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Rapid Turnover of Tryptophan Hydroxylase Is Driven by Proteasomes in RBL2H3 Cells, a Serotonin Producing Mast Cell Line[1]

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Previously we demonstrated that tryptophan hydroxylase (TPH) undergoes very fast turnover driven by ATP-dependent proteolysis in serotonin producing mast cells [Hasegawa *et al.* (1995) *FEBS Lett.* 368, 151–154]. We searched for the major proteases involved in the rapid degradation of TPH in RBL2H3 cells. Among various protease inhibitors tested, proteasome inhibitors MG115, MG101, MG132, and lactacystin effectively inhibited the intracellular degradation of TPH. Administration of the proteasome inhibitors to cultured cells caused more than a 5-fold accumulation of TPH. Administration of the inhibitors together with cycloheximide stabilized the amount of TPH with no appreciable increase or decrease. Although MG-series proteasome inhibitors could inhibit calpain, the involvement of calpain was excluded since the cysteine protease inhibitor E-64-d, which acts on calpain, had no effect. Extracts of RBL2H3 cells were shown to contain a protease that digests TPH in an ATP-dependent manner and is sensitive to proteasome inhibitors. The ubiquitination of TPH could be visualized by Western blot analysis using both anti-TPH and anti-ubiquitin antibodies. Based on these results, we conclude that 26S proteasomes are mainly involved in the degradation of TPH. In the reported amino acid sequences of TPH from various sources including human, rabbit, rat, and mouse, a PEST sequence that is widely shared among short-lived proteins has been recognized. It was noted that the PEST sequence lies within the most conserved portion of the enzyme, the pteridine binding site.

Key words: mast cell, proteasome, protein turnover, serotonin biosynthesis, tryptophan hydroxylase.

Tryptophan hydroxylase (TPH), the rate limiting enzyme in serotonin biosynthesis, is present specifically in serotonergic organs. There are two types of TPH, a brain type (*1–5*) and a peripheral type of non–neural cell origin such as from pinealocytes of the pineal gland (*6, 7*), enterochromaffin cells of the gastrointestinal mucosa (*8, 9*) and peripheral neoplastic cells from mastocytomas (*10–15*) and gastric carcinoid tumors (*16*). An established cell line derived from a mouse mastocytoma, P-815, has been a convenient source of this enzyme (*13, 14, 16–18*). In our previous study (*19*), a rapid turnover of TPH was demonstrated using cycloheximide to arrest protein biosynthesis in FMA3, a mouse mastocytoma cell line, and RBL2H3, a rat basophilic leukemia cell line. It was suggested that the rapid turnover of TPH protein (half life, 15–60 min) in these cells is driven by ATP-dependent proteolysis.

The 26S proteasome is known to be responsible for the ATP-dependent breakdown of selected proteins and the scavenging of denatured proteins. The 26S proteasome is an organized assembly of a ubiquitous ATP-dependent protease outside the lysosome (*20, 21*). The covalent attachment of ubiquitins to target proteins acts as a signal for their selective degradation, and this ubiquitin-proteasome pathway appears to be involved in the selective removal of regulatory proteins related to various cellular functions [for review (*22, 23*)]. The 26S proteasome consists of two functionally distinct units (*20, 21, 24–26*). A central cylindrical 20S proteasome with endopeptidase activities has regulatory units at both of its ends that have ATPase and deubiquitinating activities. The endoproteolytic activities of 20S proteasomes toward target proteins with basic, neutral, and acidic amino acid residues have been classified as trypsin-like, chymotrypsin-like, and V8 protease–like activities, respectively (*27, 28*). Synthetic peptide aldehydes, MG115 (carbobenzoxy-Leu-Leu-Nva-H, also called Z-LLnV), MG101 (*N*-acetyl-Leu-Leu-norleucinal, calpain inhibitor I), and MG132 (carbobenzoxy-Leu-Leu-Leu-H), are potent inhibitors of the chymotryptic activity of 20S proteasomes and therefore also of 26S proteasomes. And lactacystin, which binds covalently to 20S subunits, is a well accepted inhibitor specific to 26S proteasome (*29*). In this paper, we utilized these inhibitors to demonstrate that 26S

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; PBS, Dulbecco's phosphate-buffered saline; TPH, tryptophan hydroxylase (L-tryptophan tetrahydropteridine: oxygen oxidoreductase, EC 1.14.16.4); 5HTP, 5-hydroxy-L-tryptophan.

proteasomes are involved in the degradation of TPH. Since MG-series inhibitors also inhibit calpain to some extent, we also used E-64-d [(L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl)-L-leucine (3-methylbutyl)amide], a membrane-permeable, specific inhibitor of cysteine proteases including calpain (*22* for review). We also examined the ubiquitination of TPH and the amino acid motifs that might be involved in its degradation.

