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Regulation of Adenohypophyseal Pyroglutamyl Aminopeptidase II Activity by Thyrotropin-Releasing Hormone and Phorbol Esters

Dependence on 3,3',5'-Triiodo-L-Thyronine and Gender

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Thyrotropin-releasing hormone (TRH) is inactivated by a narrow specificity ectopeptidase, pyroglutamyl aminopeptidase II (PPII), in the proximity of target cells. In adenohypophysis, PPII is present on lactotrophs. Its activity is regulated by thyroid hormones and 17β-estradiol. Studies with female rat adenohypophyseal cell cultures treated with 3,3',5'-triiodo-L-thyronine (T_3) showed that hypothalamic/paracrine factors, including TRH, can also regulate PPII activity. Some of the transduction pathways involve protein kinase C (PKC) and cyclic adenosine monophosphate (cAMP). The purpose of this study was to determine whether T_3 levels or gender of animals used to propagate the culture determine the effects of TRH or PKC. PPII activity was lower in cultures from male rats. In cultures from both sexes, T_3 induced the activity. The percentages of decrease due to TRH or PKC were independent of T_3 or gender; the percentage of decrease due to cAMP may also be independent of gender. These results suggest that T_3 and hypothalamic/paracrine factors may independently control PPII activity in adenohypophysis, in either male or female animals.

Key Words: Thyrotropin-releasing hormone; pyroglutamyl aminopeptidase II; triiodothyronine; protein kinase C; adenohypophysis; cyclic adenosine monophosphate.

Introduction

Various lines of evidence suggest that pyroglutamyl aminopeptidase II (PPII) (EC 3.4.19.6) is the ectoenzyme responsible for inactivating released thyrotropin-releasing hormone (TRH) *(1–3)*. Analysis of rat brain and adenohypophyseal cDNA sequences demonstrated that PPII is a type II integral membrane protein *(4)* that belongs to the M1 family of aminopeptidases. It is a narrow specificity enzyme acting on TRH or closely related peptides *(5)*. PPII is mainly expressed in neurons of the brain and spinal cord *(6–9)*. It is enriched in nerve terminal preparations *(10)*. Inhibition of PPII activity increases recovery of TRH released from brain slices *(11)*.

The exact relationship between the sites of TRH release and action and the expression of PPII is under investigation. We previously suggested that in a specific TRHergic pathway, PPII is postsynaptic *(12)*. More recently, a partial overlap between the regional distributions of PPII mRNAs and TRH-receptor mRNAs was observed *(13–15)*. PPII activity is also detected, with lower values, in some organs including adenohypophysis *(6,7)*. In this latter organ, PPII is present in one of the TRH target cells, the lactotrophs *(8)*. PPII may therefore be adequately positioned to eliminate excess TRH at the surface of its targets.

The activity of PPII in adenohypophysis is first detected a few days after birth *(16)*. In adult animals, it is regulated during the estrous and circadian cycles (*[17]*; unpublished data). Various studies have identified hormonal and hypothalamic/paracrine factors that may participate in these events. One important hormonal parameter is the thyroid status: enhancement of the levels of serum thyroid hormones (THs) stringently and rapidly increases PPII activity in male or female rats; conversely, induction of hypothyroidism reduces its activity *(18–20)*. This is probably due to a control of PPII mRNA levels *(21)*. Ovariectomy increases, whereas 17β-estradiol reduces, PPII activity in female rats *(20)*; this also occurs concomitantly with control of PPII mRNA levels *(22,23)*. The effect of 17β-estradiol may explain why PPII is higher in male than in female rats *(20)*. In vitro studies using reaggregate adenohypophyseal cultures from male rat support direct effects of thyroid hormones and 17β-estradiol *(8)*.

