After electroblotting, the membranes were incubated overnight in TBS Buffer (20 mM Tris-HCl pH 7.4, 500 mM NaCl) containing 5% BSA to block nonspecific binding. Guinea pig polyclonal antibody to pyroglutamyl peptidase II (1:500 dilution) was added and membranes incubated overnight at 4 ^{0}C. The membranes were then incubated with rabbit antiguinea pig antibody for an additional hour. The immunoreactive bands were visualized by adding ^{125}I protein A (0.5 uCi/ml) in TBS containing 5% BSA and incubating for 1 h. The membranes were washed extensively several times with TTBS (TBS buffer containing 0.05% Tween-20) between each addition of antibodies and then air-dried. The dried membranes were then subjected to autoradiography.

Biosynthetic Labeling of Proteins and Immunoprecipitation of Pyroglutamyl Peptidase II :

Y-79 cells were incubated with TPA for defined times. After incubation, cells were washed twice with methionine-free RPMI medium within 30 min (starvation) and then 200 uCi ^{35}S- methionine was added to label the proteins for 30 min. Cells were rapidly spun down and washed twice with homogenizing buffer (20 mM Tris-HCl, pH 7.5, containing 2 mM PMSF and 25 ug/ml leupeptin). The cell pellets were resuspended in 250 ul of the same buffer and homogenized. Particulate fractions were separated from cytosolic fractions by centrifugation at 16.000g for 10 min. The pellets were resuspended in immunoprecipitation buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.5% SDS, 2 mM PMSF and 25 ug/ml leupeptin pH 7.5) and then 100 ul of guinea pig anti pyroglutamyl peptidase II antibodies were added, and tubes incubated overnight. Immunoprecipitates were formed by adding 100 ul of 10% protein A-Sepharose and then spun down at 16.000g for 2 min. Immunocomplexes were washed several times with immunoprecipitation buffer and washed twice in a solution of 20 mM Tris-HCl, pH 7.5 containing 150 mM NaCl and 0.05% NP-40. All steps were performed at 40 C. The pellets were resuspended in SDS sample buffer and boiled for 5 min. Samples were subjected to SDS polyacrylamide gel electrophoresis on 10% gels. The gels which contained the ^{35}S methionine-labeled proteins were dried and subjected to autoradiography.

RESULTS :

The enzymatic activity of pyroglutamyl peptidase II in Y-79 cells was inactivated by TPA, a phorbol ester, in a biphasic manner (Fig 1). We previously reported on the early rapid inhibition of pyroglutamyl peptidase II which occurred after 5 min and reached a maximum at 15 min with 10% enzyme activity remaining. The enzyme activity then returned to 70% of the control level at 60 min. Prolonged exposure to TPA ($1.6X10^{-7}M$) led to a second phase of inactivation of pyroglutamyl peptidase II. The enzyme activity declined slowly after 1 h exposure, with 15% cf the basal level remaining after 7 h (Fig 1). The activities of pyroglutamyl peptidase I, a cytosolic TRH degrading enzyme, and dipeptidyl peptidase IV, a membrane bound peptidase, were measured to determine whether the effect of prolonged exposure to TPA was specific. No significant difference in the activities of pyroglutmayl peptidase I and dipeptidyl peptidase IV after longer exposure with TPA were found (Fig 2). To determine the dose-response effect of TPA, Y-79 cells were incubated for 5h with various concentrations of phorbol ester, and pyroglutamyl peptidase II activity measured. Half-maximal inhibitory dose (IC_{50}) was about $10^{-8}M$ (Fig.3). To determine whether the TPA inactivated pyroglutamyl peptidase II was mediated by protein kinase C, cells were incubated with TPA analogs and/or the protein kinase C inhibitor, H-7, for 5h and then activities of pyroglutamyl peptidases I and II were measured. Phorbol-12,13-dibutyrate, a TPA analog, caused a similar inhibition as TPA did. On the contrary, 4 alpha-TPA , an inactive form of TPA , did not have any inhibitory effect on pyroglutamyl peptidase II (Table I). Incubation of the cells with H-7 and TPA simultaneously prevented the TPA mediated inhibition of pyroglutamyl peptidase II. H-7 alone did not change this enzyme activity (Table 1). To study the mechanism of inhibition of pyroglutamyl peptidase II after longer exposure of the cells to TPA, we quantitatively measured the amount of enzyme in the cell membrane by immunoblotting. Y-79 cells were incubated with $1.6X10^{-7}M$ TPA for various times, particulate fractions were separated, and membrane proteins were loaded onto 10% SDS-PAGE gels. After transferring the proteins to Immobilon membranes, polyclonal antibodies

to purified pyroglutamyl peptidase II were added. Immunoreactive bands were visualized by adding ^{125}I-protein A. The amount of the 48kD$_a$ protein, a subunit of pyroglutamyl peptidase II recognized by these polyclonal antibodies, decreased with increasing time of TPA incubation. To further explore this effect, the cells were labeled with ^{35}S-methionine and proteins immunoprecipitated with polyclonal antibodies to pyroglutamyl peptidase II to examine the de novo synthesis of pyroglutamyl peptidase II. The results revealed that the decrease in activity of pyroglutamyl peptidase II was due to decreased de novo synthesis of this enzyme. The immunoprecipitable 48 kD$_a$ band was quantitated by densitometry. Exposure to TPA caused a time-dependent decrease in pyroglutamyl peptidase II synthesis similar to the effect of TPA on pyroglutamyl peptidase II activity. Synthesis of pyroglutamyl peptidase II decreased to 50% of control by 3 h.