MATERIALS AND METHODS

Materials—MG115, MG132, E-64-d, and E-64 were purchased from Peptide Institute (Osaka), lactacystin from Kyowa Medex (Tokyo), MG101 (calpain inhibitor I) from Boehringer Mannheim GmbH (Mannheim), and K252b from Alomone Labs (Jerusalem, Israel). Cycloheximide, creatine kinase, and phosphocreatine were purchased from Sigma (St. Louis, MO). Anti-tryptophan hydroxylase rabbit polyclonal antiserum was raised (*8*) against tryptophan hydroxylase purified from a mouse mastocytoma P-815 according to Nakata and Fujisawa (*13*). Dihydropteridine reductase was prepared from bovine liver (purified to the second ammonium-sulfate precipitation step) (*30*). Anti-ubiquitin rabbit IgG was provided by Dr. Ueno (Juntendo University School of Medicine, Tokyo). Anti–20S proteasome antibody was provided by Dr. Tanaka (The Tokyo Metropolitan Institute of Medical Science, Tokyo). The PEST-FIND computer program (*31*) was provided by Dr. Rechsteiner (The University of Utah, Salt Lake City, Utah).

Cell Culture—RBL2H3 cells (a mast cell line derived from rat basophilic leukemia cells) were obtained from The Japanese Cancer Research Resources Bank (Tokyo). Cells were grown for five passages and then frozen as the secondary stock and kept in liquid nitrogen. Secondary stock cells were seeded and used after 10 to 70 passages. FMA3 (Furth's mastocytoma) and P-815 (Potter's mastocytoma) were maintained as described (*18*). RBL2H3 cells were kept as a monolayer culture in DMEM (Gibco BRL, Grand Island, NY) containing 10% fetal calf serum. FMA3 cells were maintained as a suspension culture in RPMI1640 medium containing 10% fetal calf serum. All cultures were maintained at 37°C under 5% CO_2/95% air. One day before experiments, cells were plated in a 96-well analytical culture plate at a density of 1×10^5 cells/well. For Western blot analysis, cells were plated at 5×10^6 cells/10-cm culture dish. Two hours before analysis, cells were placed in serum-free DMEM buffered with Hepes/NaOH containing 100 units/ml of penicillin and 100 μg/ml of streptomycin and kept at 37°C under 10% CO_2/90% air throughout the experiments. Agents with low solubility in water were dissolved in dimethylsulfoxide (DMSO) at a 100- or 200-fold greater concentration than the desired final concentration unless other-wise stated, so that the vehicle (DMSO) concentration would be the same in each experimental culture.

Tryptophan hydroxylase assay—Tryptophan hydroxylase activity in cell-free extracts was determined essentially as described (*11*): The culture medium and cells in each well of a 96-well culture plate were removed by suction, 20 μl of PBS was added, and the mixture was subjected to freezing in liquid nitrogen and thawing in water twice. The disrupted cells were incubated in a mixture of 30 mM DTT, 50 μM $Fe(NH_4)_2(SO_4)_2$, 4 mg/ml catalase in 0.1 M Tris-acetate (pH 8.0, total 50 μl) for 10 min at 30°C. Then a solution was added to give in final concentration of 250 μM tryptophan, 400 μM 6*R*-tetrahydrobiopterin, 500 μM NADH, 1 mM NSD-1015, 2 mg/ml catalase, and dihydropteridine reductase in excess in a total volume of 150 μl. The enzyme reaction was allowed to proceed for 10 min at 30°C and then terminated by the addition of K-perchloride at a final concentration of 0.84 M. The anount of 5HTP formed was determined by high performance liquid chromatography with fluorescence detection (model FP920; JASCO, Tokyo) set an excitation wavelength of 302 nm and emission wavelength of 350 nm. The solid phase was ODS (Finepak SIL C18T-5, JASCO), the mobile phase was a 90:7:5 mixture of 40 mM sodium acetate, (adjusted to pH 3.5 with formic acid), acetonitrile, and methanol, and the flow rate was 1 ml/min.