Adenohypophyseal PPII activity is also regulated in vitro by hypothalamic/paracrine factors, most notably by factors controlling prolactin (PRL) secretion. The activity of PPII

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in adenohypophyseal cell cultures from female rats is downregulated by TRH (or [3Me-His^2]-TRH, a potent agonist of TRH receptors) or pituitary adenylate cyclase activating peptide-38 (PACAP), and upregulated by bromocryptine, a D2 agonist, as well as by somatostatin (SRIF) *(24,25)*. Downregulation also occurs by activation of various cellular effectors; protein kinase C (PKC), L-type calcium channels (LVSSC), or adenylate cyclase *([24,25]*; unpublished data). Some of these effectors mediate hypothalamic/paracrine effects on PPII activity; for example, those of TRH are mediated by LVSSC activation (unpublished data), whereas those of PACAP involve protein kinase A (PKA) activity *(25)*. As for the effects of TH or 17β-estradiol, the control of PPII mRNA levels provides a substantial contribution to the actions of hypothalamic/paracrine factors and second messengers on PPII activity *(25)*.

TH, glucocorticoids, and hormones interacting with the superfamily of nuclear receptors cross talk in many ways with the transduction pathways regulated by cell-surface receptors *(26–28)*. These hormones influence transduction of hypothalamic signals at the adenohypophyseal level *(29,30)*. Gender also has significant effects on neuroendocrine responses, including thyrotropin *(31)* and PRL *(32)* secretions.

Evidence that hypothalamic or second messengers can regulate PPII activity in adenohypophysis has been obtained only with 3,3',5'-triiodo-L-thyronine (T_3)-treated cultures from female rats. The purpose of the present study was to determine whether T_3 levels determine the effects of TRH or PKC activation on PPII activity in primary cultures of adenohypophyseal cells and whether the sex of donor animals determines the effects of TRH, PKC, or cAMP on PPII activity in vitro.

Results

Effects of T_3 in Cell Cultures from Female Rats

Incubation with T_3 for 5 d in vitro induced a dose-dependent increase in PPII activity. The lower dose tested (10^{-10} *M*) induced a sixfold increase; higher doses augmented the activity further (Fig. 1). At a concentration of 10^{-8} *M*, T_3 induced a 10 ± 3-fold increase (mean ± SEM, $n = 6$ independent experiments) in PPII activity. Removal of 10^{-8} *M* T_3 at 5 d in vitro for 16 h did not decrease PPII activity (Fig. 2), but removal of T_3 for 2 d did (data not shown). Subsequent experiments were performed at 5 d in vitro with or without 10^{-8} *M* T_3 added at seeding, except where indicated.

TRH and Phorbol 12-Myristate 13-Acetate Effects in Cell Cultures from Female Rats: Dependence on T_3

To test the effect of T_3 on PPII regulation, cultures treated with or without hormone were stimulated with TRH or phorbol 12-myristate 13-acetate (TPA) (a phorbol ester

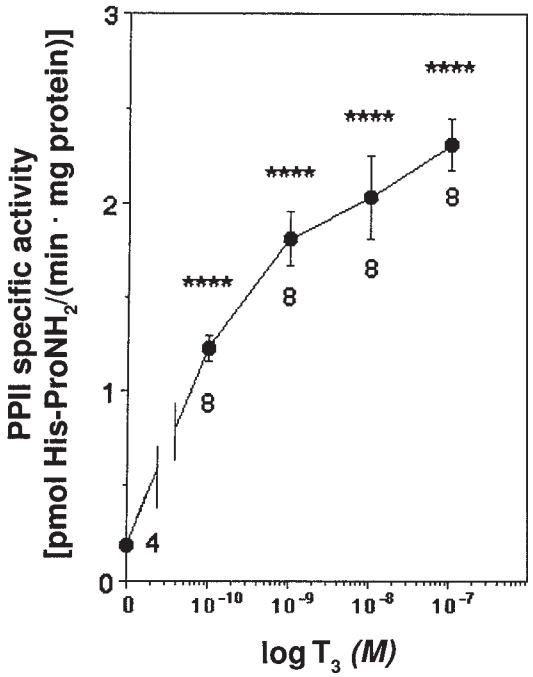

Fig. 1. T_3 induced a dose-dependent increase in PPII activity in adenohypophyseal cells from female rats. Cultures were incubated for 5 d with increasing doses of T_3 or vehicle before quantifying surface PPII activity (method 1). Data are the mean of specific activity ± SEM. The numbers of independent determinations are indicated below bars. Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan multiple range test. Each T_3 concentration was significantly different from control: $^{****}p < 0.001$.

