Stimulation of tryptophan hydroxylase production in a serotonin-producing cell line (RBL2H3) by intracellular calcium mobilizing reagents

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Abstract RBL2H3 cells showed a remarkable increase in their level of tryptophan hydroxylase (up to 25-fold), the rate-limiting enzyme in serotonin biosynthesis, by stimulation with intracellular calcium mobilizers A23187, thapsigargin, and *t*BuBHQ as well as by stimulation with an antigen in the presence of IgE. The increase in the enzyme protein was visualized by Western blot analysis using anti-tryptophan hydroxylase antiserum. The enzyme turnover (Hasegawa et al., *FEBS Lett.*, 368 (1995) 151–154) was not slowed down during the rise in tryptophan hydroxylase. Actinomycin D prevented the stimulation-induced elevation of the enzyme. These findings strongly suggest that this stimulation was achieved by the accelerated biosynthesis of tryptophan hydroxylase.

Key words: Tryptophan hydroxylase; Serotonin biosynthesis; Mast cell; Elevation, Ca^{2+}-dependent; A23187; IgE

1. Introduction

Tryptophan hydroxylase (TPH) is a tetrahydrobiopterin-dependent monooxygenase and is the rate-limiting enzyme in serotonin biosynthesis. This enzyme is located in specific neurons and secretory cells of peripheral organs including the pineal gland and gastrointestinal tract [1–3]. Studies on TPH have been hampered by difficulties in obtaining the pure enzyme in sufficient quantity and by the lack of an appropriate cell culture system, as compared with studies on phenylalanine or tyrosine hydroxylase which are also pterin-dependent monooxygenases. Rodent mast cells produce serotonin, and established cell lines of mast cell origin are available [4–8]. In our previous work, we found a very rapid turnover of TPH protein (half-life, 15–60 min) which was driven by ATP-dependent proteolysis in the mast cell lines, RBL2H3 and FMA3 [9]. This fast turnover might result in a quick increase in the enzyme level by slowing down degradation on demand. In terms of the stimulation response, however, little is known concerning the circumstances under which cells regulate the level of TPH activity or the manner in which this is accomplished. Mast cells discharge vesicular materials including serotonin. These cells might be equipped with mechanisms capable of restoring serotonin storage levels after discharge. With this possibility in mind, we sought an experimental system using mast cell lines in order to study the mechanisms involved. Although the stimulation-secretion pathway mediated by elevation of the intracellular Ca^{2+} concentration has been extensively studied [10], the restoration process has been little investigated. In the present study, an increase in the TPH level was demonstrated in RBL2H3 cells by administration of a group of reagents which are known to be intracellular-calcium mobilizers as well as by immune stimulation. The increase in the level of the enzyme was most probably driven by stimulation of de novo biosynthesis.

2. Materials and methods

Calcium ionophore A23187, actinomycin D, cycloheximide, anti-DNP IgE, and its antigen (DNP-conjugated BSA, DNP-BSA) were purchased from Sigma (St. Louis, MO). Thapsigargin and 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (*t*BuBHQ) were purchased from Alomone Labs (Israel). Dulbecco's Modified Eagle's Medium (DMEM) and Ca^{2+}-free DMEM were obtained from Gibco BRL (Gaithersburg, MD). RBL2H3 (a mast cell line derived from rat basophilic leukemia cells) was obtained from The Japanese Cancer Research Resources Bank (Tokyo). All cultures were given appropriate antibiotics from the day before and throughout the experiments. In experiments employing pharmacological agents, cells were placed in serum-free medium (buffered with HEPES/NaOH) 2 h before administration. Agents with low solubility in water were dissolved in dimethylsulfoxide (DMSO) at a concentration 100-fold greater than the final one used unless otherwise stated, so that the vehicle (mainly DMSO) would be at an equivalent level in each experimental culture with no vehicle effect.

Cell-free TPH activity was determined as described previously [9,11]. In brief, cells were placed in 20 μl of phosphate-buffered saline, then subjected twice to freezing in liquid nitrogen and thawing in water. The disrupted cells were incubated in a mixture consisting of 30 mM DTT, 50 μM $Fe(NH_4)_2(SO_4)_2$, and 4 mg/ml catalase in 0.1 M Tris-acetate (pH 8.1) for 15 min at 30°C. Then, another cocktail was added to afford a final reaction mixture of 250 μM tryptophan, 400 μM 6R-tetrahydrobiopterin, 500 μM NADH, 1 mM NSD-1015, 2 mg/ml catalase, and 4.5 μg/ml sheep liver dihydropteridine reductase (Sigma). The reaction was allowed to proceed for 10 min at 30°C. Due to substrate inhibition at higher concentrations of L-tryptophan, the enzyme exhibits its maximum activity under these conditions, where the apparent K_m is around 110 μM for L-tryptophan at a given pteridine cofactor concentration. The 5HTP formed was measured by high-performance liquid chromatography equipped with a fluorescence monitor (JASCO model, FP920) set at 302 nm and 350 nm for excitation and emission, respectively.