Western Blot Analysis—Cells were collected by centrifugation, solubilized in 1% SDS, and the cell lysates were subjected to SDS–polyacrylamide (10%) gel electrophoresis (SDS-PAGE). When immunoprecipitation was employed to concentrate the TPH protein, cells were rinsed with PBS and disrupted with 1% NP-40 in 50 mM Tris-HCl (pH 7.8) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The cell lysates were mixed with anti-TPH antiserum and left overnight at 4°C with agitation. Total IgG was collected by the addition of staphylococcal ghosts (Pansorbin; Calbiochem, La Jolla, CA) as a precipitant, solubilized in 1% SDS, and subjected to SDS-PAGE. Proteins were transferred to a nitrocellulose filter (Advantec, Tokyo) by electrophoresis across the gel (*32*). Immunoreactive proteins were visualized using their specific antisera as the primary antibody and an anti–rabbit IgG goat antibody conjugated with horse radish peroxidase (Wako, Tokyo) as the secondary antibody. The peroxidase conjugated secondary antibody was detected using an enhanced chemiluminescence detection system (ECL; Amersham, Buckinghamshire, England). In order to visualize TPH protein, of which the cellular content is very low, the amount of antibody was increased, and occasionally some unidentified protein bands appeared on the film. The protein band of TPH was identified by simultaneous electrophoresis with the purified enzyme prepared essentially according to Nakata and Fujisawa (*13*). Protein bands were scanned and quantified with NIH image ver. 1.62, a public domain software, by Wayne Rasband, National Institute of Health, USA.

Preparation of Cell Extracts—Frozen pellets of P-815 cells (5.6 g) were homogenized in 4 volumes of 50 mM Tris-acetate (pH 7.6) containing 0.1 M KCl using an Ultra-disperser (model T25; IKA Labortechnik, Staufen, Germany), and the homogenates were centrifuged at 35,000 ×*g* for 50 min. TPH in the supernatants was concentrated by ammonium sulfate fractionation between 25 and 75% saturation and then subjected to gel filtration through a column of Sephadex G-25 (1.5 × 18 cm) equilibrated with 50 mM Tris-acetate (pH 7.6) containing 0.1 M KCl and 0.06% Tween 20. Protein fractions were stored at –80°C.

Extracts from RBL2H3 cells were prepared essentially according to the method of Ugai *et al.* (*33*) for preparing 26S proteasomes from rat liver. RBL2H3 cells were homogenized in 3 volumes of 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT and 2 mM ATP using the Ultra-disperser. The homogenate was centrifuged at 1,800 ×*g* for 5 min. The fresh supernatant was used for *in vitro* proteolysis as the putative source of proteasomes.

Cell-Free Proteolysis of TPH—In vitro proteolysis was performed in a reaction mixture containing the extracts prepared from P-815 and RBL2H3 cells, 0.5 mg/ml ubiquitin, 5 mM $MgCl_2$, 2 mM ATP, 10 μg/ml creatine kinase, 10 mM phosphocreatine, and 1 mM DTT in 50 mM Tris-HCl (pH 8.0).

