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Mitogenic Effects of Tetrahydrobiopterin in PC12 Cells

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SUMMARY

(6*R*)-5,6,7,8-Tetrahydrobiopterin (BH_4), which is synthesized intracellularly from GTP, caused a concentration-dependent increase in rat pheochromocytoma (PC12) cell proliferation when added exogenously. Incubation with sepiapterin, which is converted enzymatically to BH_4 within cells, also increased PC12 cell proliferation and BH_4 levels concomitantly. These sepiapterin effects were mediated by BH_4 as inhibition of sepiapterin conversion to BH_4 by a sepiapterin reductase inhibitor, *N*-acetyl-serotonin, blocked the increase in proliferation and the elevation of BH_4 levels. 7,8-Dihydrobiopterin (BH_2) also increased BH_4 levels and PC12 cell proliferation, both of which were reversed by methotrexate, which blocks the conversion of BH_2 to BH_4 by dihydrofolate reductase. The BH_4-induced increase in PC12 cell proliferation was not related to elevated catecholamine or nitric oxide synthesis as inhibitors of tyrosine hydroxylase or nitric oxide synthase did not reduce the BH_4 effect. BH_4 and its precursors did not alter intracellular cAMP levels, suggesting that this second messenger is not involved in the enhancement of PC12 cell proliferation by BH_4. Sepiapterin and BH_4 also enhanced the proliferation of SV40-transformed human fibroblasts and rat C6 glioma cells, indicating that the stimulatory effect of BH_4 on cell proliferation is not restricted to PC12 cells.

BH_4 was originally discovered in mammals as the naturally occurring, essential cofactor for phenylalanine (1), tyrosine (2), and tryptophan (3) hydroxylases; the latter two are the rate-limiting enzymes in dopamine and serotonin synthesis, respectively. Additional roles for BH_4 were uncovered recently with the demonstrations that it is required for nitric oxide synthase in the formation of nitric oxide (4) and that BH_4 stimulates the neuronal release of dopamine (5, 6), serotonin (6, 7), acetylcholine (8), and glutamate (6).

Advances in the elucidation of the BH_4 biosynthetic pathway have helped uncover the biological roles of BH_4. The pathway of *de novo* BH_4 biosynthesis begins with the conversion of GTP to dihydroneopterin triphosphate by GTP cyclohydrolase I (EC 3.5.4.16) (9). Dihydroneopterin triphosphate is then converted enzymatically through several tetrahydropterin intermediates to BH_4. The final reaction is catalyzed by sepiapterin reductase (EC 1.1.1.153) (10, 11). Sepiapterin, a dihydropterin compound, is not a naturally occurring intermediate in *de novo* BH_4 biosynthesis. However, exogenously administered sepiapterin is converted intracellularly to BH_2, which is then converted to BH_4 by dihydrofolate reductase; this is referred to as the salvage pathway for BH_4 synthesis (12).

Although the oxidized form of BH_4, biopterin, was known to serve as a growth factor for *Crithidia fasiculata* (13, 14), it is only recently that a growth-related role for BH_4 has been suggested. Several indirect lines of evidence suggest a link between intracellular BH_4 levels and the proliferation of hemopoietic cells. For example, immature, proliferating rat red blood cells contain much more BH_4 than do nonproliferating, mature erythrocytes (15). Furthermore, during bone marrow transplantation in dogs, an increase in intracellular BH_4 levels was observed in proliferating hemopoietic cells (16). The role of BH_4 in the proliferation of hemopoietic cells was tested in cultured erythroleukemia cells with ambiguous results; exogenous BH_4 stimulated DNA synthesis and was required for the proliferation of some (15, 17) but not all mouse erythroleukemia clonal cell lines (17).

In addition to its association with hemopoesis, other observations suggest a role for BH_4 in cell replication. BH_4 biosynthesis and accumulation in rat thymocytes were transiently increased and peaked just before the early stages of the DNA synthetic (S) phase of the cell cycle (18, 19). In

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ABBREVIATIONS: BH_2, 7,8-dihydrobiopterin; BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; 6S-BH_4, (6*S*)-tetrahydrobiopterin; DMEM, Dulbecco's modified Eagle's medium; NAS, *N*-acetyl-serotonin; PC12, pheochromocytoma; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MTX, methotrexate; NAS, *N*-acetyl-serotonin; DTT, dithiothreitol; Sep, sepiapterin.