DISCUSSION :

Evidence is available that protein kinase C is a target for phorbol ester, since the tumor promoters, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) which has a diacylglycerol-like structure, directly activates this enzyme both in vitro and in vivo (11). Activation of protein kinase C delivers dual signals, which either stimulate or inhibit biological responses. In addition to a positive forward action, TPA also causes direct and/or indirect negative effects. For instance, (1) tumor necrosis factor (TNF) receptors are down-regulated by activators of protein kinase C (17) and this loss of TNF binding is concomitant with a drastic reduction of TNF cytotoxicity in an in vitro assay system (18). (2) Protein kinase C activators are potent inhibitors of heparin-binding growth-factor (HBGF)-stimulated endothelial cell growth (19) which is mediated by reduced HBGF receptors (20). (3) TPA markedly reduces glucagon-stimulated intracellular cyclic AMP concentration by inhibiting glucagon-stimulated adenylate cyclase activity (21). We report here that TPA causes a biphasic inactivation of pyroglutamyl peptidase II (Fig 1). We have previously shown that the acute inhibition of enzyme activity was caused by TPA activated protein kinase C in the cell membrane leading to a phosphorylation the 48 kD_a subunit of pyroglutamyl peptidase II (22). Recovery of pyroglutamyl peptidase II activity after 30 min is likely due to dephosphorylation. Longer exposure of Y-79 cells to TPA caused a second and slower decrease in pyroglutamyl peptidase II activity. The maximal inhibition of pyroglutamyl peptidase II occurred after 5 h incubation (Fig 1).Incubation of the cells with increasing concentrations of TPA caused a progressive decline in pyroglutamyl peptidase II activity (Fig.3) The results show that the TPA effect is time and concentration dependent. In order to examine the specificity of this effect, we measured two additional enzyme activities, dipeptidyl peptidase IV (DPP IV) and pyroglutamyl peptidase I. Exposure of the cells to 1.6 X 10^{-7}M TPA for 7 h did not change these two enzyme activities (Fig 2).

DISCUSSION :

Evidence is available that protein kinase C is a target for phorbol ester, since the tumor promoters, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) which has a diacylglycerol-like structure, directly activates this enzyme both in vitro and in vivo (11). Activation of protein kinase C delivers dual signals, which either stimulate or inhibit biological responses. In addition to a positive forward action, TPA also causes direct and/or indirect negative effects. For instance, (1) tumor necrosis factor (TNF) receptors are down-regulated by activators of protein kinase C (17) and this loss of TNF binding is concomitant with a drastic reduction of TNF cytotoxicity in an in vitro assay system (18). (2) Protein kinase C activators are potent inhibitors of heparin-binding growth-factor (HBGF)-stimulated endothelial cell growth (19) which is mediated by reduced HBGF receptors (20). (3) TPA markedly reduces glucagon-stimulated intracellular cyclic AMP concentration by inhibiting glucagon-stimulated adenylate cyclase activity (21). We report here that TPA causes a biphasic inactivation of pyroglutamyl peptidase II (Fig 1). We have previously shown that the acute inhibition of enzyme activity was caused by TPA activated protein kinase C in the cell membrane leading to a phosphorylation the 48 kD_a subunit of pyroglutamyl peptidase II (22). Recovery of pyroglutamyl peptidase II activity after 30 min is likely due to dephosphorylation. Longer exposure of Y-79 cells to TPA caused a second and slower decrease in pyroglutamyl peptidase II activity. The maximal inhibition of pyroglutamyl peptidase II occurred after 5 h incubation (Fig 1).Incubation of the cells with increasing concentrations of TPA caused a progressive decline in pyroglutamyl peptidase II activity (Fig.3) The results show that the TPA effect is time and concentration dependent. In order to examine the specificity of this effect, we measured two additional enzyme activities, dipeptidyl peptidase IV (DPP IV) and pyroglutamyl peptidase I. Exposure of the cells to 1.6 X 10^{-7}M TPA for 7 h did not change these two enzyme activities (Fig 2).

It has been described that bovine retinal rod outer segments have Ca^{+2} and phospholipid-dependent protein kinase C activity (23) and incubation of ^{32}P-labeled retinas with TPA resulted in the phosphorylation of several proteins (24). We previously demonstrated the presence of protein kinase C activity in Y-79 retinoblastoma cells and showed that TPA caused a rapid translocation of protein kinase C to the cell membrane (22). To study whether the slow decrease in pyroglutamyl peptidase II activity was protein kinase C mediated, we incubated cells with TPA, TPA analogs and/or the protein kinase C inhibitor, H-7, plus TPA. Pyroglutamyl peptidase I and II activities were measured. Table 1 shows that TPA and phorbol-12,13-dibutyrate cause a similar inhibition of pyroglutamyl peptidase II. However, 4-alpha TPA , an inactive form of phorbol ester, did not affect enzyme activity. H-7 prevented the inactivation of pyroglutamyl peptidase II activity by TPA. The enzyme activity of pyroglutamyl peptidase I did not change.

Liles reported that activation of protein kinase C by TPA induced rapid internalization and subsequent degradation of muscarinic acetylcholine receptors in neuroblastoma cells (25). Two different groups recently reported that TPA decreased both expression of neutral metallo-endopeptidase (NEP) mRNA transcripts and biosynthetically labeled immunoprecipitable NEP in rabbit synovial fibroblasts (26) and that phorbol 12-myristate-13-acetate (PMA) down-regulates enzyme activity and internalizes NEP in the neutrophils (27). There are several possibile mechanisms that may cause this late phase of inactivation of pyroglutamyl peptidase II activity, such as dissociation of pyroglutamyl peptidase II from the membrane either by internalization and degradation or by secretion to the medium, decreased pyroglutamyl peptidase II gene expression leading to decreased synthesis of new enzyme or enhanced enzyme degradation. Pyroglutamyl peptidase II activity was measured in both cytosol and medium. There was no detectable enzyme activity in either fraction. The amount of enzyme in the cell membrane was quantitated by immunoblotting. Immunoreactive pyroglutamyl peptidase II decreased with increasing time of incubation with TPA (Fig. 4). Since the cloned gene of pyroglutamyl peptidase II is currently unavailable to study the transcripitional regulation of this enzyme, ^{35}S-methionine was added to the cells to check whether new enzyme synthesis was decreased. We

