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IMP Dehydrogenase Inhibitors Reduce Intracellular Tetrahydrobiopterin Levels through Reduction of Intracellular GTP Levels

INDICATIONS OF THE REGULATION OF GTP CYCLOHYDROLASE I ACTIVITY BY RESTRICTION OF GTP AVAILABILITY IN THE CELLS*

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GTP cyclohydrolase I exhibits a positive homotropic cooperative binding to GTP, which raises the possibility of a role for GTP in regulating the enzyme reaction (Hatakeyama, K., Harada, T., Suzuki, S., Watanabe, Y., and Kagamiyama, H. (1989) *J. Biol. Chem.* 264, 21660–21664). We examined whether or not the intracellular GTP level is within the range of affecting GTP cyclohydrolase I activity, using PC-12 rat pheochromocytoma and IMR-32 human neuroblastoma cells. Since GTP cyclohydrolase I was the rate-limiting enzyme for the biosynthesis of tetrahydrobiopterin in these cell lines, the intracellular activities of this enzyme were reflected in the tetrahydrobiopterin contents. We found that the addition of guanine or guanosine increased GTP but not tetrahydrobiopterin in these cells. On the other hand, three IMP dehydrogenase inhibitors, tiazofurin, 2-amino-1,3,4-thiadiazole, and mycophenolic acid, decreased both GTP and tetrahydrobiopterin in a parallel and dose-dependent manner, and these effects were reversed by the simultaneous addition of guanine or guanosine. There was no evidence suggesting that these inhibitors inhibited other enzymes involved in the biosynthesis and regeneration of tetrahydrobiopterin. Comparing intracellular activities of GTP cyclohydrolase I in the inhibitor-treated cells with its substrate-velocity curve, we estimated that the intracellular concentration of free GTP is 150 μM at which point the activity of GTP cyclohydrolase I is elicited at its maximum velocity. Below this GTP concentration, GTP cyclohydrolase I activity is rapidly decreased. Therefore GTP can be a regulator for tetrahydrobiopterin biosynthesis.

It is well established that tetrahydrobiopterin (BH_4)[1] is an essential cofactor for three aromatic amino acid monooxygenases involved in the biosynthesis of serotonin and catecholamines (1, 2) and for *O*-alkylglycerolipid cleavage enzyme (3). Recently, BH_4 was also found to be a cofactor for both the Ca^{2+}-dependent and -independent enzyme systems producing nitric oxide (4–6). BH_4 is thus required in the synthesis of these biologically active compounds; in addition, several lines of evidence suggest a regulatory role for BH_4 in many systems. Since the administration of BH_4 enhances dopamine synthesis in rat striatum *in vivo* (7) and in cultured sympathetic neurons (8), the level of intracellular BH_4 is thought to be present in a range of concentrations substantially less than K_m for tyrosine monooxygenase, the rate-limiting enzyme in catecholamine biosynthesis. The formation of nitric oxide is also limited by the BH_4 level in murine fibroblasts (9) and murine brain endothelial cells (10). Furthermore, the differentiation of erythroid cells requires a decrease in the BH_4 level (11). It is thus important to reveal the regulatory mechanisms of the intracellular BH_4 level.

BH_4 is synthesized from GTP by the successive actions of GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase, and sepiapterin reductase (12). The rate-limiting enzyme of BH_4 biosynthesis is GTP cyclohydrolase I, which catalyzes the conversion of GTP to dihydroneopterin triphosphate (12). The activity of this enzyme is lowest among the three biosynthetic enzymes (12–14). GTP cyclohydrolase I activity is increased in a number of cells by interferon-γ treatment (13, 15) and in the adrenal glands by insulin treatment (16) and is decreased during maturation of murine reticulocytes (11, 14) and differentiation of erythroid cells (11). The mechanism of changes in GTP cyclohydrolase I activity, however, remains to be clarified.

We observed previously the positive cooperative binding of GTP to rat GTP cyclohydrolase I and raised the possibility that GTP was one of the regulatory factors for its enzyme activity *in vivo* (17). Knowledge of intracellular GTP levels has important implications for the evaluation of this hypothesis. The key question is whether intracellular GTP levels fall in the range known to affect GTP cyclohydrolase I activity. However, an accurate level of intracellular free GTP has not yet been determined because GTP may exist in separate pools in the cells (18–20). An alternative way to test our prediction is to examine the effect of change in intracellular GTP levels on BH_4 levels. Since GTP cyclohydrolase I is the rate-limiting enzyme in the biosynthesis of BH_4, changes in its activity should result in changes in BH_4 production. A number of studies have shown that GTP levels in intact cells can be lowered by treatment with IMP dehydrogenase inhibitors and raised by incubation with guanine compounds (*e.g.* Refs. 21–23); IMP dehydrogenase is the rate-limiting enzyme in the *de novo* synthesis of GTP.