2.1. Western blot analysis

Blotted membrane filters were prepared after SDS-polyacrylamide gel electrophoresis (PAGE) of the cell extracts as described previously [9,12]. TPH protein was visualized using a rabbit polyclonal anti-TPH antiserum [2] as the primary antibody and a goat antibody to rabbit IgG (horse radish peroxidase-conjugated, Wako, Tokyo) as the secondary antibody. Extended color development usually yielded non-specific signals what were presumed to be proteolytic degradation products [13]. TPH was identified by simultaneous electrophoresis of the authentic enzyme which was purified from mouse mastocytoma P815 according to Nakata and Fujisawa [7].

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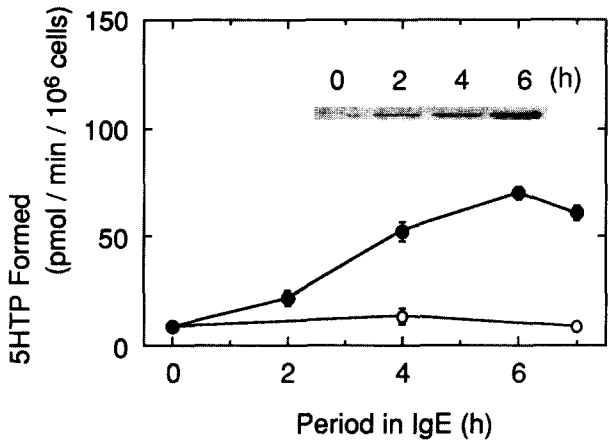

Fig. 1. Increase in TPH after addition of an antigen in the presence of specific IgE: RBL2H3 cells (1.0×10^5 cells/well of a 96-well microassay plate) were placed in serum-free DMEM buffered with HEPES/NaOH for 2 h prior to the experiment; then stimulated with anti-DNP mouse IgE (1 μg/ml) for 2 h. Thereafter, they were treated with (●) or without (○) DNP-BSA (0.1 μg/ml) and kept in culture for the indicated periods. TPH activity of the cells was determined as described in Section 2 ($n=4$). Insert: aliquots from incubations for 0, 2, 4, and 6 h were subjected to SDS-PAGE and Western blot analysis, as described in the legend to Fig. 2.

3. Results

3.1. Induction of tryptophan hydroxylase

Immune stimulation of mast cells by antigen in the presence of antigen-specific antibody (IgE) results in exocytotic discharge of the secretory vesicles. RBL2H3 cells retain this ability to respond [14,15]; the cells discharged about 20% of the cellular serotonin (total: ≈1.75 nmol/10^6 cells) within 1 h. A calcium ionophore A23187 (0.08 μM) caused serotonin release by RBL cells similar to that described for histamine release [10]. After immune stimulation (DNP-BSA/IgE), the TPH activity of RBL2H3 cells increased up to about 9-fold (Fig. 1). This increase in enzyme level occurred after certain lag period and then continued for at least 4 h, and with the highest activity observed at around 6 h of incubation. Even when DNP-BSA/IgE was washed away after 30-min incubation and the enzyme activity was determined at 6 h (from the initiation of stimulation), 85% of the maximum activity was observed and the initial 60-min incubation similarly resulted in about 98% of the maximum activity. This result suggests that 1 h stimulation seemed to be of sufficient duration to trigger the relevant increase. While serotonin release occurred soon after stimulation, the increase in the enzyme level was likely to require further elaboration inside the cells. The increase in enzyme activity was accompanied by an increase in the amount of protein which was visualized by Western blot analysis using specific antiserum to TPH (insert in Fig. 1). When the cells were placed in a Ca^{2+}-free medium, the DNP-BSA/IgE treatment was not effective. Exposure of RBL2H3 cells to high KCl medium (30 mM) did not result in a significant increase in the TPH level, suggesting that voltage gated calcium channels might not be involved in the utilization of extracellular calcium.