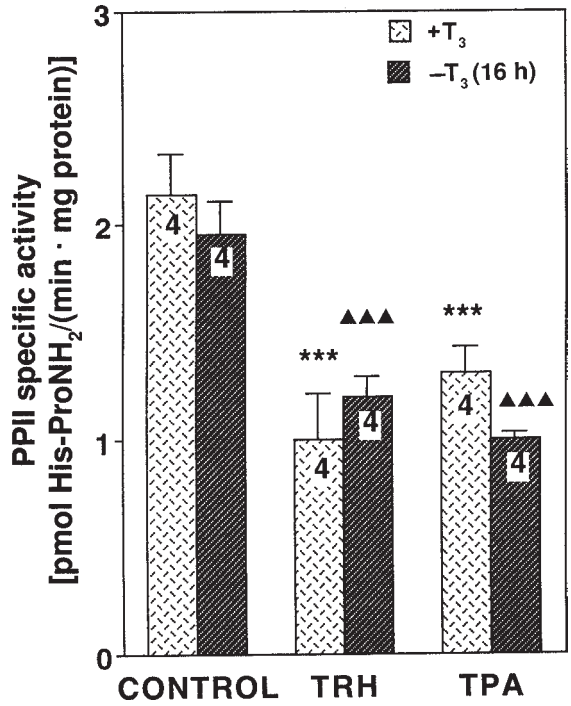

Fig. 2. TRH or TPA inhibition of PPII activity in adenohypophyseal cells from female rats was not modified by removal of T_3 at 5 d in vitro. Cultures were incubated either for 104 h with 10^{-8} *M*

activating PKC) at a maximally effective dose and time point (16 h) *(24)*. TRH (10^{-6} *M*) or TPA (10^{-6} *M*) decreased PPII activity either in control cultures or in cultures treated with 10^{-8} *M* T_3 during 5 d in vitro (Fig. 3A) or in cultures treated with 10^{-8} *M* T_3 during 5 d in vitro except during the last 16 h of culture when medium was changed for medium without T_3 (Fig. 2). In each of these groups, the percentage of decrease compared with that of controls was similar.

Effects of T_3, TRH, Phorbol Esters, and Agents Activating cAMP Production in Cell Cultures from Male Rats

PPII specific activity was lower in cultures obtained from male than from female rats (Fig. 4). Incubation for 5 d in vitro with 10^{-8} *M* T_3 increased PPII activity in cultures from male rats; the percentage of increase was similar to that observed in cell cultures from female rats, but the increase in specific activity was smaller (Fig. 4). To test the effect of gender on PPII regulation, cultures from male rats treated with or without 10^{-8} *M* T_3 for 5 d in vitro were stimulated with TRH or drugs mobilizing cAMP or activating PKC at a maximally effective dose and time point (16 h, except where indicated) *(24,25)*. The percentages of decrease in PPII activity due to 16 h of incubation with TRH or TPA were similar to those observed in cultures from female rats (Fig. 3B, Table 1). The effect of 10^{-6} *M* TPA was slightly reduced when TPA was present for 24 h; the effects of 10^{-7} *M* TPA or phorbol-12,13 dibutyrate (PDBu) (another phorbol ester that activates PKC) for 16 h mimicked those obtained with 10^{-6} *M* TPA (Table 1). The percentages of reduction obtained with these treatments were similar to those previously described for cultures from female rats *(24)*. Drugs increasing intracellular cAMP levels, i.e., 10^{-6} *M* forskolin or 10^{-4} *M* 3-isobutyl-1-methylxanthine (IBMX), reduced PPII activity (Table 1); the percentage of decrease was as previously described in cultures from female rats *(25)*. As observed for cultures from female rats, percentages of reduction in PPII activity by TRH and TPA were independent of T_3 (Fig. 3B).