In the present study, we used three IMP dehydrogenase inhibitors (tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide), 2-amino-1,3,4-thiadiazole, and mycophenolic acid) to manipulate GTP levels in PC-12, derived from rat pheochro-

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[1] The abbreviations used are: BH_4, 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium.

mocytoma, and IMR-32, from human neuroblastoma, and examined their effects on BH_4 levels in these cells. PC-12 cells were previously reported to synthesize BH_4 (24), and IMR-32 cells were shown to synthesize BH_4 in this study.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the indicated sources: Eagle's minimum essential medium containing kanamycin and Dulbecco's modified Eagle's medium (DMEM) from Nissui (Japan); horse serum, fetal calf serum, and nonessential amino acids from GIBCO; penicillin, streptomycin, bovine insulin, human transferrin, progesterone, putrescine dihydrochloride, sodium selenite, mycophenolic acid, neopterin, pterin, and biopterin from Sigma; 2-amino-1,3,4-thiadiazole from Aldrich; trypsin (1:250) from Difco; guanine and guanosine from Kohjin (Kyoto, Japan); GTP from Yamasa (Chiba, Japan); Hepes from Dojindo Laboratories (Kumamoto, Japan); Cosmosil columns from Nacalai Tesque (Kyoto, Japan); and Partisil columns from Whatman. Sepiapterin and tiazofurin were generous gifts from Dr. M. Masada, University of Chiba, Chiba, Japan and Drs. Y. Natsumeda and G. Weber, Indiana University, respectively.

Cell Culture—PC-12 cells and IMR-32 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and the Japanese Cancer Research Resources Bank (Tokyo, Japan), respectively. PC-12 cells were maintained at 37 °C and a CO_2 concentration of 10% in DMEM containing 10% horse serum, 10% fetal calf serum, 50 units/ml penicillin, 100 μg/ml streptomycin, and 4 mM glutamine; culture medium was changed three times a week, and subculturing was done once a week using 0.25% trypsin in phosphate-buffered saline. IMR-32 cells were maintained at 37 °C and a CO_2 concentration of 5% in Eagle's minimum essential medium containing 10% fetal calf serum, 0.1 mM nonessential amino acids, and 2 mM glutamine; culture medium was changed three times a week, and subculturing was done once a week using trypsin solution. For experimental use, PC-12 and IMR-32 cells were plated at a density of 5×10^5 cells/well (9.6 cm^2) of 6-well tissue culture plates (Falcon) and grown for 2 days. Thereafter, the cells were washed with Hanks' balanced salt solution and incubated for 20 h in serum-free defined media containing IMP dehydrogenase inhibitors and/or guanine compounds as indicated in the text. The serum-free medium used for PC-12 cells contained DMEM (high glucose) supplemented with 15 mM Hepes buffer pH 7.2, 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM selenium, 50 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine as described by Bottenstein and Sato (25, 26). IMR-32 cells were cultured in Eagle's minimum essential medium containing 0.1 mM nonessential amino acids, 2 mM glutamine, 5 μg/ml bovine insulin, 5 μg/ml human transferrin, and 30 nM selenium.

High Performance Liquid Chromatographic Analysis of Nucleotides and Pteridines—Cells were harvested by aspirating the medium and immediately adding 500 μl of ice-cold 0.4 N perchloric acid to extract nucleotides and pteridines. Thereafter, cells were kept on ice for 30 min. Then the solutions of extracts were collected and centrifuged, and the supernatant solutions were subjected to analyses for nucleotides and pteridines. The residual cells were solubilized with 700 μl of 0.1 N NaOH for 30 min on ice for protein quantitation.