3.2. Effect of various calcium mobilizers

An increase in TPH activity was observed after a 2-h lag period and proceeded for the next 4 h (Fig. 2a). The initial enzyme level was typically about 5 pmol 5HTP formed per min per 10^6 cells (the level can vary from culture to culture, as mentioned previously [9]), and administration of 0.08 μM A23187 caused it to rise to about 115 pmol 5HTP/min/10^6 cells, a level roughly 20-fold higher than the basal level. Removal of the ionophore from the medium after 1 h did not interfere with the subsequent increase, as was the case with DNP-BSA/IgE. The above observation suggests that intracellular calcium mobilization was crucial for the increase in TPH. An appreciable augmentation of TPH was also ob-

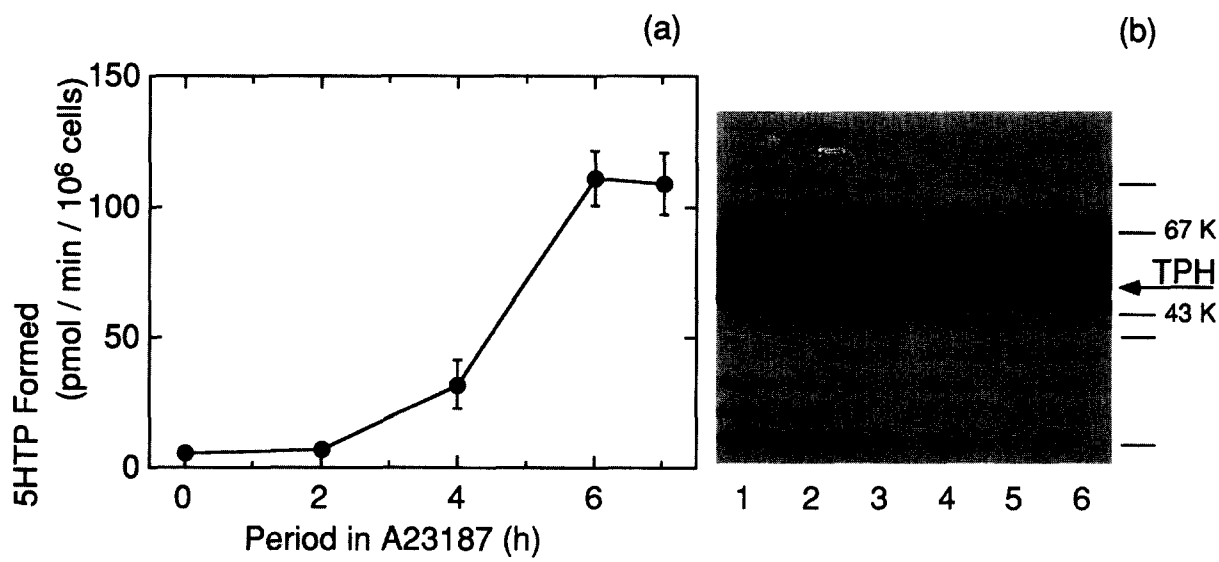

Fig. 2. Increase in TPH by the Ca^{2+}-ionophore A23187: (a) RBL2H3 cells were exposed to 0.08 μM A23187 for the indicated periods. TPH activity of the cells was determined as described in Section 2 ($n=4$). (b) RBL2H3 cells exposed to A23187 as above were washed with PBS and disrupted in 1% NP40 containing 1 mM PMSF. The supernatant solution was subjected to SDS-PAGE for Western blot analysis. Lanes 1–4; A23187 for 0, 2, 4, and 6 h (10 μg each of extracted protein). Lane 5; 60 ng of purified TPH from mouse mastocytoma P-815. Lane 6; 4.5 ng of TPH + 2.5 μg of protein extract from a 6-h incubation. Thin lines represent marker proteins (disregard a thin line next to 43 k), from top to bottom; 94 k, 67 k, 43 k, and 30 k.

served by administration of thapsigargin (peak at 3 nM), *t*BuBHQ (peak at 10 μM) as well as A23187 (peak at 0.08 μM) (Fig. 3). All the described increases in TPH activity in RBL cells coincided with the increases in the amount of protein visualized by Western blot analysis (see Fig. 2b and the insert in Fig. 3 for A23187 and thapsigargin, respectively, not shown for *t*BuBHQ).