Drosophila melanogaster, certain mutations in the *Punch* gene encoding GTP cyclohydrolase, the initial and rate-limiting enzyme in BH_4 biosynthesis, prevented early embryonic nuclear division (20); this suggests a requirement for BH_4 biosynthesis in nuclear division and mitogenesis.

Although there is some evidence for the involvement of BH_4 in the proliferation of mouse-derived hemopoietic cells, there is no evidence that BH_4 is involved in the proliferation of other nonhemopoietic cells or cells from species other than mouse. Reduction of endogenous BH_4 levels did not alter the growth of either MOLT-4 T cell leukemia or MCF-7 breast adenocarcinoma cells in culture (21). To test the hypothesis that BH_4 plays a role in regulating the proliferation of nonhemopoietic cells, we investigated the effects of BH_4 on rat PC12 cell proliferation. PC12 cells are tumor cells derived from adrenal medullary chromaffin cells and have the capacity to synthesize BH_4 and catecholamines. To identify the potential involvement of BH_4 in regulating proliferation of human and other nonhemopoietic cells, we also tested the effects of BH_4 on human SV40-transformed fibroblasts and rat C6 glioma cells.

Materials and Methods

Cell culture. Rat PC12 cells were maintained in tissue culture flasks (150 cm^2) in a humidified (90%) atmosphere of 95% air/5% CO_2 at 37°. The medium consisted of DMEM (4500 mg/L glucose with L-glutamine), 44 mM sodium bicarbonate, and 24 mM HEPES. pH was adjusted to 7.25 before the addition of 7% heat-inactivated horse serum, 7% fetal bovine serum, 100 units/ml penicillin per 100 mg/ml streptomycin, and subsequent filtration. Cells were harvested by mechanical dislodging and collected by centrifugation at room temperature (7 min, 500 × *g*). The supernatant was removed, and the pellet was resuspended in DMEM. Cell viability was determined by Trypan blue (0.01%) exclusion, and cells were resuspended to the desired final density in the appropriate media and replated.

SV40-transformed human skin fibroblasts (GM0637) were cultured in DMEM containing 10% fetal bovine serum and penicillin/streptomycin (100 units/ml per 100 mg/ml). Rat C6 glioma cells were cultured in Ham's F-10 media, pH 7.4, containing 15% heat inactivated horse serum, 2.5% fetal bovine serum, and penicillin/streptomycin (100 units/ml per 100 mg/ml). Both human fibroblasts and C6 glioma cells were harvested by trypsinization and resuspended in appropriate medium, and viable cells were counted before replating.

Measurement of BH_4. PC12 cells were cultured in 24-well plates (100,000 cells/well), washed with Dulbecco's phosphate-buffered saline, and homogenized in 200 ml of 0.1 N perchloric acid containing 1 mM DTT. Homogenates were centrifuged at 40,000 × *g* for 20 min at 4°. Supernatant BH_4 levels were determined by high pressure liquid chromatography with electrochemical detection (22).

