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Rapid Turnover of Tryptophan Hydroxylase: The Turnover Rate is Unaffected after Elevation of the Enzyme Amount Induced by Treatment of Cells with Calcium Ionophores and Protease Inhibitors

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Introduction

The largest reservoirs of serotonin in mammals are the blood circulation and the gastrointestinal mucosa (1,2). Tryptophan hydroxylase is the rate limiting enzyme in serotonin biosynthesis and is localized specifically in serotonin producing cells. The main producers of body serotonin are gastrointestinal enterochromaffin-cells and, in rodents, mast cells. In our previous study (3,4), a rapid turnover of tryptophan hydroxylase (half life; 15-60 min) was demonstrated using cycloheximide to metabolically arrest protein biosynthesis in RBL2H3 cells of a cultured mast cell line derived from a rat basophilic leukemia cell. In addition, this process required ATP production, suggesting that it was enhanced by metabolic ATP. Since the intracellular tryptophan hydroxylase level must be maintained by its biosynthesis counterbalanced to the rapid degradation, cells might be able to set the desired enzyme level by attenuating the rate either of biosynthesis or degradation. We have demonstrated a dramatic rise in the cellular enzyme level in response to antigen-IgE stimulation through the calcium-triggered signal transduction pathway (5). Antigen-IgE stimulation was replaceable by calcium ionophores or low doses of a group of protease inhibitors. These stimulating effects were inhibited by relatively high doses of the same inhibitors. In the present study, we examined whether the rapid degradation was attenuated in setting the amount of tryptophan hydroxylase to various intracellular levels.

Materials and Methods

MG115 (carbobenzoxy-Leu-Leu-Norvalinal), and PSI (carbobenzoxy-Ile-Glu (*O*-t-Bu)-Ala-leucinal) were purchased from Peptide Institute (Osaka). Calpain inhibitor-1 was obtained from Boehringer Mannheim GmbH (Germany). Calpain inhibitor-2, cycloheximide, and A23187 were purchased from Sigma (St.Louis, MO). RBL2H3, a mast cell line derived from rat basophilic leukemia cells, was obtained from The Japanese Cancer Research Resources Bank (Tokyo). RBL2H3 cells were kept as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. All cultures were maintained at 37°C under 5% CO_2/95% air.

The degradation rate of tryptophan hydroxylase in living cells was estimated as described (3). Cells were exposed to A23187 and protease inhibitors for 6 hours to induce the enzyme. Then the cells were administered cycloheximide (10 μg/ml) and enzyme activity was measured after termination of the culture and disrupting the cells at the indicated time. 5HTP formation was determined using a high performance liquid chromatography,

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and the apparatus was equipped with a fluorescence monitor (JASCO, model FP920) set 302 nm and 350 nm for excitation and emission, respectively.

Results

Under resting conditions of RBL2H3 cells in monolayer culture, the tryptophan hydroxylase level, measured in a cell-free assay system, was around 5-10 pmol 5HTP formed per min per 10^6 cells. However, this level is maintained by continuous biosynthesis of this enzyme counterbalanced by rapid degradation. The degradation proceeded as a first order-like reaction. The rate varied from culture to culture but had half life of between 15 and 60 min. When the cells were stimulated with antigen/IgE or exposed to a calcium ionophore A23187 for more than 2 hours, the enzyme activity typically rose 7 or 20 fold over the initial level, respectively. For example, enzyme activity increased up to around 120-150 pmol/min/10^6 cells after 6 hours of incubation with 0.1 μM A23187. In our research to understand the intracellular signal transduction mechanisms which brought about this increase in enzyme level, we found that this process was sensitive to a group of protease inhibitors, Calpain inhibitor-1, Calpain inhibitor-2, MG115, and PSI, all known as calpain inhibitors and proteasome in-

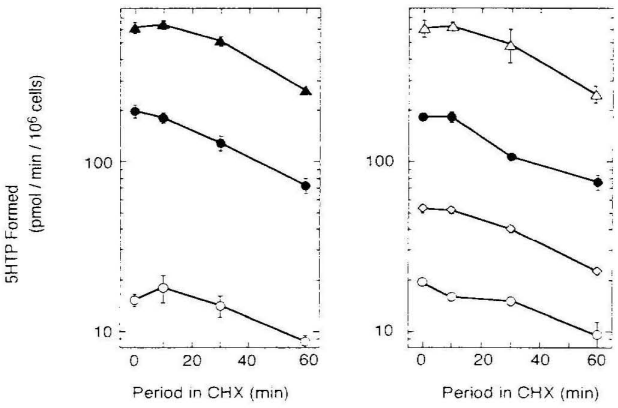

Figure 1. In vitro degradation of tryptophan hydroxylase: RBL2H3 cells (10^5 cells per well in a 96-well microassay plate) were placed in serum-free DMEM buffered with Hepes/NaOH 2 hours prior to the experiment; then the cells were exposed to A23187 (0.1 μM) with or without calpain inhibitor for 6 hours. Right and left panels represent results of two independent experiments. Thereafter they were placed in 10 μg/ml of cycloheximide (CHX) for the indicated periods. Non-treated (open circle), treated with A23187 (closed circle), A23187 plus Calpain inhibitor-1 at 1 μM (closed triangle; left panel) and at 10 μM (open diamond; right panel), and A23187 plus Calpain inhibitor-2 (open triangle; right panel).