RESULTS

Effect of Protease Inhibitors on TPH Degradation in RBL2H3 and FMA3 Cells—The steady state activity of TPH in RBL2H3 cells was found to be very low and variable (roughly 0.2 to 8 pmol/min per 10^6 cells) between stocks for unknown reasons. The rapid ATP-dependent turnover of TPH was first demonstrated with this cell line (*19*). Since the degradation was counter balanced by an equally rapid *de novo* synthesis of TPH, the enzyme activity appeared constant. Upon the administration of the peptide aldehyde proteasome inhibitor, MG115, to a culture of RBL2H3 cells, the TPH level increased almost linearly up to 5-fold within 90 min after a lag period of about 30 min (Fig. 1). When cycloheximide (10 μg/ml) alone was administered, the enzyme level decreased to zero without an appreciable lag, as reported previously (*19*). The lag period observed after MG115 administration is presumably a reflection of its slow permeation into the cells. When cycloheximide was added 30 min after MG115, the TPH level remained nearly constant (Fig. 1), an indication of the arrest of both TPH biosynthesis and degradation. Based on this result, the concentration dependence of the inhibition of TPH degradation was examined under similar conditions using MG132, which is more specific for proteasome (Fig. 2a). About 10 μM MG132 completely prevented TPH degradation in RBL2H3 cells with an IC_{50} for MG132 of roughly 0.4 μM (Fig. 2a). Lactacystin also effectively prevented TPH degradation in RBL2H3 cells with an IC_{50} of roughly 10 μM (Fig. 2b). A cysteine protease inhibitor, E-64-d, did not significantly inhibit TPH degradation in RBL2H3 cells at concentrations from 0.001 to 10 μM (Fig. 2c) under conditions similar to those in the experiment shown in Fig. 2a, suggesting that it is unlikely that calpain is involved in TPH degradation. In an experiment similar to that shown in Fig. 1, the increase in TPH activity in FMA3 cells appeared 60 min after MG115 administration (data not shown), 30 min later than in the case of RBL2H3 cells. A similar experiment as in Fig. 2a was performed using FMA3 cells in which MG115 was added 60 min before cycloheximide. The apparent IC_{50} of MG115 with FMA3 cells was approximately 0.4 μM (data not shown). Another inhibitor, MG101, had a similar effect on TPH degradation except that its IC_{50} was approximately 3 μM in both cell lines (data not shown). Classical inhibitors of lysosomal proteases, chymostatin, leupeptin, pepstatin, and aprotinin, were tested with FMA3 cells in the presence of cycloheximide (Table I). Most of them were only poorly effective, although chymostatin was moderately effective, consistent with the idea that proteasomes act as chymotrypsin-like proteases. Furthermore, up to 50 mM of the lysosomotropic

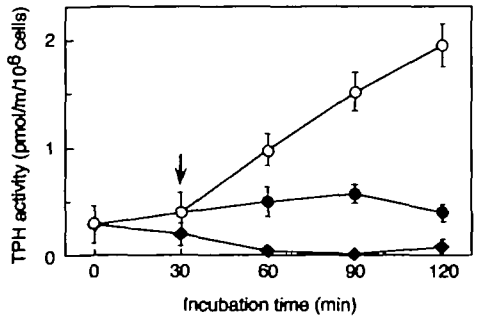

Fig. 1. **Perturbation of TPH turnover in RBL2H3 cells by a proteasome inhibitor and/or a protein synthesis inhibitor.** RBL2H3 cells were treated with 3 μM MG115 for 120 min (○ and ●), and with 10 μg/ml cycloheximide at time 0 (◆) or from 30 to 120 min (●). In the last case, cycloheximide was added after 30 min of MG115 exposure (indicated by the arrow). TPH activity determined as described in "MATERIALS AND METHODS." Each experiment was performed with cells in a single 96-well plate (1 × 10^5 cells /well). Data were acquired from cells in individual wells and are expressed as averages with standard deviations (n = 4).