measured the immunoprecipitable pyroglutamyl peptidase II band (48 kD_a) as relative optical density. Maximal inhibition of de novo synthesis of pyroglutamyl peptidase II occurred after the cells were incubated with TPA for 5 to 7 h (Fig. 5). The time course of decrease in enzyme activity paralleled the time course of decline in immunoblottable and immunoprecipitable pyroglutamyl peptidase II. These findings indicate that the decrease in pyroglutamyl peptidase II activity in the cells after long term exposure to TPA is mediated neither by internalization nor by degradation but rather by a decrease in new enzyme synthesis.The activity of pyroglutamyl peptidase II is therefore subject to multiple mechanisms of regulation. Thyroid hormone treatment in vivo induces new synthesis of this enzyme (10) whereas prolonged exposure to TPA in vitro decreases de novo enzyme synthesis. Brief exposure to TPA leads to a protein kinase C mediated phosphorylation and down regulation of this enzyme (22). These studies all suggest a key role for pyroglutamyl peptidase II in terminating the biological activity of TRH.

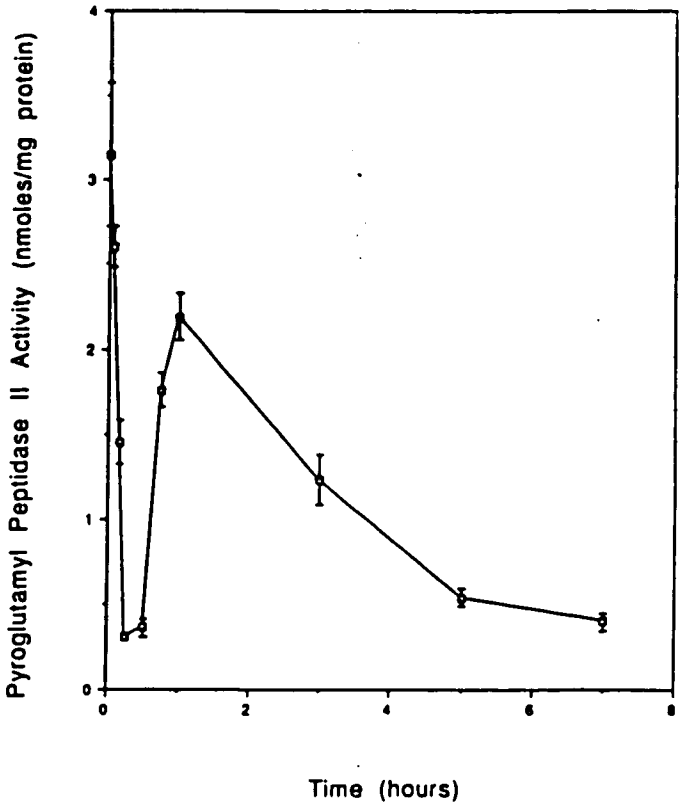

Fig 1. Time course of the effect of TPA on the pyroglutamyl peptidae II activity in Y-79 retinoblastoma cells : After treatment of the cells with TPA ($1.6X10^{-7}M$) for different times, the reactions were stopped and membrane fractions were collected. Pyroglutamyl peptidase II activity was measured as described in methods. Each point represents the mean +/- SEM of five determinations.

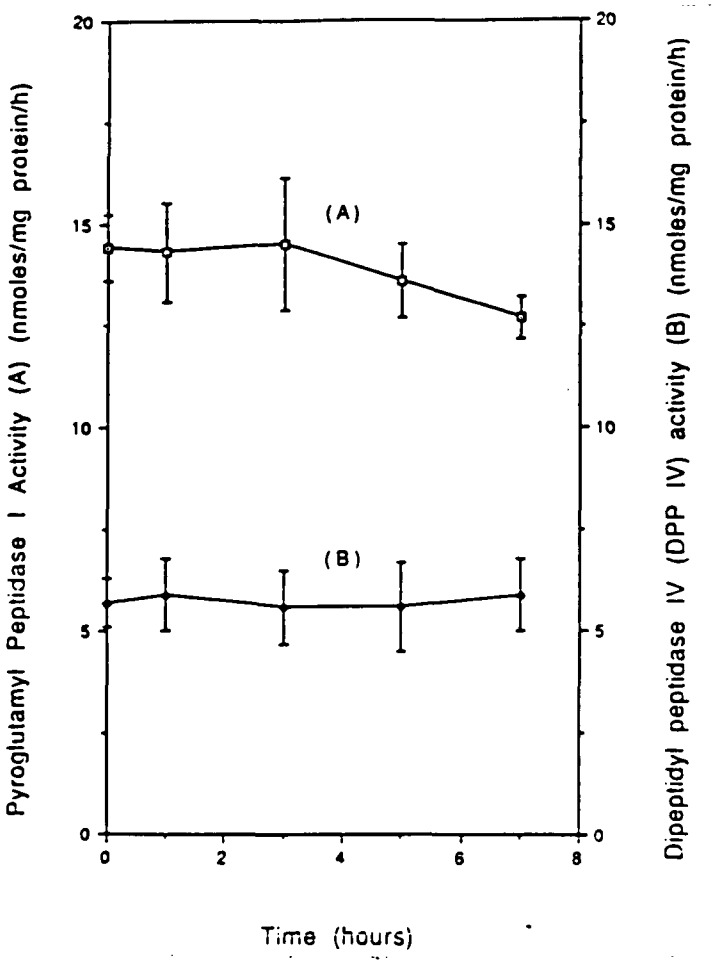

Fig 2. Enzymatic activities of pyroglutamyl peptidase I and dipeptidyl peptidase IV (DPP IV) following TPA treatment : Y-79 cells were treated with 1.6X10-7M TPA for various times. Pyroglutamyl peptidase I activity in the cytosol and dipeptidyl peptidase IV activity in the membrane fraction were measured as described in methods. Each point represents the mean +/- SEM of five determinations.