For nucleotide analysis, aliquots of the perchloric acid extracts were neutralized with KOH, and the precipitated potassium perchlorate was removed by centrifugation. The resulting supernatant was applied to a Cosmosil 5 C_{18} column (4.6 × 250 mm) connected to a Cosmosil 10 C_{18} column (4.6 × 50 mm). Nucleotides were eluted isocratically with a solvent of 0.2 M sodium phosphate buffer (pH 6.0) at a flow rate of 1.0 ml/min. The column temperature was maintained at 25 °C. The eluate was monitored by ultraviolet absorbance at 254 nm.

For pteridine analysis, aliquots (300 μl) of the perchloric acid extracts were oxidized with 50 μl of iodine solution (2.5% I_2, 10% KI in 0.2 N perchloric acid) for 1 h at room temperature (24, 27). After the addition of 50 μl of 5% ascorbic acid, the sample was applied to a Partisil 10 ODS column (4.6 × 250 mm). Biopterin and pterin were eluted isocratically with a solvent of 50 mM sodium acetate buffer (pH 5.0) containing 0.1 mM EDTA and 5% methanol at a flow rate of 0.8 ml/min. The column temperature was maintained at 25 °C. The eluate was monitored with a fluoromonitor (excitation, 350 nm; emission, 440 nm). BH_4 was determined as described previously (28, 29) with minor modifications as follows. The extraction of BH_4 was performed as described above, except the extraction solution contained 5 mM dithiothreitol in 0.2 N trichloroacetic acid. After centrifugation, the trichloroacetic acid extract was treated twice with ethyl ether and applied to a Partisil SCX-10 strong cation-exchange column (4.6 × 250 mm). BH_4 was eluted isocratically with a solvent of 0.1 M ammonium acetate buffer (pH 3.8) containing 5% methanol at a flow rate of 1.0 ml/min. The eluate was monitored electrochemically with an amperometric detector (oxidation potential, 300 mV).

Enzyme Assay—The cells were cultured in 25-cm^2 dishes. After aspirating the medium, the cells were washed with 5 ml of Hanks' balanced salt solution and dipped into 0.25% trypsin in phosphate-buffered saline. After aspirating the trypsin solution, the cells were incubated at 37 °C for 5 min, and the detached cells in each dish were then suspended in 2 ml of ice-cold culture medium. The cells were collected with a centrifuge and washed with 4 ml of phosphate-buffered saline. The collected cells were soaked in 400 μl of ice-cold 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml bovine serum albumin and were lysed by sonication at 50 Hz with a Branson sonifier. The cell lysate was centrifuged, and the resulting supernatant was used for enzyme assay. The enzyme solution was filtered through a column of Sephadex G-25 equilibrated with 20 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.1 M KCl, and 0.2 mM phenylmethylsulfonyl fluoride, and the eluate was used for the determination of GTP cyclohydrolase I activity.

GTP cyclohydrolase I activity was assayed by the method of Duch *et al.* (30) with some modifications (17). The 6-pyruvoyl tetrahydropterin synthase activity was assayed as described previously (31). Sepiapterin reductase activity was assayed by the method of Ferre and Naylor (32) except that the mobile phase (5% methanol) used for high performance liquid chromatography was replaced with 50 mM sodium acetate (pH 5.0) in 5% methanol. Dihydropteridine reductase activity was assayed by the method of Craine *et al.* (33).

Protein Assay—The concentration of protein was determined by the method of Lowry *et al.* (34), as modified by Peterson (35). Bovine serum albumin was used as the standard.

RESULTS

IMP Dehydrogenase Inhibitors Decrease GTP and Biopterin Levels in PC-12 Cells—The effects of three IMP dehydrogenase inhibitors on the GTP levels in PC-12 pheochromocytoma cells are shown in Fig. 1. The GTP levels were reduced in the presence of these inhibitors in a dose-dependent manner. The respective EC_{50} values for tiazofurin, mycophenolic acid, and 2-amino-1,3,4-thiadiazole were approximately 100, 0.1, and 200 μM. The IMP levels increased in inverse proportion to the decrease in GTP levels (data not shown). As observed in leukemia cell lines and hepatoma cells (21–23), the ATP levels decreased slightly to 91, 91, and 84% of control values after treatment with 2-amino-1,3,4-thiadiazole (500 μM), tiazofurin (100 μM), and mycophenolic acid (0.1 μM), respectively. These