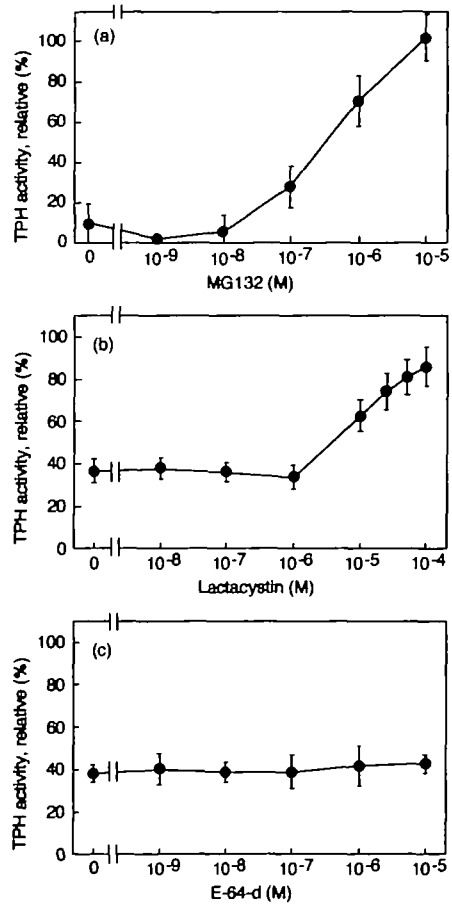

Fig. 2. **Effect of protease inhibitors, MG132, lactacystin, and E-64-d, on TPH degradation in RBL2H3 cells.** RBL2H3 cells (1×10^5 cells/well) were treated with 10 μg/ml cycloheximide 30 min after the administration of various concentrations of protease inhibitors administered previously to allow penetration into the cells. Activities remaining after 60 min in the presence of cycloheximide and the indicated concentrations of protease inhibitors [(a) MG132, (b) lactacystin, and (c) E-64-d] are presented as the percentage of the activity at zero-time of cycloheximide administration. TPH activities at zero-time of cycloheximide administration were (a) 0.54 ± 0.11, (b) 3.65 ± 0.55, and (c) 12.49 ± 0.37 pmol/min/10^6 cells. Values are means ± SD (n = 4).

TABLE I. **Effect of protease inhibitors on TPH degradation in FMA3 cells.** FMA3 cells (10^5 cells/well) were exposed to protease inhibitors at the indicated concentrations together with 10 μg/ml cycloheximide for 60 min. Then the TPH activity of the cells was determined as described in "MATERIALS AND METHODS." Data are expressed as means ± SD (n = 3) and the effect of protease inhibitors was calculated from the reagent-dependent increase in the remaining activity.

Treatment		TPH activity	
	(μM)	(pmol/min/10^6 cells)	(inhibition (%)*)
Control (vehicle)		8.07±0.19	(100)
Cycloheximide (alone)		3.99±0.1	(0)
Cycloheximide			
+ leupeptin	10	4.22±0.19	5.6
	100	4.36±0.07	9.1
+ aprotinin	10	4.32±0.15	8.1
+ chymostatin	10	4.80±0.09	20
	100	5.12±0.08	28
+ pepstatin	10	4.07±0.04	2.0
	100	4.26±0.15	6.6
+ MG115	10	6.46±0.09	61

*Decrease in TPH activity by cycloheximide alone represents endogenous protease activity. The increase in activity due to added protease inhibitors (+cycloheximide) represents the effect of protease inhibitors. The inhibitory effect is presented as a percentage using these values.

reagent NH_4Cl was given to FMA3 cells for 60 min, but TPH degradation was not affected. All these results suggest that 26S proteasomes, but not lysosomal proteases, are involved in the rapid degradation of TPH.

Ubiquitination of TPH—In most cases, proteolysis by 26S proteasomes targets ubiquitinated proteins. In order to examine whether TPH is ubiquitinated before degradation, we looked for ubiquitinated TPH after the administration of a proteasome inhibitor. In FMA3 cells, which have abundant TPH compared with RBL2H3, the TPH protein band was easily detected by Western blot analysis. The TPH protein band became denser with increasing concentration of MG132 up to 0.1 μM (60-min incubation) and reached a plateau (a densitometry analysis showed 1.6–1.8-fold over the control, Fig. 3a). The TPH activity increased along with the increase in TPH-immunoreactive protein caused by MG132 (data not shown). This indicates that the accumulated protein is the active enzyme and is likely to be a substrate for protease. If TPH undergoes ubiquitination, the molecular mass of the protein should increase according to the amount of ubiquitin bound. However, a higher molecular mass band of immunoreactive protein was scarcely observed in extracts of FMA3 cells, providing little evidence for the ubiquitination of TPH. The effect of MG132 on RBL2H3 cells was examined after immunoprecipitation of TPH with anti-TPH antiserum. With RBL2H3 cells, in contrast to FMA3 cells, a diffuse but higher molecular mass immunoreactive band was seen around 80–94 kDa, consistent with addition of 3 to 5 ubiquitin molecules (8.6 kDa each) to the TPH protein (53 kDa). The strongest signal was observed with 0.1 μM MG132, higher concentrations of MG132 gave smeared signals that were not stronger. When the same immunoprecipitates were visualized using an anti-ubiquitin IgG, the same band was observed when RBL2H3 cells were treated with 0.1 μM MG132 (Fig. 4). The administration of E-64, a cysteine protease inhibitor, showed no significant difference from the control with