3.3. Turnover of tryptophan hydroxylase

Since the level of TPH in RBL cells is maintained by rapid biosynthesis counter-balanced by rapid degradation, we examined whether the rise in the enzyme level was due to a slowdown in the protein turnover. Turnover was estimated by tracing enzyme activity after administration of cycloheximide (10 μg/ml), as reported previously [9], both before and during the ionophore-induced increase in the enzyme (Fig. 4). Before stimulation, the steady-state level was 8.2 ± 0.9 pmol 5HTP/min/10^6 cells, and the apparent half-life of TPH was about 60 min. At 6 h after calcium ionophore administration, the enzyme activity rose roughly 28-fold (232 ± 15 pmol 5HTP/min/10^6 cells), and in this case, the half-life was about 30 min. Thus, proteolysis after stimulation was no slower than the fast degradation which occurs in the resting stage of the cell. This feature was also observed in the case of immune stimulation in vitro (data not shown). Generally, the steady-state rate of biosynthesis is equal to that of degradation, and in the present system, the degradation rate was not slower. Therefore, the elevated level of TPH activity was judged to result from an increase in the rate of biosynthesis.

3.4. Effect of Actinomycin D

When actinomycin D (10 μg/ml) was administered to RBL2H3 cells, TPH activity appeared not to be affected for at least 4 h, suggesting that the mRNA of TPH is more stable than the protein. In the presence of actinomycin D, administration of A23187 (0.1 μM) did not raise TPH activity. When actinomycin D was administrated at 2 h after addition of A23187, TPH activity did not rise. When the inhibitor was added half way during the increase in enzyme level (at 4 h, Fig. 5), the rise was terminated.

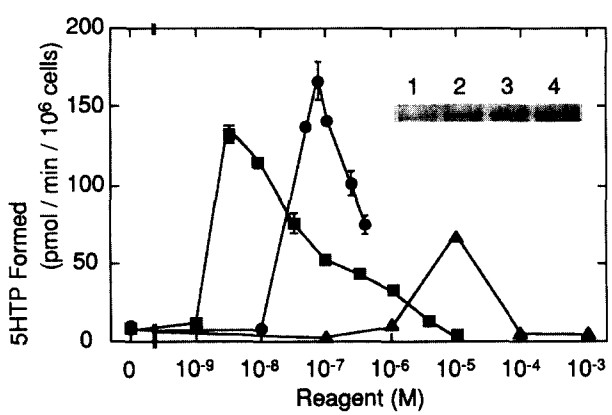

Fig. 3. Effect of intracellular calcium mobilizers on cellular TPH activity: RBL2H3 cells were placed in serum-free DMEM 2 h before the experiment. The cells were exposed to Ca^{2+} mobilizing agents at the indicated concentrations for 6 h. Thapsigargin (■), A23187 (●), and *t*BuBHQ (▲) were dissolved in DMSO as the vehicle (final 1% in the medium). TPH activity of the cells was determined as described in Section 2 ($n = 4$). Insert: Cell extracts from thapsigargin treatment were subjected to SDS-PAGE and Western blot analysis; 1–4 correspond to 10^{-9}, 3×10^{-9}, 10^{-8}, and 3×10^{-8} M thapsigargin, respectively.

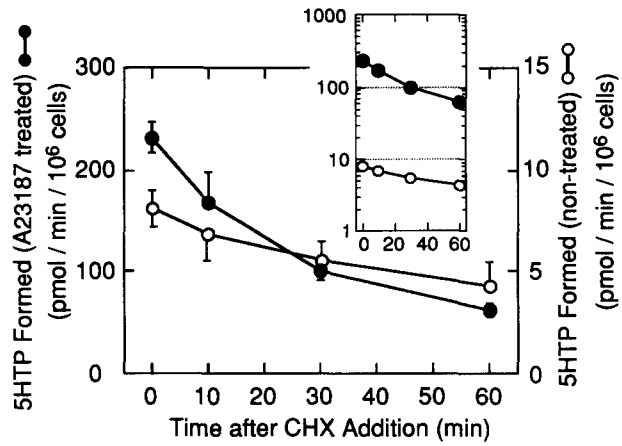

Fig. 4. Degradation of TPH before and during A23187-induced elevation: RBL2H3 cells were exposed to 0.1 μM A23187 for 6 h. Protein degradation was shown by blocking protein synthesis with cycloheximide (CHX, 10 μg/ml) before (○, see right ordinate) and during (●, left ordinate) A23187-induced elevation of TPH activity ($n = 4$). Insert: the same data were plotted on a semi-logarithmic scale.