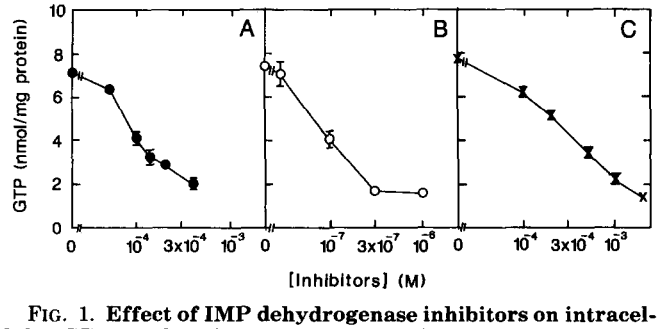

FIG. 1. **Effect of IMP dehydrogenase inhibitors on intracellular GTP levels.** PC-12 cells were treated with various concentrations of tiazofurin (*A*), mycophenolic acid (*B*), and 2-amino-1,3,4-thiadiazole (*C*) for 20 h. GTP amounts were measured by high performance liquid chromatography as described under "Experimental Procedures." Data shown are representative of four independent experiments with similar results. Each *point* represents means from two identical experiments performed in separate cultures; the *bars* represent the standard error.

results indicate that IMP dehydrogenase inhibitors are able to reduce selectively GTP levels in PC-12 cells.

The biopterin levels in PC-12 cells were determined using the same perchloric acid extracts as those used for the measurement of ribonucleotides. As shown in Fig. 2, all three inhibitors reduced the levels of the cellular biopterin. The data presented in Fig. 2 represent the total biopterin content because the analysis was carried out with acid-oxidized samples. By analyzing nonoxidized samples, we found that more than 98% of the biopterins were tetrahydrobiopterin (data not shown). The amount of biopterin released into the medium was also decreased concomitantly with the decrease in intracellular BH_4. These results suggested that the biopterin production in PC-12 cells declined as a result of the treatment with IMP dehydrogenase inhibitors.

In addition to biopterin, we also measured the amounts of other pteridines, neopterin and pterin, in PC-12 cells. Neopterin is derived from dihydroneopterin triphosphate, the substrate for 6-pyruvoyl-tetrahydropterin synthase. If this enzyme is inhibited by the IMP dehydrogenase inhibitors, dihydroneopterin triphosphate would accumulate in the cells. Pterin is a degradation product of BH_4 (36) and can also be derived from 6-pyruvoyl-tetrahydropterin, a substrate for sepiapterin reductase (29). Therefore, if sepiapterin reductase is inhibited by the IMP dehydrogenase inhibitors, an increase in pterin levels should be observed. In neither treated nor untreated PC-12 cells did we detect neopterin and pterin. In the medium, neopterin was not found but pterin was detected. The amount of pterin in the medium was decreased with increasing concentrations of the inhibitors, concurrent with the reduction in biopterin in the cells. Thus, it is unlikely that IMP dehydrogenase inhibitors inhibit 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase involved in the BH_4 biosynthesis.

Reversal of the Effects of IMP Dehydrogenase Inhibitors by Guanine and Guanosine—If the decrease in the biopterin level by IMP dehydrogenase inhibitors was the result of the decrease in GTP level in PC-12 cells, the decline of biopterin production should be overcome by the simultaneous supply of guanosine or guanine. Since there is a known salvage synthesis of guanine nucleotides by hypoxanthine-guanine phosphoribosyltransferase, guanine or guanosine would supply GTP without IMP dehydrogenase. As shown in Table I, guanosine or guanine added simultaneously with the inhibitors completely reversed the decrease in biopterin level caused by the inhibitors. This result suggests that there is a close link between the reductions in biopterin and GTP levels by IMP dehydrogenase inhibitors in PC-12 cells.

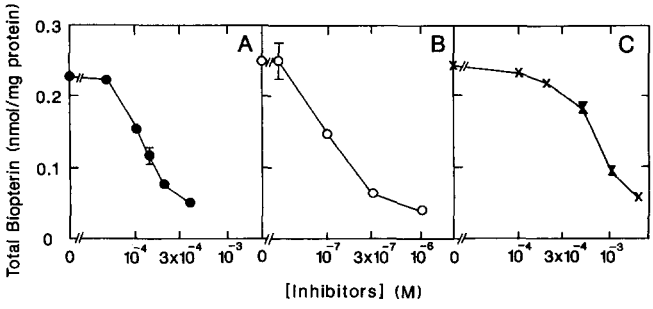

FIG. 2. **Effect of IMP dehydrogenase inhibitors on intracellular biopterin levels.** PC-12 cells were treated with various concentrations of tiazofurin (*A*), mycophenolic acid (*B*), and 2-amino-1,3,4-thiadiazole (*C*) for 20 h. The procedures for extraction and measurement of biopterin were described under "Experimental Procedures." Data shown are representative of four independent experiments with similar results. Each *point* represents means from two identical experiments performed in separate cultures; the *bars* represent the standard error.