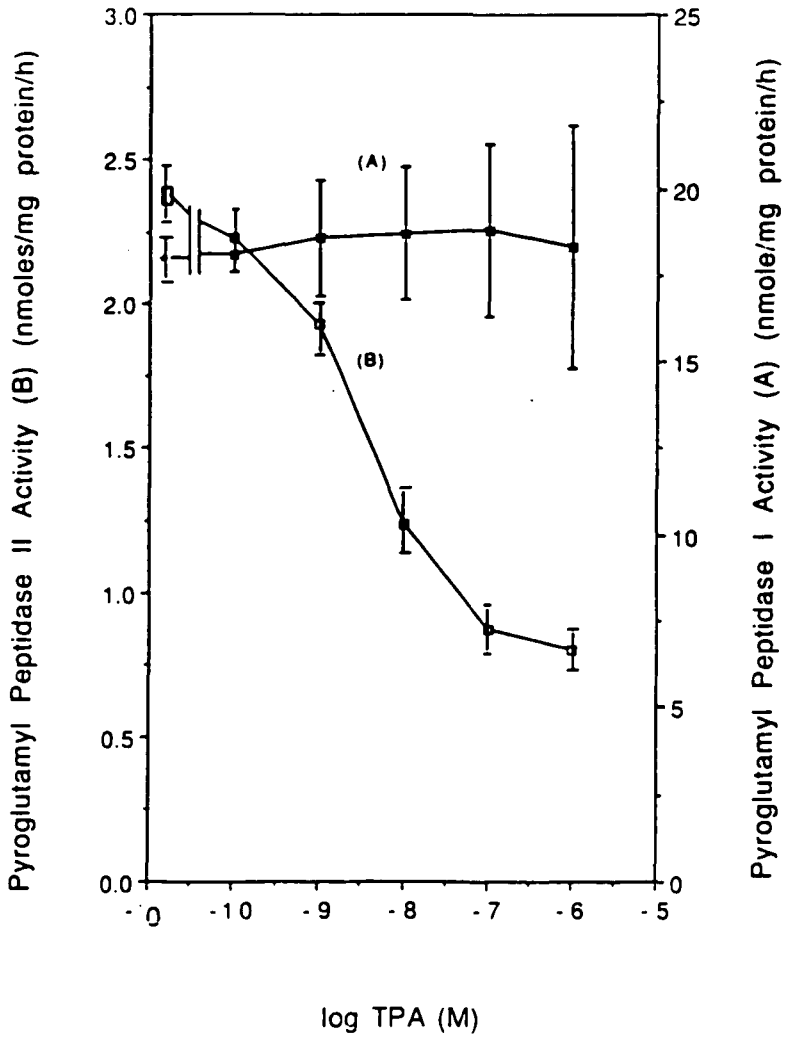

Fig 3. Concentration dependence of TPA effect on the pyroglutamyl peptidase I and II activities in Y-79 cells : Cells were treated with TPA (concentrations from10^{-10}M to 10^{-6}M) for 5 hours. Pyroglutamyl peptidase I (cytosol) and II (membrane fraction) activities were then measured. Each point represents the mean +/- SEM of five determinations.

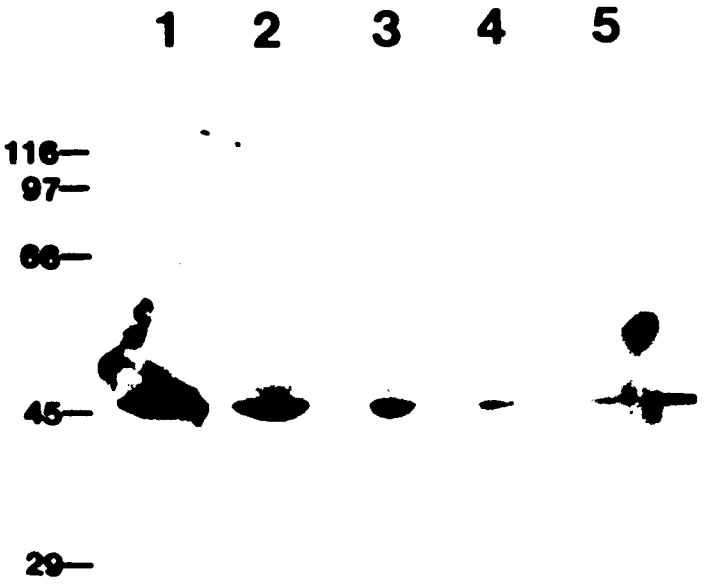

Fig 4. Immunoblot measurement of pyroglutamyl peptidase II in Y-79 cells following exposure to TPA : After treatment of the cells with $1.6X10^{-7}M$ TPA for various times, membrane proteins were collected and separated on 10% SDS-PAGE gels. The proteins were then transferred to Immobilon membranes and polyclonal antibody to pyroglutamyl peptidase II was added. Immunoreactive proteins were visualized by autoradiogrphy after adding [^{125}I]-protein A. lane 1: control. lane 2 to 5 : 1 h, 3 h, 5 h, 7 h respectively.