Discussion

TRH or PACAP downregulate PPII activity in T_3-treated adenohypophyseal cell cultures obtained from female rats *(24,25)*. The second-messenger systems participating in these responses have been investigated. The activation of various cellular effectors, i.e., PKC, LVSSC, or adenylate cyclase downregulates PPII activity *([24,25]*; unpublished data). The effects of TRH are mediated by LVSSC activation (unpublished data) whereas those of PACAP involve PKA activity *(25)*. However, the ligands using the PKC

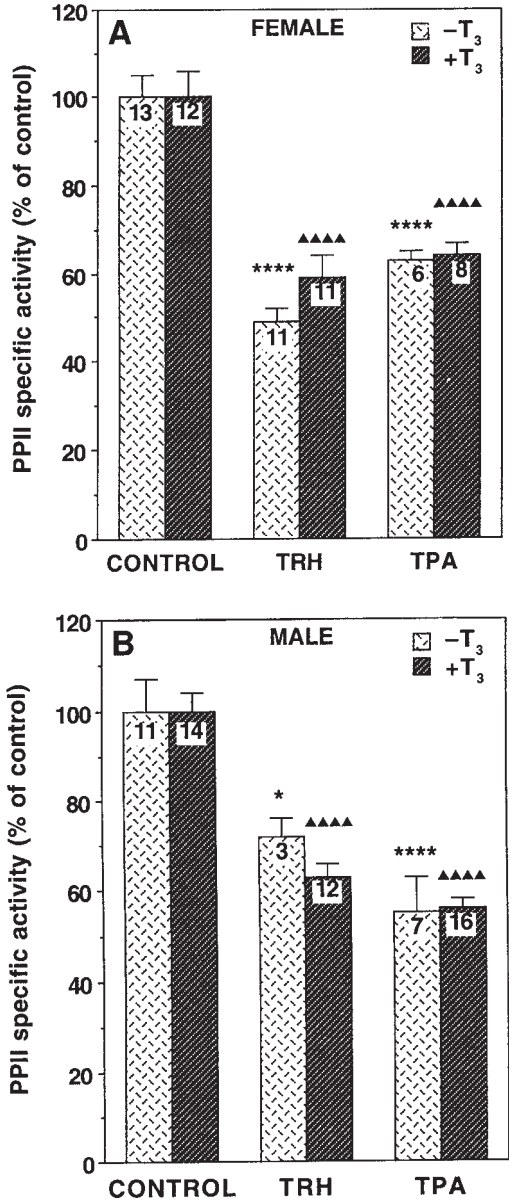

Fig. 3. Relative effects of TRH and TPA on PPII activity in adenohypophyseal cells were independent of T_3 addition and sex of donor animal. Cultures were incubated for 5 d either with or without 10^{-8} *M* T_3. Cells were treated with 10^{-6} *M* TRH or 10^{-6} *M* TPA for 16 h before determination of surface PPII activity (method 1). Data are the mean of specific activity (in percentage of control) ± SEM. **(A)** The mean of PPII specific activity in T_3-treated cultures from female rats was 1.9 pmol of His-ProNH_2/(min·mg protein). **(B)** The mean PPII specific activity in T_3-treated cultures from male rats was 0.4 pmol of His-ProNH_2/(min·mg protein). The numbers of independent determinations are indicated inside bars. Data were analyzed by two-way ANOVA followed by Duncan multiple range test. Significant differences vs respective control: $^{*}p < 0.05$; $^{****}p < 0.001$; $^{\text{mmmm}}p < 0.001$.

(continued from opposite page) T_3 and medium without T_3 added during the last 16 h or for 5 d with 10^{-8} *M* T_3. Cells were treated with vehicle, or 10^{-6} *M* TRH, or 10^{-6} *M* TPA for 16 h before determination of PPII activity in cell surface (method 1). Data are the mean of specific activity ± SEM. The numbers of independent determinations are indicated inside bars. Data were analyzed by two-way ANOVA followed by Duncan multiple range test. Significant differences vs respective control: $^{***}p < 0.01$; $^{\text{mm}}p < 0.01$.