Close Relationship between GTP and Biopterin Levels in PC-12 Cells—Fig. 3 shows the relationships between the GTP and biopterin levels in PC-12 cells. The GTP values were obtained from Fig. 1, and the corresponding biopterin values were obtained from Fig. 2. The curves for the three inhibitors exhibited common sigmoidal shapes. These shapes were similar to the sigmoidal saturation kinetics of purified GTP cyclohydrolase (17). In view of the fact that GTP cyclohydrolase I is the rate-limiting step in the biosynthesis of BH_4 in PC-12 cells (Table II), the similarity between the substrate-velocity curve of GTP cyclohydrolase I (Fig. 5*A*) and the GTP-biopterin curves (Fig. 3) suggests that the enzyme also behaves in a positive cooperative manner in the cells.

We then treated PC-12 cells only with guanine or guanosine in order to examine the effect of increased intracellular GTP levels upon intracellular biopterin levels. The treatment with guanine or guanosine increased the GTP level but did not increase the biopterin levels (Table I). On the other hand, on addition of the inhibitors, the level of biopterin started to decrease as the GTP levels decreased slightly from the normal level found in the untreated cells (7–8 nmol/mg of protein) (Fig. 3). These results suggest that the cellular GTP concentration in PC-12 cells is at the marginal level to allow maximum BH_4 production.

The Simultaneous Decreases in GTP and Biopterin Levels in IMR-32 Cells—In order to generalize the above relationship between GTP and biopterin levels, we examined the effect of mycophenolic acid on IMR-32 cells that were derived from human neuroblastoma. As shown in Fig. 4, mycophenolic acid lowered both GTP and biopterin levels in the cells in a manner similar to that observed for PC-12 cells. Therefore, the intracellular concentrations of GTP were found in the range effectively altering the biopterin level in neuronal cell lines from two different species. As noted in the legend for Fig. 4, each biopterin level of the two neuronal cell lines was different. However, when the biopterin levels were plotted as a percentage of each basal level (Fig. 4), the shape of the GTP-biopterin curve observed on IMR-32 cells closely resembled that observed on PC-12 cells. It should be noted that the amounts of GTP (7–8 nmol/mg of protein) were nearly equal among the neuronal cells of the above two species (Fig. 4).

Similarity between in Vivo and in Vitro Kinetic Behavior of GTP Cyclohydrolase I—The shapes of the GTP-biopterin curves observed on IMR-32 cells were sigmoidal as observed on PC-12 cells (Fig. 4). Since the rate-limiting step of BH_4 biosynthesis in IMR-32 cells was also the reaction catalyzed by GTP cyclohydrolase I (Table II), the GTP-biopterin curves would reflect the properties of their GTP cyclohydrolase I. To see if human GTP cyclohydrolase I also exhibits allosteric behavior as does the rat enzyme, we performed a kinetic analysis of GTP cyclohydrolase I activity in the cell lysates from IMR-32 cells. The saturation curves shown in Fig. 5*A* revealed that human GTP cyclohydrolase I also showed a positive cooperativity against GTP. The data obtained *in vivo* and *in vitro* were thus closely correlated (Figs. 4 and 5). Hill's coefficient, a cooperativity index, of the human enzyme was 2.5, a value that was nearly equal to the coefficient of 2.9 for the rat enzyme (Fig. 5*B*). The GTP concentration that produced half-maximal velocity (70 μM) of the human enzyme was nearly equal to that of the rat enzyme (60 μM) (Fig. 5*A*). Thus, the properties of rat and human GTP cyclohydrolase I were very similar. Furthermore, the GTP levels under normal conditions were nearly the same in both PC-12 and IMR-32 cells as described above. Another similarity between the hu-

TABLE I

Effects of guanine compounds on IMP dehydrogenase inhibitor-induced decreases in GTP and biopterin contents in PC-12 cells

PC-12 cells were treated with IMP dehydrogenase inhibitors in the absence and presence of guanine or guanosine. Values shown represent means ± S.E. from two identical experiments performed in separate cultures.