Determination of cell proliferation. PC12 cell number was determined with the use of a colorimetric proliferation assay (Celltiter 96 AQeous). PC12 cells were cultured in 96-well plates (10,000 cells/well) and allowed to attach to the wells for 24 hr before the addition of test compounds. Cells were incubated in the presence of test compounds for 24 hr unless otherwise specified. Because exogenously administered BH_4 is in the dihydrochloride form, control and BH_4-treated cells were incubated in medium containing a final concentration of 1.6 mM HCl to normalize for the HCl in the different incubation media; 1 mM DTT was also added to the incubation media to reduce oxidative degradation of BH_4. In some cases, PC12 cells were treated with methotrexate (10 μM) to inhibit dihydrofolate reductase. To prevent the impairment of folate metabolism and mitosis by methotrexate, methotrexate-treated cells and their controls were incubated in the presence of 100 μM hypoxanthine and 40 μM thymidine. After incubation of PC12 cells with the appropriate test condition, the medium was removed and replaced with medium containing the Celltiter 96 AQeous dye. Exogenous BH_4 (but not sepiapterin) caused cells to detach from plates in a concentration-dependent manner, yet the cells remained viable as determined by Trypan blue exclusion (data not shown). To prevent loss of cells during aspiration of medium, the medium was collected and centrifuged so that fresh medium containing dye could be added to the pelleted cells; cells were then added back to appropriate wells. After a 2-hr incubation at 37°, absorbance at 490 nm was measured in a Biotek 340 plate reader. Color formation was directly proportional (correlation coefficient of 0.999) to the number of viable cells over a range of 3,125–100,000 cells/well and was not influenced by any of the test conditions. Results of the colorimetric assay were confirmed by direct cell counting (hemacytometer) of Trypan blue-excluding cells and by measuring the effects of test compounds on the total genomic DNA levels with the use of the DNA binding fluorochrome H33258 (23). Although the effects of pterins on PC12 cell proliferation were comparable for all methods, the Celltiter assay was used routinely because of its high sensitivity and convenience of use in large assays.

Other methods. Protein was measured according to the method of Bradford (24). cAMP levels were measured in PC12 cells with the use of a radioimmunoassay kit.

Materials. BH_4, 6S-BH_4, BH_2, sepiapterin, methotrexate, and 6-biopterin were purchased from Schircks Laboratories (Jona, Switzerland). Rat PC12 cells were obtained from Dr. Gordon Guroff (National Institutes of Health, Bethesda, MD). SV40-transformed human skin fibroblasts (GM0637) were obtained from the National Institutes of Health Human Genetic Mutant Cell Repository (Camden, NJ). Rat C6 glioma cells were purchased from American Type Culture Collection (Rockville, MD). cAMP assay kits were purchased from Amersham. Culture flasks and DMEM were purchased from GIBCO-BRL. Celltiter 96 AQeous cell proliferation assay kit was purchased from Promega. The DNA binding fluorochrome, H33258, was purchased from Calbiochem. All other chemicals and reagents were of the highest grade available from commercial suppliers.

Statistical analysis. Statistical analysis was carried out by one-way analysis of variance. Posthoc comparisons were made with the Newman-Keuls test.

Results

The effects of various pterins on the proliferation of PC12 cells after a 24-hr exposure are shown in Fig. 1. BH_4 caused a concentration-dependent increase in PC12 cell number to 150% of control, which peaked at 400 μM. The synthetic isomer 6S-BH_4 stimulated PC12 cell proliferation to a lesser extent, and biopterin had no effect. BH_2 was as effective as BH_4 in enhancing PC12 cell proliferation. Sepiapterin, which is converted intracellularly to BH_2 and then to BH_4, was the most effective pterin in stimulating proliferation, causing a 2-fold increase in cell number. Similar increases in total cell number (cells in the media and on the plate) were detected for all tested compounds in direct cell-counting experiments with a hemacytometer and Trypan blue exclusion used as an estimate of cell viability (data not shown).

Long term incubation of PC12 cells with sepiapterin (100 μM) resulted in enhanced proliferation rates for the first 72 hr of incubation compared with control (Fig. 2). Cell number reached confluence at 72 hr in the presence of sepiapterin, whereas cells growing under control conditions reached confluence at 96 hr. PC12 cells grown in the presence or absence of sepiapterin reached growth arrest at the same cell densities (saturation density). At 96 hr, no significant change in cell viability was evident in either control or sepiapterin-