4. Discussion

A novel increase in TPH activity by immune stimulation in vitro or by administration of a group of agents known as intracellular calcium mobilizers was demonstrated with the mast cell line RBL2H3. We previously demonstrated that a steady-state level of TPH is maintained by continuous biosynthesis counter-balanced by fast degradation due to ATP-dependent proteolysis [9]. With the increase observed in this study, however, the rate of degradation was shown to be unaffected. In addition, administration of actinomycin D prevented ionophore-induced elevation of the enzyme level whereas the reagent alone did not affect the level of the enzyme for at least 4 h, which suggests that the increase likely involved accelerated transcription of the *TPH* gene. However, the mechanism involved in this acceleration is not yet clear. In any case, this rise in the steady-state level was driven by an increased rate of biosynthesis of the enzyme protein rather than by a slowdown in enzyme degradation.

Administration of an antigen and its specific antibody (IgE) is a useful physiological model of stimulation of cells with characteristics of mast cells. In this study, stimulation was carried out with the calcium mobilizers thapsigargin, *t*BuBHQ, and A23187. Although the mechanisms of action differ, these reagents are well known to elevate the intracellular calcium concentration; thapsigargin is an inhibitor of vesicular Ca^{2+}-ATPase [16] and *t*BuBHQ causes release of Ca^{2+} from an inositol 1,4,5-*tris*-phosphate-sensitive Ca^{2+} pool [17]. These results suggest that the intracellular rise in the Ca^{2+} concentration, simultaneously triggered the relevant signal transduction cascade, resulting in the observed increase in TPH and vesicular secretion. It was noted that all of the effective agents, thapsigargin, calcium ionophore A23187, and *t*BuBHQ, exhibited a narrow effective dose range (Fig. 3). The negative effect at high doses might have counter-

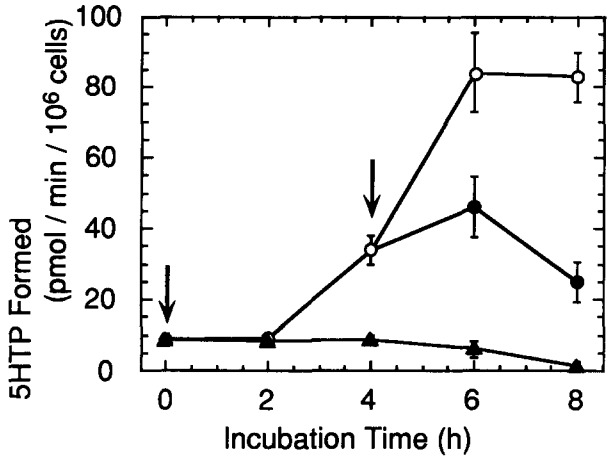

Fig. 5. Effect of actinomycin D on the increase in TPH induced by A23187: RBL2H3 cells were treated with 0.1 μM A23187 (○). Actinomycin D was administered at 10 μg/ml to RBL2H3 cells just before the addition of ionophore (▲) and after 4 h of A23187 exposure (●). Arrows indicate the time of actinomycin D addition.

balanced the positive effect, resulting in the observed dose dependent inhibition.

Other cells used in our laboratory, FMA3 (Furth's mastocytoma) and P-815 (Dan Potter's mastocytoma), maintain a high enzyme level, (100–200 pmol 5HTP/min/10^6 cells, which is 10–20-fold greater than RBL cells under non-stimulating conditions). However, these cells showed little increased in enzyme activity in response to immune stimulation in vitro (DNP-BSA/IgE) or to the calcium ionophore A23187. RBL cells show very low enzyme activity under non-stimulating conditions but can be upregulated. The mouse mastocytoma P-815 retains little activity of L-aromatic amino acid decarboxylase [8], presumably because it was lost at some point since first established more than three decades ago. FMA3 and P-815 cells might also have lost the ability to maintain TPH activity at a low level for later up-regulation. On the other hand, RBL cells appear to have been maintained selectively as high responders to immune stimulation. However, the strong response of RBL cells seen in this study could be attributed to a loss in the ability of natural mast cells for masking it. Therefore, the extent to which non-neonatal cells might be capable of this response remains to be studied.