Treatment	GTP		Biopterin	
	nmol/mg protein	%	pmol/mg protein	%
Control	8.3 ± 0.1	100	194 ± 21	100
+10 μM guanine	10.5 ± 0.7	127	192 ± 3	99
+10 μM guanosine	11.0 ± 0.3	133	191 ± 16	98
100 μM tiazofurin	4.1 ± 0.0	49	104 ± 5	54
+10 μM guanine	6.5 ± 0.1	78	174 ± 4	90
+10 μM guanosine	8.5 ± 0.1	102	216 ± 7	111
0.1 μM mycophenolic acid	2.8 ± 0.1	34	70 ± 1	36
+10 μM guanine	10.7 ± 0.4	129	189 ± 6	97
+10 μM guanosine	10.9 ± 0.3	131	187 ± 6	96
300 μM 2-amino-1,3,4-thiadiazole	6.1 ± 0.2	73	153 ± 6	79
+10 μM guanine	11.1 ± 0.3	134	203 ± 10	105
+10 μM guanosine	10.7 ± 1.2	129	207 ± 20	107

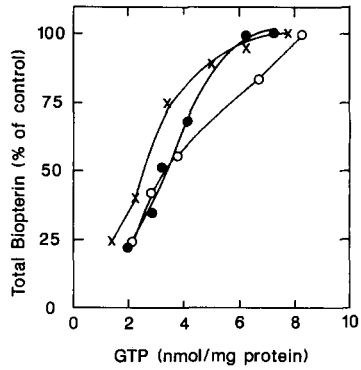

FIG. 3. **Relationship between intracellular GTP and biopterin levels in PC-12 cells.** The values of GTP shown in Fig. 1 were plotted against the corresponding values of biopterin shown in Fig. 2 for tiazofurin (●), mycophenolic acid (○), and 2-amino-1,3,4-thiadiazole (×). Both values were expressed as a percentage of the control values.

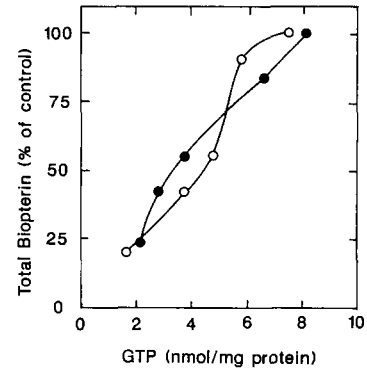

FIG. 4. **Relationship between intracellular GTP and biopterin levels in neuronal cell lines.** PC-12 (●) and IMR-32 (○) cells were treated with various concentrations of mycophenolic acid for 20 h. Then GTP and biopterin were extracted and quantitated as described under "Experimental Procedures." The amounts of GTP were plotted against the amounts of biopterin expressed as a percentage of the control values. The control values of biopterin content in PC-12 and IMR-32 cells were 250 ± 1 and 126 ± 9 pmol/mg of protein, respectively. Data shown are representative of two to four experiments.

TABLE II

Activities of BH_4 biosynthetic enzymes in neuronal cell lines

Enzymes were extracted and assayed as described under "Experimental Procedures." Values shown represent means ± S.E. from two identical experiments performed in separate cultures.

Enzyme	Enzyme activities	
	PC-12	IMR-32
	nmol/h·mg protein	
GTP cyclohydrolase I	0.228 ± 0.002	0.037 ± 0.002
6-Pyruvoyltetrahydropterin synthase	2.02 ± 0.08	0.317 ± 0.008
Sepiapterin reductase	92.8 ± 1.2	2.99 ± 0.02
Dihydropteridine reductase	597 ± 57	522 ± 10

man cells and the rat cells was that no increase in biopterin level was observed upon addition of guanine or guanosine (data not shown). These results, together with the observation that the initiation of decrease in biopterin levels correlated with a slight decrease in GTP levels (Fig. 4), suggest that concentration of the intracellular free GTP in IMR-32 cells is also at the minimum level where GTP cyclohydrolase I activity is elicited at a velocity near its V_{max} value such as that found in PC-12 cells. By comparing Figs. 4 and 5*A*, we estimated the intracellular free GTP concentration in IMR-32 and PC-12 cells to be approximately 150 μM.