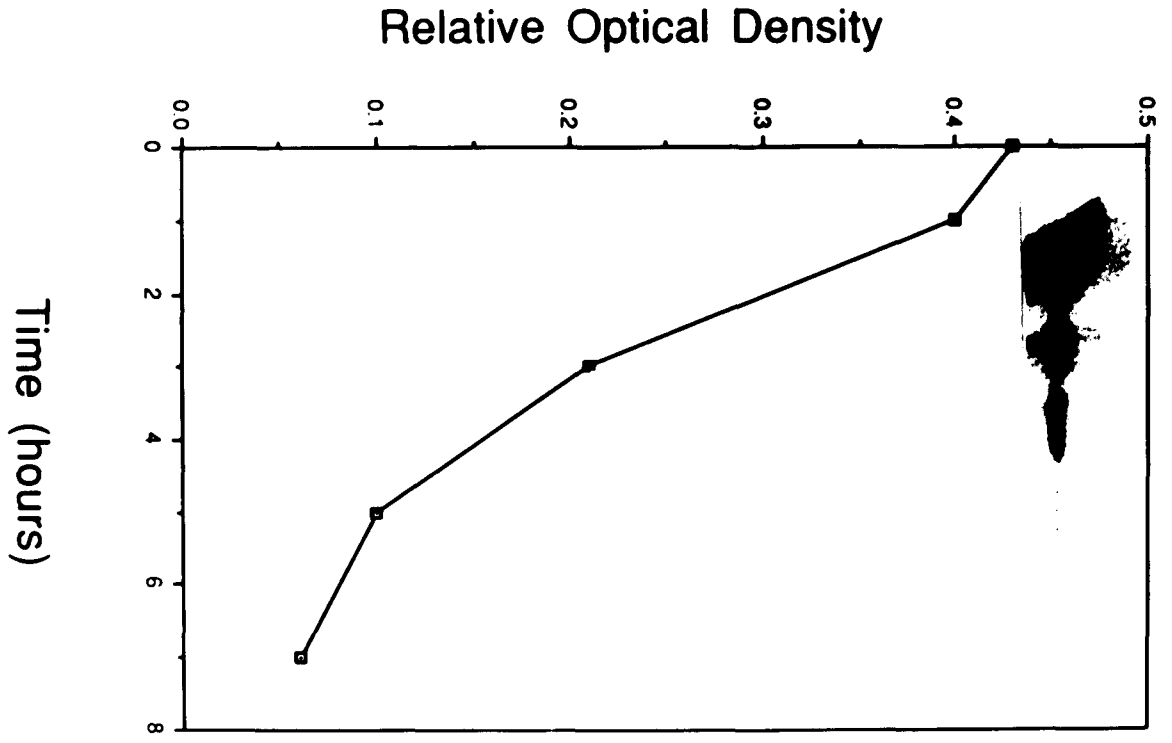

Fig 5. Immunoprecipitation of ^{35}S-methionine labeled pyroglutamyl peptidase II in Y-79 cells : Cells were incubated with 1.6X10^{-7}M TPA for different times, and then labeled with 200 uci ^{35}S-methionine for 30 min. Membrane fraction proteins were isolated and immunoprecipitated with polyclonal antibody to pyroglutamyl peptidase II as described in methods. Immunoprecipitable pyroglutamyl peptidase II bands were quantitated by densitometry.

Table 1:

The Effect of Phorbol Ester, Phorbol Ester Analogs and Protein Kinase C Inhibitor (H-7) on the Enzymatic Activities of Pyroglutamyl Peptidase I and II :

	Enzymatic Activity (nmoles/mg protein/h) +/- SEM	
	Pyroglutamyl Peptidase I.	Pyroglutamyl Peptidase II.
Additions*		
1) Control	17.0 +/- 0.9 (3)	2.6 +/- 0.2 (3)
2) TPA	16.5 +/- 1.8 (3)	0.7 +/- 0.3 (3)[a]
3) Phorbol-12, 13 -Dibutyrate	14.6 +/- 1.2 (3)	1.2 +/- 0.1 (3)[a]
4) 4-alpha-TPA	15.5 +/- 1.5 (3)	2.5 +/- 0.3 (3)
5) H-7	16.9 +/- 1.7 (3)	2.4 +/- 0.2 (3)
6) TPA + H-7	17.0 +/- 2.1 (3)	2.4 +/- 0.3 (3)

* TPA ($1.6X10^{-7}M$), Phorbol-12,13-Dibutyrate ($1.6X10^{-7}M$), 4-alpha-TPA ($1.6X10^{-7}M$), H-7 ($1.0X10^{-5}M$). Each incubation was carried out in duplicate. Numbers in parentheses indicate number of separate experiments. [a] $p < 0.05$; compared to control. Cells were incubated with these compounds for 5 h.

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DISCUSSION :

The termination of classical neurotransmitter action may occur by either extracellular metabolism or re-uptake followed by intracellular metabolism (63). In the cholinergic system, acetylcholine (ACh) is hydrolyzed by acetylcholinesterase, a membrane bound enzyme, at a turnover time of 150 microseconds, equivalent to hydrolyzing 5000 molecules of ACh per molecule of enzyme per second. In the adrenergic system, uptake experiments have indicated that in addition to the reuptake of 40 to 60% of released catecholamine by the presynaptic terminal, two enzymes, monoamine oxidase and catechol-0-methyltransferase (COMT), metabolize the catecholamine within adrenergic neurons and extraneuronally. Advances made in the study of protease degradation of neuropeptides over the past decade have indicated that termination of the biological action of neuropeptides appears to occur predominantly through extracellular metabolism, although the involvement of uptake mechanisms can not yet be rigorously excluded for neuropeptide inactivation (64,65). A large body of literature exists on the enzymatic hydrolysis of virtually all of the known neuropeptides by a variety of preparations, from crude homogenates to purified enzymes. Most studies have been largely biochemical in nature. Little work has been done to show that regulation of neuropeptide degrading enzymes can play a important role in controlling the biological action of neuropeptides. In this thesis project, TRH has been used as a model system to study the role of neuropeptide degrading enzymes in controlling the biological activity of neuropeptides.