Table 1. **Increase in Tryptophan Hydroxylase in the presence of A23187 and Protease Inbibitors**: RBL2H3 cells were kept as monolayer culture (10^5 cells/well in a 96-well microassay plate). Cells were treated with the indicated reagent for 6 hours. Then tryptophan hydroxylase activity was measured of disrupted cells by freezing and thawing for 2 times. Enzyme activity was expressed as 5HTP formation under standard assay conditions. Each experiment was conducted with different cultures. As described previously, tryptophan hydroxylase activity at a resting stage was variable between cultures. Data given are means±S.E(n=4). CPI-1: Calpain inhibitor-1, CPI-2: Calpain inhibitor-2, MG115: carbobenzoxy-L-Leucyl-L-Leucyl-L-Norvalinal, PSI: carbobenzoxy-Ile-Glu(O-t-Bu)-Ala-leucinal.

Stimulation of cells (6 hours)	Tryptophan hydroxylase activity
	pmol 5HTP/min/10^6cells
1Expreriment 1	
control	11.3±0.9
A23187 (0.1 μM)	178±11
A23187+CPI-1 (1.0 μM)	463±17
A23187+CPI-2 (10 μM)	367±41
Expreriment 2	
control	12.7±1.4
A23187 (0.1 μM)	54.4±11.8
A23187+MG115 (0.15 μM)	243±19
A23187+PSI (0.05 μM)	252±31
Experiment 3	
control	9.32±1.34
A23187 (0.1 μM)	85.5±21.2
MG115 (0.05 μM)	109±2
PSI (0.05 μM)	102±1
CPI-1 (1 μM)	76.9±8.9
CPI-2 (30 μM)	49.6±9.4

hibitors. Although calpains and proteasomes are known as extra lysosomal proteases and are obviously different, these inhibitors more or less overlap in their ability to inhibit these proteases (6). By stimulating RBL2H3 cells with the calcium ionophore and the protease inhibitors together or alone, we were able to observe various increases in levels of tryptophan hydroxylase activity after 6 hours of culture as depicted in Table 1 and Fig. 1. These changes coincided with changes in the amount of protein determined by Western blot analysis using anti-trytophan hydroxylase antibody as the primary probe (data not shown). As shown in Table 1, A23187 at 0.1 μM alone increased the enzyme activity by about 5-16 fold. A23187 plus protease inhibitor enhanced the increase up to about 10-40 fold. For example, CPI-1 at 1 μM alone also stimulated an enzyme increase up to 8 fold whereas A23187 plus CPI-1 enhanced it by 41 fold. And as depicted in Fig. 1 (right panel), the same inhibitor at more than 10 μM completely prevented the A23187-induced increase. A similar effect was observed with other reagents although the effective doses differed widely. Since the enzyme turnover by proteolysis, the inhibitors could interfere with the degradation. This was ture but a much higher dose was needed to elevate enzyme activity the resting conditions. Hence the present experiment using inhibitors dealt only with relatively low doses of the inhibitor.

Since the steady state level of the enzyme is determined by an equilibrium between fast degradation and equally fast biosynthesis, the observed increase in the enzyme level could either be accomplished by accelerated biosynthesis of the enzyme protein or by a reduced rate of degradation. We examined which process, biosynthesis or degradation, determines the level by measuring the degradation rate after arresting protein biosynthesis. Cells were treated with A23187 and Calpain inhibitors-1 and -2 for 6 hours. Then the cultures were given 10 μg/ml cycloheximide and enzyme activity was assayed with time (Fig. 1). The observed half lives of each case were all between 40 to 60 min. This result indicates that treatment with A23187 and/or calpain inhibitors did not affect the degradation rate of tryptophan hydroxylase. Therefore, the elevation of tryptophan hydroxylase was more likely driven by accelerated biosynthesis of this protein.

Discussion

Rapid turnover of trytophan hydroxylase is driven by proteolysis. The shortest half life of the enzyme so far observed was 10 min and most often was around 20 min. Moreover, and even slower decreases were observed. Little is known about the mechanism controlling proteolysis. This fast turnover could provide the cells with the ability to change the steady state level by attenuating the rate of degradation. We recently found the enzyme level rises in response to stimulation with antigen in the presence of the specific antibody (IgE) (5). In addition to the mechanism involved in this process, we wanted to know whether the rate of degradation was primarily attenuated in the change in enzyme level of the cells. The observed degradation of tryptophan hydroxylase was, however, almost constant under various conditions; at least, the difference was judged not to be sufficient to afford the changes in the enzyme level. As long as this experiment was concerned, the enzyme degradation was constitutive rather than inducible.

This judgement is based on the kinetics of the first order reaction. Namely, the enzyme half life was determined to be almost the same under resting and stimulated conditions. Since the initial enzyme level was 10 times higher in the stimulated cell, the actual processing was also 10 times faster. Degradation, therefore, takes place in a hit-and-react or non-saturable manner suggesting that the protease was abundant and the reaction velocity was determined by availability of the substrate. One problem that appears is what is the signal that the protease used to recognize the target protein. This problem is still open to investigation.

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