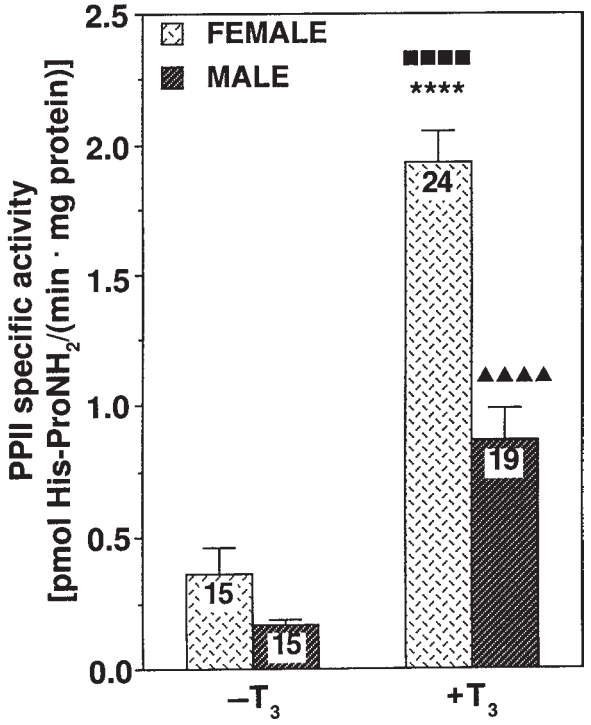

Fig. 4. PPII specific activity was lower in adenohypophyseal cells from male compared with female rats, independent of T_3 addition. Cultures were incubated for 5 d either with or without 10^{-8} *M* T_3 before determination of surface PPII activity (method 1). Data are the mean of specific activity ± SEM. The numbers of independent determinations are indicated inside bars. Data were analyzed by two-way ANOVA followed by Duncan multiple range test. Significant differences: $^{****}p < 0.001$ or ^{mmmm} $p < 0.001$ vs group without T_3; ^{jjjj} $p < 0.001$ vs cells from male rat group treated with T_3.

pathway that control PPII activity are still unknown. The present study demonstrates that the percentage of reduction in adenohypophyseal PPII activity by transduction pathways elicited by either TRH or PKC does not depend on the gender of the animals from which the cell culture is performed or on the levels of T_3 present in the culture medium. The data also suggest that the effect of cAMP is independent of the gender of the animals; however, a direct gender comparison is required to confirm this conclusion.

These observations were obtained at one time point, corresponding to the maximum long-term effect of various treatments, and using maximal effective doses of regulatory substances *(24,25)*. It is therefore possible that the effects of lower doses can be influenced by T_3 levels or gender. However, the available evidence suggests that T_3 and hypothalamic/paracrine factors independently modulate PPII activity, in either male or female rats.

Our data show that as demonstrated in cultures from male rats *(8)*, T_3 has a direct action on cultures from female rats; the effect of T_3 is very potent, in agreement with previous in vitro or in vivo studies *(8,18–20)*. We had previously shown that in T_3-treated cultures from female rats,

Table 1
Effects of Agents Regulating PKC Activity or Intracellular cAMP Levels on PPII Specific Activity in Primary Cultures of Male Adenohypophyseal Cells[a]

	Treatment	PPII specific activity
Experiment 1	Control	100 ± 13 (3)
	TPA (10^{-6} *M*) 16 h	53 ± 6 (4)*
	TPA (10^{-6} *M*) 24 h	74 ± 10 (4)*
	TPA (10^{-7} *M*) 16 h	56 ± 3 (4)**
	PDBu (10^{-6} *M*) 16 h	48 ± 6 (4)**
Experiment 2	Control	100 ± 6(4)
	Forskolin (10^{-6} *M*)	68 ± 13 (4)*
	IBMX (10^{-4} *M*)	75 ± 6 (4)*

[a]Cultures were incubated for 5 d with 10^{-8} *M* T_3 before cells were treated with drugs for 16 h (except where indicated) and the activity of PPII was measured in the cell surface (method 1, i.e., experiment 1) or membranes (method 2, i.e., experiment 2). Data are the mean specific activity (in percentage of control) ± SEM. Means of control PPII specific activities were 1.3 pmol of His-$ProNH_2$/(min·mg protein) for experiment 1 and 55 pmol βNA/(min·mg protein) for experiment 2. The numbers of independent determinations are indicated in parentheses in the last column. Data were analyzed by one-way ANOVA followed by Duncan multiple range test. Significant differences vs control: $^{*}p < 0.05$; $^{**}p < 0.01$.