As previously described, initial degradation of TRH is mediated by three different enzymes (42). Two cytosolic enzymes, pyroglutamyl peptidase I and prolyl endopeptidase, cleave a variety of different peptides in addition to TRH. Pyroglutamyl peptidase II, a membrane bound ectoenzyme, is a TRH-specific degrading enzyme. This laboratory previously reported that pyroglutamyl peptidase I and prolyl endopeptidase, and very low pyroglutamyl peptidase II activities are present in GH_3 cells (66). GH_3

cells, a rat pituitary tumor cell line synthesize and secrete prolactin in response to nanogram amounts of TRH. This cell line offers an excellent in vitro system for investigating the regulation of TRH degrading enzymes. Friedman. et al, have shown that exposure of GH_3 cells to the pyroglutamyl peptidase I inhibitor, 5-oxoprolinal for three days, followed by washing to remove inhibitor, produced an unexpected three fold increase in pyroglutamyl peptidase I activity, whereas prolyl endopeptidase activity was unaltered ($EC_{50}=10^{-7}$ M) (67). The increase in pyroglutamyl peptidase I activity was not blocked by cycloheximide, a protein synthesis inhibitor, indicating that increased enzyme activity may be due to an increase in the half-life of pyroglutamyl peptidase I. Exposure of cells to the prolyl endopeptidase inhibitor Z-Pro-Prolinal did not elevate activities of prolyl endopeptidase or pyroglutamyl peptidase I. Sodium butyrate, a short chain fatty acid, can exert a variety of effects in GH_3 cells, such as changing cell morphology, increasing both GH and PRL synthesis 2- to 4-fold, and decreasing specific [^{3}H]-TRH binding (68,69). We found that the specific activity of pyroglutamyl peptidase I increased upon exposure to sodium butyrate in a time- and concentration-dependent manner, whereas the specific activity of prolyl endopeptidase was unchanged. The maximal effect occurred at a concentration of 1 mM sodium butyrate and at 16 h after exposure. The sodium butyrate-induced increase in pyroglutamyl peptidase I activity was completely reversible. Cycloheximide totally blocked this stimulation, indicating that the increase was due to new protein synthesis. These studies suggest that the activity of pyroglutamyl peptidase I in GH_3 cells is under regulation by different mechanisms.

Analysis of actively secreting endocrine glands suggests that intracellular degradation of the hormonal polypeptide may be regulated and/or may contribute to the control of the amount of hormone secretion and synthesis. There are several possible mechanisms which have been proposed. First, the enzyme or enzymes catalyzing the degradation may be under regulation. In the parathyroid gland, a substantial proportion of newly synthesized parathyroid hormone (PTH) is degraded and the rate of PTH degradation decreases when circulating calcium levels drop below a set point. Evidence exists that cathepsin B, which cleaves the Ala^{36}-Leu^{37} bond of PTH may be principally responsible for intracellular PTH degradation (70,71). Second, regulation could occur by fusion of secretory

granules with lysosomes, as has been proposed to occur with prolactin in the anterior pituitary (72) and insulin in pancreatic islet beta cells (73). Third, peptide hormone can be continuously produced at a high level and degraded after production, with degradation being inhibited during stimulation of secretion. Thus, enzymatic degradation of peptide signals could be either increased or decreased in the peptide-secreting cell when functional demand is placed on that cell. Finally, the internalization of hormone-receptor complexes and the presence of degrading activity for a given peptide in the target cell appear to be of great potential significance in the regulation of peptide hormone actions. The ability of thyroid hormones to control the degradation of TRH by serum was first noted by Redding and Schally (74). Subsequent studies by White et al (59) and Bauer (60) have documented a decreased degradation of TRH by serum from hypothyroid rats, and an increased degradation of TRH by serum from hyperthyroid rats. The effect of thyroid hormone on the action of TRH degrading enzymes in GH_3 cells was considered. Exposure of the cells to T_3 led to a time- and dose-dependent increase in the pyroglutamyl peptidase I activity ($EC_{50}=5X10^{-10}M$), whereas the specific activity of prolyl endopeptidase was unaffected. The increase in pyroglutamyl peptidase I was not due to a decrease in the K_m for the substrate but rather to an increase in V_{max}. Experiments with cycloheximide indicated that the increase in V_{max} was due to induction of new enzyme synthesis. We have also found an elevation in the activity of pyroglutamyl peptidase I in a number of brain regions and in the pituitary following chronic administration of T_3. This effect was most marked in hypothalamus and pituitary where pyroglutamyl peptidase I activity was increased two to three fold. Because the substrate specificity of this enzyme is not restricted to TRH, changes in the activity of pyroglutamyl peptidase I are not necessarily related to TRH. Other pyroglutamyl peptides such as LHRH and neurotensin are also substrates of this enzyme. However, it is reasonable to postulate that the increased level of pyroglutamyl peptidase I in pituitary may contribute to the intracellular degradation of TRH molecules after the internalization of hormone-receptor complexes. It has been a long-standing controversy as to whether thyroid hormone negatively regulates TRH in the hypothalamus. Recently Segerson et al (75) have shown that thyroid hormone can play a role in controlling hypothalamic TRH . Their studies demonstate an increase in proTRH mRNA and immunoreactive TRH in the paraventricular nucleus of the hypothyroid

rat and a decrease both in proTRH mRNA and immunoreactive TRH after administration of T_3 for 3 weeks. We speculate that elevation of pyroglutamyl peptidase I activity in the hypothalamus following chronic treatment with T_3 increases the degradation of internalized TRH and is an important factor in controlling the amount of TRH molecules in the hypothalamus.