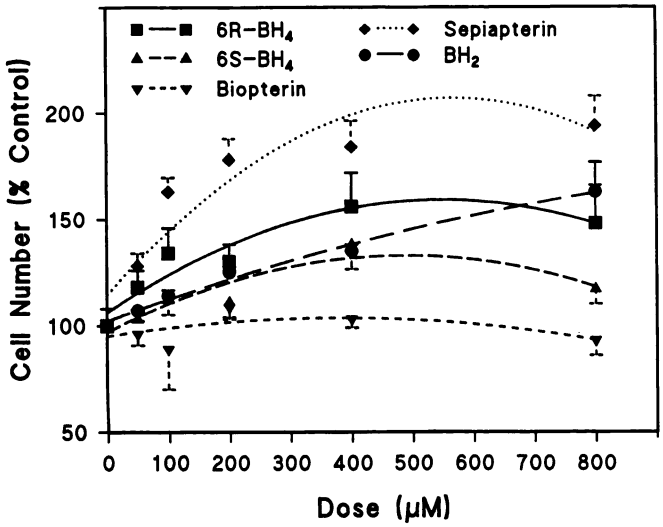

Fig. 1. Effects of various pterins on PC12 cell proliferation. PC12 cells were plated in 96-well plates and incubated for 24 hr in the presence of various pterins before determination of cell number by the colorimetric proliferation assay as described in Materials and Methods. BH_4, 6S-BH_4, BH_2, and sepiapterin significantly enhanced PC12 cell proliferation ($p < 0.05$ at 100, 400, 200, and 50 μM, respectively; analysis of variance). Biopterin did not alter cell proliferation from control. Values represent the mean ± standard error of three to six independent determinations performed in duplicate.

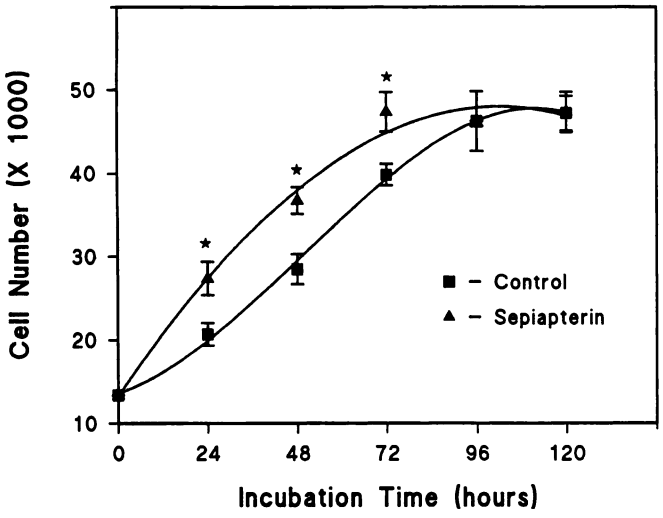

Fig. 2. Effect of sepiapterin on the growth curve of cultured PC12 cells. PC12 cells were plated in 96-well plates and incubated for 24, 48, 72, 96, or 120 hr in control media or in the presence of 100 μM sepiapterin before determination of cell number by the colorimetric cell proliferation assay described in Materials and Methods. Sepiapterin significantly ($p < 0.05$; analysis of variance) enhanced the growth rate of PC12 cells during the first 3 days in culture. PC12 cells reached saturation density and stopped dividing at 72 hr of incubation with sepiapterin. In comparison, control cells reached saturation density at 96 hr. Media and conditions were replenished every 24 hr. Values represent the mean ± standard error of four independent determinations performed in duplicate.

treated cells after direct cell counting and Trypan blue exclusion.

Fig. 3 shows that the enhancement of PC12 cell proliferation after a 24-hr exposure to precursors of BH_4 (200 μM each of sepiapterin or BH_2) specifically requires metabolism of these precursors to BH_4. NAS, an inhibitor of sepiapterin