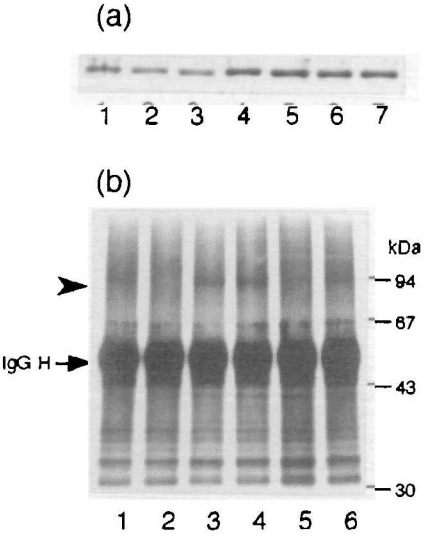

Fig. 3. **Effect of MG132 on the TPH-immunoreactive protein in cultured cells.** (a) FMA3 cells (5×10^6 cells/10-cm culture dish) were exposed to MG132 (lanes 2–7, at 0, 0.01, 0.03, 0.1, 0.3, and 1 μM, respectively) for 60 min, and then the cell lysates (10 μg each) were subjected to SDS-PAGE for Western blot analysis. Lane 1 represents 50 ng of purified TPH from mouse mastcytoma P-815. (b) RBL2H3 cells (5×10^6 cells/10-cm culture dish) were preincubated with various concentrations of MG132 for 30 min, and then exposed to 10 μg/ml cycloheximide for 60 min. The cells were disrupted, subjected to TPH immunoprecipitation, and Western blots were made as described in "MATERIALS AND METHODS." Lane 1: vehicle control. Lanes 2–6: MG132 at 0, 0.01, 0.1, 1, and 10 μM, respectively.

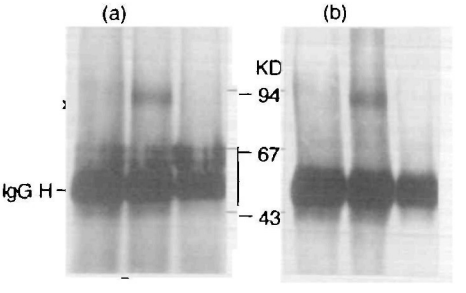

Fig. 4. **Detection of ubiquitinated TPH protein.** RBL2H3 cells (5×10^6 cells/10-cm culture dish) were exposed to 0.1 μM MG132 or 3 μM E-64 for 60 min. The cells were treated as in Fig. 3 and TPH-immunoreactive protein was visualized by Western blot analysis using (a) anti-TPH IgG and (b) anti-ubiquitin IgG. For both (a) and (b), left to right, vehicle control (left), MG132 (center), and E-64 (right). Numerals represent relative molecular masses. The arrowhead represents the relative molecular mass of TPH bound with 3–5 ubiquitins. Thick bands around the arrow represent IgG heavy chain.

respect to the appearance of the high molecular mass protein. The results indicate that ubiquitinated TPH accumulates as a result of the inhibition of the proteasomes.

Degradation of TPH in Cell-Free System—To investigate further the mechanism of the degradation of TPH, we designed an *in vitro* proteolysis system using extracts of mouse mastocytoma P-815 cells as a source of TPH. P-815 cells exhibited high TPH activity, around 150 pmol 5HTP/min/10^6 cells, roughly 300-fold higher than RBL2H3 cells, and the half-life of TPH was much longer than 60 min.

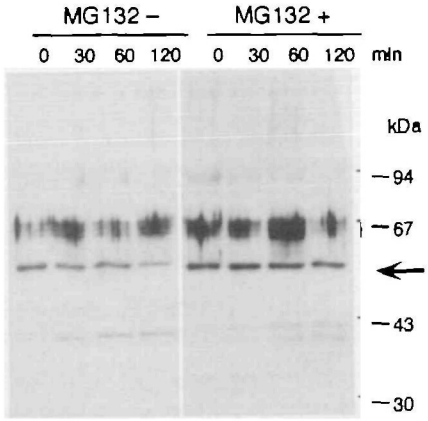

Fig. 5. **Effect of MG132 on the degradation of TPH in cell-free system.** P-815 cells were the source of substrate and RBL2H3 cells the source of the presumed protease. Cell-free extracts of P-815 and RBL2H3 cells, prepared as described in "MATERIALS AND METHODS."were mixed (600 and 400 μg protein, respectively) and incubated in the presence of 5 mM $MgCl_2$, 2 mM ATP, 1 mg/ml ubiquitin, and 10 μM MG132 in a total volume of 500 μl at 30°C for the indicated times. Aliquots of the reaction (40 μl each) were then quenched at the indicated times in SDS sample buffer and subjected to SDS-PAGE and Western blot analysis using anti-TPH antiserum as the primary antibody. The arrow shows the relative molecular mass of TPH (53 kDa).