DISCUSSION

IMP Dehydrogenase Inhibitors Reduce Intracellular Tetrahydrobiopterin Levels via Reduction of Intracellular GTP Levels—In the present study, we have demonstrated that IMP dehydrogenase inhibitors lower the levels of intracellular tetrahydrobiopterin in PC-12 rat pheochromocytoma and IMR-32 human neuroblastoma cells. We have concluded that the cause of this decrease in biopterin content is attributable to the restriction in the availability of GTP, a substrate for GTP cyclohydrolase I. This conclusion is substantiated by the following findings. (i) Concurrent addition of the IMP dehydrogenase inhibitor and either guanine or guanosine restored the inhibition of biopterin synthesis caused by the inhibitors; (ii) IMP dehydrogenase inhibitors did not inhibit any enzymatic activities of BH_4-synthesizing enzymes except GTP cyclohydrolase I; (iii) the effects (on the cellular GTP and biopterin levels) of three inhibitors with different structures were similar. Thus, the depletion of biopterin was due to the actions of these inhibitors capable of decreasing GTP levels.

In addition, several lines of evidence ruled out the possibility that there are other causes involved in the reduction of BH_4 levels besides the effect of IMP dehydrogenase inhibitors on the GTP cyclohydrolase I activity via altering the GTP levels. The IMP dehydrogenase inhibitors have been reported

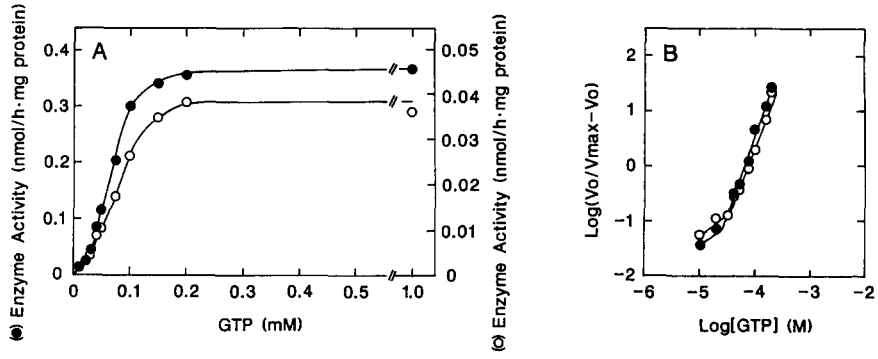

FIG. 5. *A*, effect of various concentrations of GTP on GTP cyclohydrolase I activities of neuronal cell lines. The enzymes were extracted from 10^8 cells of PC-12 (●) and IMR-32 (○) cells and assayed as described under "Experimental Procedures." Data shown are representative of two experiments. *B*, Hill plots were drawn using the data presented in *A*. *Symbols* are the same as in *A*.

to affect RNA and DNA syntheses in some cell lines, probably through changing the levels of GTP and, to a lesser extent, other nucleotides (37, 38). This raised the possibility that the inhibitors could influence the synthesis of the enzymes of the BH_4 biosynthetic pathway through changes in RNA and DNA syntheses. The treatment of PC-12 cells with tiazofurin did not significantly change the specific activities in the cell lysates for the BH_4 biosynthetic enzymes, *i.e.* GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase, and sepiapterin reductase. This result indicated that the IMP dehydrogenase inhibitors did not either depress the synthesis of these enzymes or alter the enzyme activities through covalent modifications. Besides biosynthesis, the concentration of biopterin in the cells could be affected by its supply from the culture medium or its intracellular degradation. However, since the serum-free medium we used did not contain biopterin, we eliminated the possibility that biopterin was supplied by the medium. It was also unlikely that the degradation of BH_4 was enhanced by the treatment of IMP dehydrogenase inhibitors because the total amount of biopterin and pterin, the main degradation products, was found to be decreased. Furthermore, we did not find any differences in the degradation rates of BH_4 between the cell lysates from the inhibitor-treated and untreated cells (data not shown).