It is well established that there is inverse relationship between plasma concentrations of TSH and thyroid hormones. The mechanism of this phenomenon is partially due to inhibition of TSH secretion and synthesis by thyroid hormone. Thyroid hormones also exert a powerful negative feedback control over the pituitary response to TRH. At least part of this regulation occurs at the level of the TRH receptors. Thyroid hormones cause a slow decline in TRH receptor density in vivo (76). In parallel, the TRH response is diminished. This component of thyroid hormone feedback control requires 24-48 hours and is fully reversible. Because of the unique properties of pyroglutamyl peptidase II i.e. narrow substrate specificity and occurrence as an ectoenzyme, this enzyme can be a candidate for the study of the importance of neuropeptide degrading enzymes in the termination of neuropeptide actions. Several groups (77,78) have investigated whether an increase in TRH degradation could contribute as one of the mechanisms in thyroid hormone-mediated negative feedback control. Male Sprague Dawley rats received a single injection of T_3. TRH degrading enzyme activities in the anterior pituitary gland were measured after different time intervals. Only pyroglutamyl peptidase II activity, but not pyroglutamyl peptidase I or prolyl endopeptidase, was increased in a time-dependent manner. Activity was stimulated three fold after 24 hours. When the animals were treated with the mild goitrogenic agent propylthiouracil (PTU), a rapid loss of pyroglutamyl peptidase II activity was observed. We also found that acute treatment with T_3 produces a similar increase of pyroglutamyl peptidase II in pituitary and a 70% increase in the activity of this enzyme in frontal cortex. Schulz and Bauer recently reported that thyroid hormones regulate the extracellular degradation of TRH in GH_3 cells (79). These findings collectively indicate that the biological activity of TRH at the pituitary is terminated by pyroglutamyl peptidase II and that this increased enzymatic activity contributes to the negative feedback regulation of thyroid status by

thyroid hormones.

It is known that TRH stimulates phosphatidyl inositol turnover to produce inositol triphosphate (IP_3) and diacylglycerol (DG). The rapid formation of IP_3 causes a mobilization of intracellular Ca^{+2} which triggers a burst release of prolactin in GH_3 cells. TRH also produces a sustained formation of diacylglycerol (DG), which is the assumed endogenous activator of protein kinase C (PKC). Because pyroglutamyl peptidase II activity in the pituitary gland is elevated after administration of thyroid hormones, the possibility of regulation of pyroglutamyl peptidase II by TRH activated protein kinase C was considered. Because GH_3 cells do not contain easily measurable amounts of pyroglutamyl peptidase II, nine different neuroblastoma and endocrine cell lines were screened in order to identify a cell line expressing this specific TRH degrading enzyme for study. One cell line, the human retinoblastoma (Y-79) derived from a primitive neuroectodermal cell, contained relatively high amounts of this enzyme. We also observed that membranes of rabbit retina, a tissue known to contain relatively high levels of immunoreactive TRH (80) and of TRH receptors, exhibit pyroglutamyl peptidase II activity at a level exceeding all non-CNS tissues studied. Since Y-79 cells do not contain the TRH receptor, phorbol ester was used instead of TRH to directly stimulate protein kinase C. Exposure of Y-79 human retinoblastoma cells to 12-0-tetradecanoyl phorbol 13-acetate (TPA) caused a biphasic inactivation of pyroglutamyl peptidase II but did not affect pyroglutamyl peptidase I. The rapid and marked inactivation of pyroglutamyl peptidase II was evident as early as 5 min declining to 10% of control after 15 min treatment. The enzyme activity returned to 70% of the control at 1 h. A second phase of inactivation followed after longer exposure of the cells to TPA. In order to rule out the possibility that inactivation of this membrane-bound enzyme was not due to a nonspecific membrane effect produced by TPA, another membrane-bound peptidase, dipeptidyl peptidase IV (DPP IV) was examined. TPA tested at different concentrations and at different times of exposure did not significantly alter the activity of DPP IV. To determine whether the TPA effect was mediated by protein kinase C, pyroglutamyl peptidase II activity was measured after Y-79 cells were incubated with phorbol ester analogs and/or protein kinase C inhibitors. 4-

alpha-TPA, an inactive form of phorbol ester, did not change the activity of pyroglutamyl peptidase II in either of the phases. Pretreating the cells with the protein kinase C inhibitors, H-7 or sphingosine, abolished the TPA mediated inactivation of pyroglutamyl peptidase II. The two inhibitors by themselves did not affect this enzyme activity. Protein kinase C was also rapidly translocated from cytosol to membrane as determined by measuring the enzyme activity and by quantitatively immunoblotting PKC molecules. The mechanism of the biphasic inactivation of pyroglutamyl peptidase II was studied. Immunoblot analysis of pyroglutamyl peptidase II demonstrated that inactivation of this enzyme at the early phase was not due to dissociation and/or internalization of this enzyme molecule. Immunoprecipitation showed that a 48 kDa subunit of pyroglutamyl peptidase II, was phosphorylated in a time-dependent manner. Phosphorylation of this subunit increased with increasing time and reached a maximum after 30 min. Preincubation of the cells with H-7, a protein kinase C inhibitor, caused a reduction of the TPA-mediated phosphorylation of this subunit. These results demonstrated that rapid and marked inactivation of pyroglutamyl peptidase II is due to phosphorylation of this enzyme by TPA-activated protein kinase C. Because the immunoreactive subunit (48 kDa) of pyroglutamyl peptidase II in the membrane fractions was decreased after longer times of exposure to TPA, cells were biosynthetically labelled with 35S-methionine to measure new protein synthesis. The results showed a parallel time course of decreasing amounts of pyroglutamyl peptidase II as measured by immunoblotting and decreased 35S-methionine labelling of this enzyme as measured by immunoprecipitation. Decreased pyroglutamyl peptidase II activity after longer exposure of the cells to TPA resulted from decreasing new enzyme synthesis.