TRH and TPA slowly downregulate PPII activity *(24)*. The new data demonstrate that PPII activity is independently regulated by T_3 and TRH or PKC in spite of T_3 regulation of TRH binding sites in vitro *(33)*. We also have shown that the donor animals' sex does not affect the percentages of PPII activity change induced by T_3, TRH, TPA, and, possibly, agents increasing intracellular cAMP levels. In vivo, maximal doses of T_3 increased PPII activity up to a similar level in male and female animals; however, the percentage of increase was higher in female than in male rats *(22)*. Our data suggest that this difference is not owing to a differential sensitivity of adenohypophyseal cells to T_3.

Female rats have lower adenohypophyseal PPII activity than male rats, probably reflecting the inhibitory influence of estrogens. By contrast, adenohypophyseal cells from female rats have a higher basal or T_3-induced activity of PPII. This gender-related difference may be intrinsic to the ability of adenohypophyseal cells to express PPII, because the in vitro conditions were identical for both sexes, in contrast to the sex-related difference in the in vivo hormonal milieu. Because PPII is enriched in lactotrophs *(8)*, the sexual difference in vitro could be due to the lower percentage of lactotrophs present in cultures obtained from male rats *(34)*.

In conclusion, maximal and long-term relative effects of TRH or PKC on adenohypophyseal PPII activity in vitro are independent of sex of animal or of T_3 levels. Therefore, some of the previously described regulatory pathways of hypothalamic-paracrine origin may operate both in male and female adenohypophysis independently of T_3 levels.

Materials and Methods

Reagents

TRH was purchased from Peninsula Laboratories (Belmont, CA). [L-2,3,4,5-^{3}H-Pro]-TRH ([^{3}H]-TRH; 83 Ci/mmol) was obtained from New England Nuclear (Boston, MA); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), vitamins, and antibiotics-antimycotic from Gibco-BRL (Grand Island, NY); 4-well multidishes from Nunc (Roskilde, Denmark); TRH-βNA (TRH βnaphtylamide) from Bachem Bioscience (King of Prusia, PA); and solvents from J. T. Baker (D. F., México). All other reagents (including T_3, PDBu, forskolin, insulin, and TPA) were from Sigma (St. Louis, MO). Dipeptidyl aminopeptidase IV (DAPIV) was purified from goat cortical kidney according to Yoshimoto and Walter *(35)*.

Animals

Male or random cycle female Wistar rats (80–100 d old, 260–300 g) were kept under controlled lighting conditions (light from 7:00 AM to 7:00 PM) and fed ad libitum. They were killed by decapitation between 10:00 AM and 11:00 AM The *Guidelines for the use of animals in neuroscience research* of the Society for Neuroscience were followed.

Cell Culture

Adenohypophyseal cells were cultured as previously described *(24)*. Briefly, 15 adenohypophyses separated from the neurointermediate lobe were placed in DMEM containing bovine serum albumin (BSA)(0.3%) (DMEM-BSA), and were minced, washed, and incubated in 3 mL of DMEM-BSA containing trypsin (5 mg/mL) for 18 min at 37°C. Then 4.5 mL of DNase (12 μg) was added to the medium and the reaction proceeded for 2 min. Tissue was allowed to settle, the supernatant was discarded, and the adenohypophyses were incubated for 5 min in 2 mL of DMEM-BSA containing trypsin inhibitor (1.5 mg) followed by incubation in Ca^{2+}-, Mg^{2+}-free medium with EDTA (2 m*M*) for 8 min, with EDTA (1 m*M*) for 15 min, and mechanically dispersed. Viable cells, determined by trypan blue exclusion, were plated at a density of 5000–6000 cells/mm^2 on 16-mm diameter culture dishes with 1 mL of DMEM supplemented with FBS (10%; the lot used contained 180 n*M* T_4), vitamins, penicillin (50 U/mL), streptomycin (50 μg/mL), fungizone (125 ng/mL), glutamine (2 m*M*), glucose (14 m*M*), insulin (0.07 m*M*), and T_3 (10^{-8} *M*) except where indicated. After 3 d of incubation at 37°C in a water-saturated atmosphere of 95% O_2 and 5% CO_2, the medium was changed.