We have considered another mechanism of IMP dehydrogenase inhibitors. Tiazofurin is known to inhibit IMP dehydrogenase by its conversion to tiazofurin adenine dinucleotide, an analogue of NAD (39). Since sepiapterin reductase and dihydropteridine reductase utilize NADPH and NADH as a cofactor, respectively, we assumed that the fraudulent dinucleotide, tiazofurin adenine dinucleotide, might inhibit the activities of the two reductases competitively. However, this was not the case. Because the effect of tiazofurin was reversed by the addition of the guanine compounds (Table I), the BH_4 biosynthetic system should be functional. Therefore, it is unreasonable to assume that tiazofurin inhibition is due to the inhibitory effect on the above reductases. On the other hand, 2-amino-1,3,4-thiadiazole was shown to be converted to 2-amino-1,3,4-thiadiazole mononucleotide, which competed with IMP but did not compete with NAD for IMP dehydrogenase (40). Accordingly, the anabolite might not inhibit sepiapterin reductase and dihydropteridine reductase. In fact, the effect of 2-amino-1,3,4-thiadiazole was reversed by the addition of guanine or guanosine (Table I). In contrast with these two thiazole compounds, mycophenolic acid inhibited IMP dehydrogenase directly without any anabolization (41). The addition of guanine and guanosine was able to reverse the effect of mycophenolic acid (Table I). Furthermore, we found that these three inhibitors did not inhibit any activities of the three BH_4 biosynthetic enzymes as well as dihydropteridine reductase even at a concentration 50-fold higher than the EC_{50} value sufficient to reduce intracellular biopterin levels (data not shown). It is, thus, unlikely that IMP dehydrogenase inhibitors directly affect the activities of the enzymes responsible for biosynthesis and/or catabolism of BH_4.

Relationship between Intracellular GTP Levels and GTP Cyclydrolase I Activity in the Cells—In the present study we showed pharmacologically that changes in GTP levels could elicit changes in biopterin levels. To determine the physiological relevance of this type of regulation, we must refer to the issue of changes in GTP concentration under physiological circumstances. Pall (42) indicated a possible role of GTP in regulating anabolic processes. In yeast, declines in GTP pools were observed in conjunction with sporulation (43). In eukaryotes, there were few studies concerning physiological changes in GTP levels; however, it has been reported very recently that p53, a cellular anti-oncogene, regulates the synthesis of GTP, thereby controlling cell growth (44). Physiological alteration of GTP levels has been reported in studies on the biosynthesis of biopterin in T cells and macrophages (45). The induction of activity of GTP cyclohydrolase I was observed with a simultaneous increase in the level of GTP when the T cells and macrophages were activated by phytohemagglutinin and interferon-γ, respectively (45). If the GTP levels in these cells before activation were below the concentration needed to elicit a maximum reaction rate of GTP cyclohydrolase I, the elevation of GTP levels could accelerate the GTP cyclohydrolase I-catalyzed reaction synergistically with the elevation of the number of active GTP cyclohydrolase I molecules *per se*.

The Estimations of Concentrations of Intracellular Free GTP—We estimated the intracellular concentrations of free GTP to be approximately 150 μM by comparing the change in biopterin production in the IMP dehydrogenase inhibitor-treated cells (Fig. 4) with the kinetics of GTP cyclohydrolase I (Fig. 5*A*). Several studies showed that the decrease in intracellular GTP levels by IMP dehydrogenase inhibitors lowered the activity of adenylate cyclase (18–20). In these studies the intracellular concentrations of free GTP were estimated by comparing the activity of adenylate cyclase in intact cells in the presence of IMP dehydrogenase inhibitors with saturation kinetics of the homogenates with varying concentrations of GTP. The estimated concentrations of free GTP were 1–100 μM, values much lower than the values obtained by dividing the total amount of cellular GTP by the cell volume: C6 glioma cells, 300 μM (18); rat kidney cells, 500 μM (19); and Ehrlich ascites tumor cells, 600 μM (20). To explain this discrepancy, a separate pool of GTP has been postulated, *e.g.* a pool of free GTP and a pool of GTP accommodated in microsomes or bound to abundant GTP-binding proteins. The values we estimated as intracellular levels of free GTP in neuronal cells fell in between these two groups of values estimated in a different way. The discrepancies in these values for intracellular free GTP may be due to the

differences in cell types and/or in the milieu around GTP cyclohydrolase I and adenylate cyclase in the cells. On the other hand, the intracellular concentrations of free GTP in cardiac myocytes have recently been reported (using a patch clamp technique) to be in the range of 50–150 μM (46–48), very close to the value estimated in this study for neuronal cells.

In conclusion, we revealed that the intracellular GTP levels were present in the ranges affecting the activity of GTP cyclohydrolase I, the rate-limiting enzyme of BH_4 biosynthesis, and that GTP cyclohydrolase I appeared to behave in a positive cooperative manner against GTP in the cells.

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