In order to support the idea that biological actions of neuropeptides are predominantly terminated by enzymatic hydrolysis, several criteria are considered; 1) neuropeptides are degraded by neuropeptide-specific enzymes, 2) These neuropeptide-degrading enzymes should be strategically located at either its target cells or its secreting cells and possess a high affinity towards their substrates, 3) specific inhibitors of neuropeptide-degrading enzymes should increase either the concentration or biological actions of neuropeptides by preventing their

degradation, 4) Potentation of the biological activity of neuropeptide analogues reflects their resistance against degradation rather than changes at the receptor level. In general, insufficient information is available to fulfill all these criteria. Some studies have shown that the effect of inhibitors of neuropeptide-degrading enzyme can modulate either the concentration or biological actions of neuropeptides. Mauborgne et al (81) have shown that inhibitors of endopeptidase-24.11, such as phosphoramidon, can protect substance P from degradation when released from slices of rat substantia nigra. Inhibitors of endopeptidase-24.11 and of angiotensin converting enzyme have also been shown to potentiate substance P induced bronchoconstriction (82). Faivre-Bauman et al reported that CBZ-Gly-Pro-diazomethyl ketone, which inhibits both pyroglutamyl peptidase I and prolyl endopeptidase, increased intracellular TRH levels in hypothalamic cell cultures (83). Molineaux et al demonstrated that coadministration of LH-RH and cFP-AAF-PAB, a specific active site-directed inhibitor of endopeptidase-24.15, resulted in levels of LH-RH in brain that were up to an order of magnitude greater than controls. (84). Recently, Charli et al, indicated that CPHNA, an inhibitor of pyroglutamyl peptidase II, enhances the basal and K+ stimulated recovery of TRH from brain slices (49). These reports suggest a role in vivo for these enzymes in regulating some physiological responses of neuropeptides.

Since almost all neuropeptides are rapidly inactivated in vitro by enzymatic hydrolysis, it has been postulated that these enzymes may serve a dynamic control function by regulating the amount of neuropeptides available at the receptor level. If this hypothesis is true, it should also be expected that a) the activities of the neuropeptide-degrading enzymes represent a limiting factor within the regulatory mechanisms, b) changes in enzyme activities correlate in time with physiological, neuroendocrine or behavioral events, c) the activity of neuropeptide-degrading enzymes is finally controlled by either feed-foreward- or feedback-regulatory mechanisms. For opioid peptides, [Leu]enkephalin and [Met]enkephalin, it is well accepted that endopeptidase-24.11 and aminopeptidase N are two principal enzymes which participate in the inactivation of opioid peptides and its physiological actions. Recently, two different groups have reported that phorbol ester down

regulates membrane-bound endopeptidase-24.11 by either decreasing expression of this enzyme's mRNA and thereby decreasing de novo synthesis of this enzyme(85), or by increasing internalization and proteolytic degradation (86) at different cells. To explore the biological significance of this regulation requires further experimental work. Like endopeptidase-24.11, pyroglutamyl peptidase II is an ectoenzyme. From the substrate specificity and the tissue distribution studies, pyroglutamyl peptidase II has been considered the first characterized neuropeptide-specific peptidase. We and other groups have shown that thyroid hormone stimulates pyroglutamyl peptidase II activity in the pituitary gland leading to an increased hydrolysis of TRH. Decreased TRH biological responses by T3-stimulated TRH degradation may contribute to the negative feedback control of thyroid status. In the Y-79 retinoblastoma cell, activation of protein kinase C by phorbol ester causes a rapid and marked inactivation of pyroglutamyl peptidase II. We speculate that TRH activated protein kinase C may provide a mechanism for potentiation of the action of TRH at its target sites. Bauer also documented that there is a sex difference in the enzymatic activity of pyroglutamyl peptidase II in adenohypophyseal membrane preparations. Compared with the enzyme activity from male rats, only 25% of the pyroglutamyl peptidase II activity was found in female rats. After ovariectomy, the enzyme activity increased 3-fold and rapidly decreased to basal levels when the ovariectomized rats were treated with estradiol benzoate. These reports provide evidence to support the hypothesis that regulation of neuropeptide-degrading enzymes play a crucial role in the control of neuropeptide actions.

In summary, regulation of TRH degrading enzymes currently provide the best examples of the importance of neuropeptide degrading enzymes in controlling the biological activities of neuropeptides. We have shown that 5-oxoprolinal, thyroid hormones and sodium butyrate can increase pyroglutamyl peptidase I activity in GH_3 cells by different mechanisms. In vivo studies also demonstrated a marked elevation of pyroglutamyl peptidase I activity in the pituitary gland and hypothalamus after chronic administration of thyroid hormones. This suggests that the levels of neuropeptides in the cell can be modified by either an increase or a decrease in intracellular neuropeptide degrading enzyme activities. An

ectoenzyme, pyroglutamyl peptidase II, is also regulated in different ways. Several groups documented an acute increase in pyroglutamyl peptidase II activity in the pituitary gland after a single injection of T_3. Bauer reported that thyroid hormones increase extracellular degradation of TRH by pyroglutamyl peptidase II in GH_3 cells. These studies indicate that increased extracellular TRH degradation is one of the mechanisms for the effect of thyroid hormone in decreasing TRH responses at the pituitary gland. We also found that exposure of Y-79 retinoblastoma cells to phorbol ester leads to a biphasic inactivation of pyroglutamyl peptidase II. The acute decrease in this enzyme activity is due to phosphorylation of pyroglutamyl peptidase II by phorbol ester activated protein kinase C. Prolonged exposure to TPA caused an inhibition of de novo synthesis of pyroglutamyl peptidase II. Activation of protein kinase C by TRH is believed to be responsible for TRH actions and phorbol ester can mimic some of these actions. We speculate that the biological avtivties of TRH can be potentiated at target sites by decreasing TRH degradation at the cell surface. Finally, because of the advantage of having an antiserum and purified pyroglutamyl peptidase II in this laboratory, cloning of this enzyme is underway by using oligonucleotide probes and by using antiserum to screen the expression cDNA library. After having the cDNA clone of pyroglutamyl peptidase II, cellular distribution of this enzyme can be determined by in situ hybridization. Regulation of pyroglutamyl peptidase II by thyroid hormones and phorbol ester at the molecular level can also be studied. The molecular interaction of pyroglutamyl peptidase II and TRH can eventually be elucidated by using a bacterial expression system to produce and then crystallize this enzyme.

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