Tyrosine hydroxylase is well known to be activated by phosphorylation, mainly by a cyclic AMP-dependent protein kinase (lowered K_m for BH_4 which is sub-saturating in the tissue). Phenylalanine hydroxylase is also activated by phosphorylation with the A-kinase (heightened V_{max}). TPH in brain is known to increase activity by about 2-fold as a result of phosphorylation with calcium–calmodulin-dependent protein kinase II, but only in the presence of 14–3–3 protein, a group-activating protein affecting various neural proteins [18]. Therefore, we were very interested in the phosphorylation of this enzyme in RBL2H3 cells. However, there have been no reports on phosphorylation of any peripheral TPH. Since the strong increase in enzyme activity (15–25-fold) was accompanied by a rise in enzyme protein, phosphorylation of the enzyme, if any, may not be the predominant mechanism for stimulating enzyme activity as seen in the present study. With regard to cAMP-related up-regulation of gene expression, increases in immunoreactive substances [19] or in the relevant mRNA [20] were reported to occur in cultured cells of neural origin. These increases which took place over the course of several days of culture, seemed to be due to cell differentiation. Recently, studies appeared in which transformed culture cells were used with the recombinant *TPH* gene upstream combined with reporter genes. The gene expression measured by CAT activity was demonstrated to be accelerated with cAMP [21]. No positive effect of cAMP-related agents, including muscaline, bethanechol, forskolin, theophylline, or dibutyryl cyclic AMP, have been observed in our short-term experiment (less than 8 h, data not shown).

In conclusion, the increase in TPH observed in this study was not driven by enhanced degradation of the enzyme protein, and can be attributed to accelerated biosynthesis. This is the first report demonstrating that a serotonin-producing cell is equipped with mechanisms for increasing the TPH level in response to extracellular stimulation.

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References

[1] Ichiyama, A., Hasegawa, H., Tohyama, C., Dohmoto, C. and Kataoka, T. (1976) in: Iron and Copper Proteins, Vol. 74, pp. 103–117 (Yasunobu, K.T., Mower, H.F. and Hayaishi, O., eds.), Plenum Press, New York.
[2] Hasegawa, H., Yanagisawa, M., Inoue, F., Yanaihara, N. and Ichiyama, A. (1987) Biochem. J. 248, 501–509.
[3] Inoue, F., Hasegawa, H., Yamada, M. and Ichiyama, A. (1987) Biomed. Res. 8, 53–59.
[4] Sato, T.L., Jequier, E., Lovenberg, W. and Sjoerdsma, A. (1967) Eur. J. Pharmacol. 1, 18–25.
[5] Hosoda, S. (1975) Biochim. Biophys. Acta 397, 58–68.
[6] Kuhn, D.M., Rosenberg, R.C. and Lovenberg, W. (1979) J. Neurochem. 33, 15–21.
[7] Nakata, H. and Fujisawa, H. (1982) Eur. J. Biochem. 124, 595–601.
[8] Yanagisawa, M., Hasegawa, H., Ichiyama, A., Hosoda, S. and Nakamura, W. (1984) Biomed. Res. 5, 19–28.
[9] Hasegawa, H., Kojima, M., Oguro, K. and Nakanishi, N. (1995) FEBS Lett. 368, 151–154.
[10] Foreman, J.C., Hallett, M.B. and Mongar, J.L. (1977) J. Physiol. (Lond.) 271, 193–214.
[11] Hasegawa, H. and Ichiyama, A. (1987) Methods Enzymol. 142, 88–92.
[12] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[13] Hasegawa, H., Kojima, M., Oguro, K., Watabe, S. and Nakanishi, N. (1995) Pteridines 6, 138–140.
[14] Barsumian, E.L., Isersky, C., Petrino, M.G. and Siraganian, R.P. (1981) Eur. J. Immunol. 11, 317–323.
[15] Kulczycki, A., Jr., Isersky, C. and Metzger, H. (1974) J. Exp. Med. 139, 600–616.
[16] Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R. and Dawson, A.P. (1990) Proc. Natl. Acad. Sci. USA 87, 2466–2470.
[17] Kass, G.E., Duddy, S.K., Moore, G.A. and Orrenius, S. (1989) J. Biol. Chem. 264, 15192–15198.
[18] Isobe, T., Ichimura, T., Sunaya, T., Okuyama, T., Takahashi, N., Kuwano, R. and Takahashi, Y. (1991) J. Mol. Biol. 217, 125–132.
[19] Mary, J.E. and Scott, R.W. (1995) J. Neurobiol. 28, 465–481.
[20] Foguet, M., Hartikka, J.A., Schmuck, K. and Lubbert, H. (1993) EMBO J. 12, 903–910.
[21] Boularand, S., Darmon, M.C., Ravassard, P. and Mallet, J. (1995) J. Biol. Chem. 270, 3757–3764.