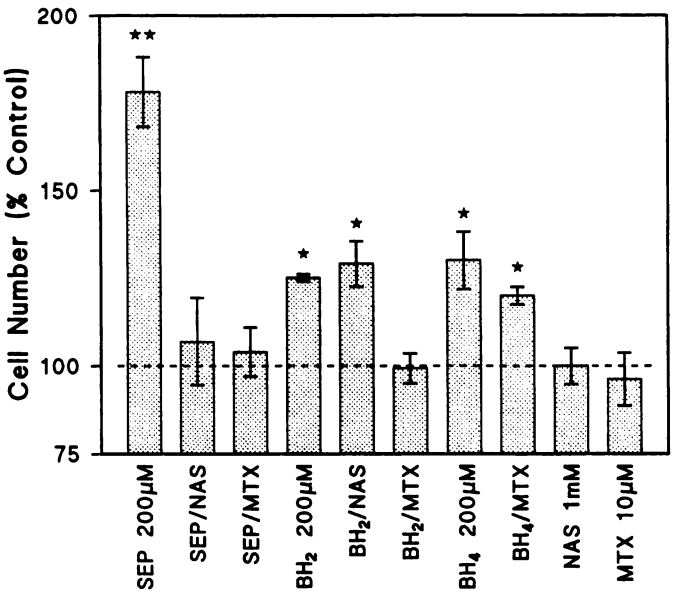

Fig. 3. Effects of NAS and methotrexate on sepiapterin-, BH_2-, and BH_4-induced PC12 cell proliferation. PC12 cells were plated in 96-well plates and incubated for 24 hr in the presence of various pterins before determination of cell number by the colorimetric proliferation assay as described in Materials and Methods. Values represent the mean ± standard error of three to five independent determinations performed in duplicate. *, **, ***, $p < 0.05$, < 0.01, and $< 0,001$, respectively; Student's t test.

reductase, reduced the 80% increase in PC12 cell number caused by sepiapterin essentially to control levels. Methotrexate, which inhibits the conversion of BH_2 to BH_4 by dihydrofolate reductase, also antagonized the sepiapterin effect. The enhancement of PC12 cell proliferation by BH_2 was blocked by methotrexate but not by NAS (does not inhibit conversion of BH_2 to BH_4). The effect of BH_4 on cell number was not significantly reduced by methotrexate. Finally, neither NAS nor methotrexate altered control PC12 cell proliferation. This demonstrates that BH_4 is an enhancer of PC12 cell proliferation and may not be required for the maintenance of the basal proliferation rate.

Table 1 shows the effects of pterin treatments (200 μM each) with and without inhibitors of BH_4 biosynthesis on the levels of BH_4 in PC12 cells after 24 hr. The control intracellular BH_4 concentration (118 pmol/mg protein) was reduced to below the detection limit of the assay (80 pmol/mg protein) when PC12 cells were incubated with either NAS or methotrexate alone. Intracellular BH_4 levels were increased 4-fold in the presence of exogenous BH_4. In the presence of BH_4 and methotrexate, PC12 cell BH_4 levels were decreased below control at 24 hr. Sepiapterin elevated intracellular BH_4 levels ~19-fold, which was returned to control by NAS at early time points (data not shown) and blunted at 24 hr to 27% of the effect with sepiapterin alone (Table 1). This delayed elevation of intracellular BH_4 was not sufficient to increase proliferation measured at 24 hr above control (Fig. 3). The higher level of BH_4 at 24 hr after this coincubation with NAS and sepiapterin compared with early time points is presumably due to the instability of NAS in the medium such that sepiapterin reductase activity is less inhibited at later time points. BH_2 increased PC12 cell BH_4 levels by ~4-fold. Coincubation of NAS with BH_2 had no effect on this elevation,

TABLE 1

Intracellular BH_4 levels after pterin treatments alone or in combination with BH_4 biosynthetic inhibitors

PC12 cells were plated in 24-well plates and incubated for 24 hr in the presence of various combinations of the following: 200 μM of either BH_4, BH_2, or Sep, 10 μM MTX, and 1 mM NAS. Cells were washed and harvested, and intracellular BH_4 levels were determined as described in Materials and Methods.

Condition	Amount
	pmol/mg protein
Control	118 ± 12[a]
NAS	<80 (detection limit)
MTX	<80
BH_4	464 ± 118[b]
BH_4/MTX	<80
Sep	2226 ± 306[c]
Sep/NAS	381 ± 89[d]
Sep/MTX	<80
BH_2	488 ± 108[b]
BH_2/NAS	502 ± 101[b]
BH_2/MTX	<80

[a] Values represent mean ± standard error of three independent determinations performed in duplicate.
[b] $p < 0.01$, Student's *t* test.
[c] $p < 0.001$, Student's *t* test.
[d] $p < 0.05$, Student's *t* test.

whereas coincubation with methotrexate caused a decline of BH_4 to below control levels.