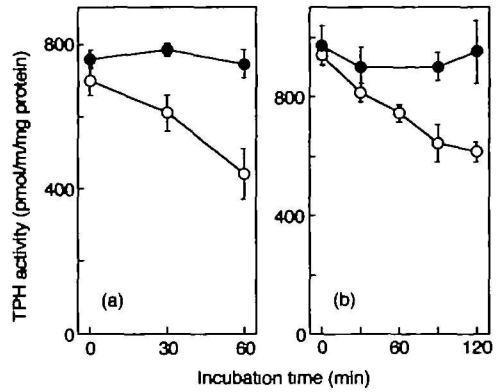

Fig. 6. **Blocking of the degradation of TPH *in vitro* with inhibitors of proteasomes or protein kinases.** Cell-free extracts of P-815 and RBL2H3 cells were prepared as described in "MATERIALS AND METHODS" and 600 μg P-815 and 400 μg RBL2H3 protein were incubated at 30°C for the indicated times with 5 mM $MgCl_2$, 2 mM ATP, 1 mg/ml ubiquitin, and either 10 μM MG132 (a) or 0.1 mM K252b (b) in a total volume of 500 μl. Aliquots (40 μl) of the reaction were removed at the indicated times, and TPH activity was determined as described in "MATERIALS AND METHODS." Values are means ± SD ($n = 4$).

When P-815 extracts alone were incubated, no apparent degradation of TPH was observed. Extracts of RBL2H3 cells were used as the putative source for the proteasome system including ubiquitinating enzymes if required. In order not to allow the dissociation of 26S proteasomes, extraction was performed in the presence of 2 mM ATP and 1 mM DTT (*33*). For effective degradation in this system, ATP and an ATP regenerating system, creatine and creatine kinase, were unavoidable, although addition of ubiquitin did not significantly enhance TPH degradation. Using this *in vitro* system, TPH degradation was also observed and it was clearly prevented by the addition of MG132 (10 μM) as shown in Fig. 6a. MG115 was equally effective but E64 was not effective (data not shown). As shown in Fig. 5, the TPH signal visualized by Western blot decreased during *in vitro* incubation (TPH signal at 120-min decreased to 32% of the 0-timesignal), while 10 μM MG132 efficiently prevented this (81% prevention at 120-min). We tried to remove 26S-proteasome from the extracts. An aliquot of the extracts (200 μg protein) was subjected to immunoprecipitation by mixing with 100 μg of IgG for 90 min, then subjected to the *in vitro* TPH degradation system. The initial activity of the added TPH was 13.0 pmol 5HTP formed per minute per single reaction. The remaining TPH activity after 180 min incubation with the IgG-treated extracts of RBL2H3 cells was 11.5 pmol/min, while in control incubations using preimmune IgG the TPH activity decreased to 4.51 pmol/min; about 82% of the TPH degradation was prevented by the anti-(20S-proteasome) IgG and larger amounts had no further effect. Some protein kinase inhibitors completely prevented the *in vitro* degradation of TPH, including 100 μM K252b (Fig. 6b) and 0.1 μM staurosporine (data not shown). The administration of these reagents to cultured cells was unsuccessful mainly due to uncontrollable detachment of the cells from the culture dishes.