Cell Treatments

Forskolin (10^{-2} *M*), PDBu, and TPA (2×10^{-3} *M*) were dissolved in dimethylsulfoxide; TRH (10^{-3} *M*) in acetic acid (0.001%); and T_3 (10^{-2} *M*) in NaOH (10^{-1} *M*). Stock solutions of drugs were diluted with culture medium to the appropriate concentration just before addition to cultures. Vehicles had no effect on PPII activity. T_3 was added when cells were plated and maintained until determination of PPII activity at 5 d in vitro. Except where indicated, TRH or agents modifying intracellular transduction were added at 5 d in vitro and maintained for 16 h until determination of PPII activity. Cells were observed under a Zeiss inverted light microscope on the day of plating as well as before and after treatments. Cell cultures were maintained healthy for at least 5 d, and we did not observe a morphologic change with any of the drugs tested.

PPII Assays

Enzymatic determinations were performed on the d 5 of culture. Either method 1 or method 2 was used. Most experiments were performed with method 1; however, in the course of this study, method 2 was introduced because it is less expensive and more rapid than method 1. We have previously shown that the relative effects of various agents on PPII activity are similar for either method *(25)*.

Method 1

Surface PPII activity was detected on intact cells as previously described *(24)*. After drug treatment, dishes (10^6 cells) were washed twice with Krebs Ringer bicarbonate containing 10 m*M* glucose (KRBG) and incubated in 250 μL of KRBG with treatment drug and substrate ([^{3}H]-TRH, 10^{-6} *M*, 200,000 cpm). Aliquots of the medium (10 μL) were taken at 0, 20, 40, and 60 min to ensure measurement of initial velocity. [^{3}H]-His-ProNH_2 produced was detected after separation from [^{3}H]-TRH by ion-exchange paper chromatography on Whatman cellulose phosphate P81 sheets. Activity was referred to cell protein content.

Method 2

PPII activity was measured in membranes from 4.5×10^6 cells, essentially as described *(25)* except for a few modifications. Briefly, after drug treatment, cells were washed twice with KRBG, scraped on ice in a total volume of 230 μL of lysis medium (10 m*M* $NaPO_4$, pH 7.5; 1 m*M* phenylmethysulfonyl fluoride, 1 μ*M* pepstatin A, 5 m*M* iodoacetamide, 1.5 m*M* DNase, 2.5 m*M* $MgCl_2$) and ruptured by three cycles of freezing and thawing. They were centrifuged at 120,000*g* for 60 min at 4°C. The pellets were rinsed with 1 *M* NaCl and resuspended in 200 μL of 50 m*M* $NaPO_4$ buffer remove; insert, pH 7.5. Membrane PPII activity was determined with the substrate TRH-βNA in a coupled assay in the presence of excess DAPIV according to Friedman and Wilk *(6)* except for a few modifications. *N*-ethyl maleimide (0.2 m*M*), bacitracin (0.2 m*M*), and DAPIV (4 nmol of Gly-Pro-βNA hydrolyzed/min) were added to the membrane preparation (150–250 μg of protein/assay). The enzymatic reaction was initiated in duplicate, by the addition of 450 μ*M* pGlu-His-ProβNA to the reaction mixture (total volume of 250 μL) and carried out

at 37°C; every 30 min 50 μL was withdrawn from 30 to 120 min and the reaction was stopped by the addition of 50 μL of 100% methanol. The volume was completed to 400 μL with 50% methanol before detecting βNA in a fluorometer (excitation: 335 nm; emission: 410 nm). An aliquot was kept for protein quantification. The activity was linear during 2 h and refereed to membrane protein content.

Protein Quantification

Cultured cells washed with KRB (for method 1) or an aliquot of the membrane preparation (for method 2) were digested with NaOH (1 *N*) at room temperature for 24 h, and proteins were determined according to Lowry et al. *(36)*.

Statistical Analyses

Data are the mean ± SEM values of independent cultures (each performed in triplicate or quadruplicate). Either one- or two-way ANOVA followed by Duncan multiple range test was used to determine statistical significance between individual means. Differences were considered significant at $p < 0.05$.

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