The methotrexate-induced reduction of intracellular BH_4 to below control levels after 24-hr exposure led us to examine the time course of changes in intracellular BH_4 after incubation of PC12 cells with BH_4 either alone or in the presence of methotrexate (Fig. 4). The rate of decline of elevated intracellular BH_4 at 1 and 3 hr after incubation of PC12 cells with 200 μM BH_4 was not different in the presence and absence of methotrexate. However, after 6 and up to 24 hr, methotrexate caused a decrease in BH_4 levels below the amount found with BH_4 alone; inhibition of dihydrofolate reductase by methotrexate blocks the regeneration of BH_4 from BH_2 (a major oxidative byproduct of BH_4), which is the likely mechanism for the decrease in intracellular BH_4 levels. Incubation of PC12 cells with 200 μM sepiapterin elevated BH_4 levels to a greater extent than BH_4 did after 3 hr and maintained these high BH_4 levels throughout the 24-hr incubation. When sepiapterin was removed after 6 hr from the incubation media and cells were incubated for an additional 18 hr in DMEM (total incubation time of 24 hr), the level of intracellular BH_4 returned to control levels rapidly after 6 hr and remained at this level for the remainder of the 24-hr period; furthermore, the elevation of PC12 cell number after 6 hr of sepiapterin plus 18 hr of DMEM was only 130% of control (data not shown), as opposed to 180% control with 24-hr exposure to sepiapterin. The 30% increase in PC12 cell number caused by 6-hr exposure to sepiapterin is equivalent to the increase caused by 24-hr exposure to BH_4 (Fig. 3). In contrast to incubation with BH_4 where intracellular BH_4 levels declined after several hours (Fig. 4), sepiapterin maintained elevated intracellular BH_4 levels throughout the 24-hr period. This indicates that a continued presence of elevated intracellular BH_4 is required for maximal enhancement of proliferation.

The involvement of the second messenger cAMP in mediating the effects of BH_4 and sepiapterin on PC12 cell proliferation was also investigated as shown in Fig. 5. Neither BH_4 (200 μM) nor sepiapterin (100 μM) altered PC12 cell cAMP content after 5-, 10-, 20-, or 40-min exposures (10 min shown). In contrast, VIP (10 μM), which has been shown to raise PC12 cell cAMP levels (25–27), caused a 3.5-fold increase in cAMP content after a 10-min incubation.

We also tested whether BH_4 induced cell proliferation by serving as a cofactor for tyrosine hydroxylase or nitric oxide synthase and enhancing catecholamine or nitric oxide syn-

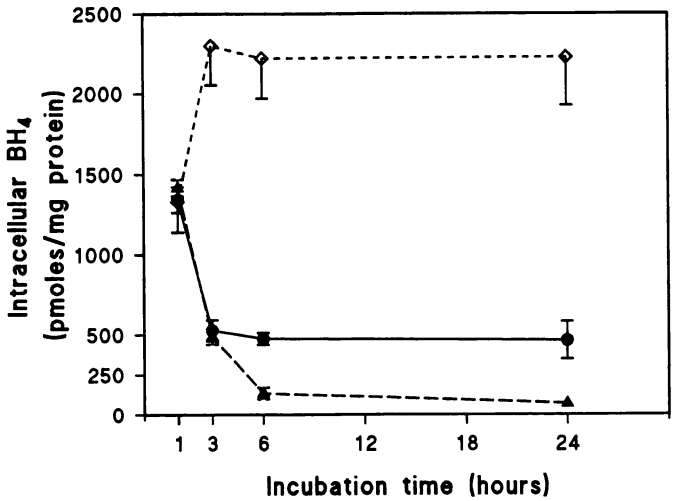

Fig. 4. Effects of incubation time on intracellular BH_4 levels after treatment of PC12 cells with sepiapterin, BH_4, or BH_4 plus methotrexate. PC12 cells were plated in 6-mm tissue culture dishes (1×10^6 cells/dish) and incubated for 1, 3, 6, or 24 hr with sepiapterin (◇), BH_4 alone (●), or BH_4 plus methotrexate (▲). BH_4 levels were estimated as described in Materials and Methods. Values represent the mean ± standard error of two independent determinations performed in duplicate. BH_4 plus methotrexate was significantly lower than BH_4 alone at 6 and 24 hr ($p < 0.01$; analysis of variance).