DISCUSSION

In our previous work, we demonstrated that the turnover of TPH in the mast cell lines RBL2H3 and FMA3 is driven by a very rapid, ATP-dependent proteolysis (*19*). In this work, we present evidence that TPH is mainly cleaved by 26S proteasomes MG-series peptide analogs are potent inhibitors of 26S proteasomes, but also inhibit calpain, a calcium-dependent cysteine protease. The degradation of TPH was effectively inhibited by MG-series inhibitors as well as lactacystin, but not by E-64-d, a specific inhibitor of cysteine proteases, suggesting that the effect of MG series inhibitors arises from inhibition of the action of 26S proteasomes. The prevention of TPH degradation by the inhibitors was also demonstrated by Western blot analysis. We encountered serious difficulties, however, in visualizing ubiquitinated TPH. Although the ubiquitination of TPH was finally detected, as shown in Fig. 3b, the ubiquitinated TPH signal was always smaller than expected, and the ubiquitinated TPH immunoreactive signal did not accumulate during extended incubations with MG132. *In vitro* proteolysis was also carried out with cell-free extracts in which P-815 cells were the source of TPH and RBL2H3 cells the source of the putative proteasomes. The disappearance of the major band of TPH immunoreactivity was effectively inhibited by 10 μM MG132 (Fig. 5), but no inhibitor-dependent accumulation of ubiquitinated TPH was visualized. It is possible that ubiquitinated TPH is rapidly deubiquitinated by ubiquitin isopeptidases (*34*) resulting in the accumulation of 53 kDa TPH subunits in the presence of MG132. Although the ubiquitination of TPH obviously takes place, at least in part (Fig. 4), a non-ubiquitinating proteasome pathway has not been ruled out. A proteasome pathway without ubiquitination has been reported for ornithine decarboxylase, which is digested in the presence of its antizyme (*35*).

TPH cDNA from several sources has been cloned (*36–40*) and the predicted amino acid sequences are available. We

examined the primary structure of TPH for the presence of any motifs related to protein degradation. A sequence called PEST has frequently been found in various short-lived proteins, and has been proposed as a target of ubiquitination (*31, 41*). Using the computer program PEST-FIND (*31*), we recognized PEST sequences at residues 261–275 in mouse and 258–272 in human, rabbit, and rat TPH (PEST scores are 9.7 for mouse and rat, and 10.4 for the others). It was noted that the PEST sequence was within the most conserved portion of the enzyme, the pteridine binding site (*42, 43*). In order to see whether pteridine binding regulates the rate of TPH degradation, sepiapterin, which greatly increases intracellular BH_4, was added to cultured cells to see whether it would affect the rate of TPH degradation. However, the turnover rate of TPH was unchanged (data not shown). Moreover, the addition of tryptophan to cultured cells also had no effect. RAALGNISN, another putative recognition sequence, has been reported to be required for the ubiquitin-mediated degradation of cyclins (*44, 45*), but this sequence was not found in TPH. In the cases of CLN3 (*46*) and I-κB (*47*), multiple phosphorylation sites are required in addition to PEST. In this regard, it was noted that protein kinase inhibitors were effective in preventing TPH degradation, at least *in vitro* (Fig. 6). Therefore, we were interested in seeing whether TPH phosphorylation in RBL2H3 is involved in 26S proteasome digestion. However, attempts to obtain direct evidence for the phosphorylation of TPH from mast cells have not been successful. Furthermore, there are no reports to date of the intracellular phosphorylation of any peripheral TPH. However, the possibility has not been ruled out that the phosphorylation of TPH triggers, at least in part, its ubiquitination, or its digestion by 26S proteasomes without ubiquitination.

In this work, we used three established cell lines of rodent mast cell origin: RBL2H3 cells, which have very low levels of TPH, but show the most rapid turnover among the three cell lines; FMA3, which contain high TPH activity and show an appreciable but slower rate of turnover than RBL2H3 cells; and P-815, which also contain high TPH activity, but whose turnover rate is slow. We assume that the ancestors of these cells share common metabolic pathways and regulatory systems and that they have deviated by losing different parts of these systems. RBL2H3 cells retain the ability to respond to physiological secretagogues for the exocytotic release of the active substances characteristic of mast cells, while the other cell lines have virtually lost this function. Furthermore, P-815 cells have lost the 5HTP decarboxylase activity that is rather abundant in serotonergic cells (*18*). Therefore, we can assume that RBL2H3 cells are the least deviated from the original mast cells or basophiles. In this work, we employed appropriate cell lines for our purpose of demonstrating what might happen in the original cells. Based on the similarity of TPH in various peripheral tissues, including the pineal gland (*6–9, 16*), the rapid turnover of TPH might be common to peripheral tissues. Since neural TPH is different in many respects when analyzed *in vitro*, we are not certain that the features of TPH degradation we demonstrated are applicable to neurons. There is little information about TPH turnover in the CNS.

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