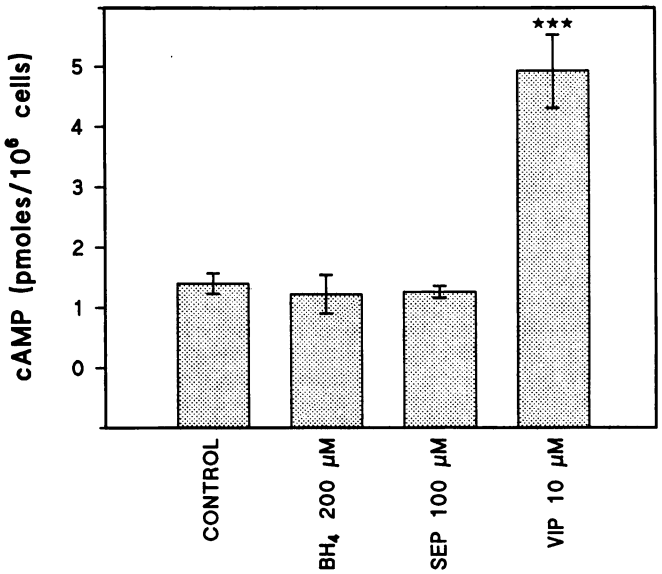

Fig. 5. The effect of BH_4, sepiapterin, and vasoactive intestinal polypeptide on PC12 cell cAMP levels. PC12 cells were collected in 1.5-ml plastic tubes (2×10^6 cells/tube) and incubated for 10 min at 37° in the presence of BH_4 (200 μM), sepiapterin (*SEP*, 100 μM), or vasoactive intestinal polypeptide (*VIP*, 10 μM). Cells were homogenized in 0.05 M Tris/4 mM EDTA buffer, and cAMP was determined in the homogenate as described in Materials and Methods. Values represent the mean ± standard error of two or three independent determinations performed in duplicate. ***, $p < 0.001$; Student's *t* test.

thesis, respectively. PC12 cells treated for 24 hr with the tyrosine hydroxylase inhibitor, α-methyl-*para*-tyrosine (20 μM), exhibited a 75% reduction in catecholamine levels (data not shown), whereas cell proliferation was unaffected. α-Methyl-*para*-tyrosine also did not inhibit the increase in cell number caused by 100 μM sepiapterin. PC12 cells were also treated for 24 hr with concentrations of N^{ω}-nitro-L-arginine that have been shown to inhibit nitric oxide synthase (28). N^{ω}-Nitro-L-arginine (0.1–4 mM) did not alter control or the sepiapterin (100 μM) enhancement of PC12 cell proliferation. Supplementing PC12 cells for 24 hr with L-arginine (100 μM), the substrate that can enhance nitric oxide synthase activity and nitric oxide production, also did not change PC12 cell number.

To examine whether enhancement of cell proliferation by BH_4 was restricted to rat PC12 cells, human fibroblasts and rat C6 glioma cells were incubated with 200 μM sepiapterin, as shown in Fig. 6. Sepiapterin significantly enhanced the proliferation of both human fibroblasts and C6 glioma cells after 24 hr, although the percentage increase was greater in C6 glioma cells (250% of control) than in human fibroblasts (115% of control). This effect of sepiapterin was reversed in both cell lines by NAS. NAS alone did not affect basal proliferation rates (data not shown).

Discussion

Our results demonstrate that elevating intracellular BH_4 levels significantly enhances the proliferation of rat PC12 cells, rat C6 glioma, and human fibroblasts above basal proliferation rates. In PC12 cells, only pterins that elevated intracellular BH_4 levels were effective, as biopterin had no effect on either BH_4 levels or proliferation. This was confirmed by enzyme inhibitors that block the conversion of sepiapterin and BH_2 to BH_4, which prevented the rise of intracellular BH_4 and blocked the enhancement of proliferation (Fig. 3).

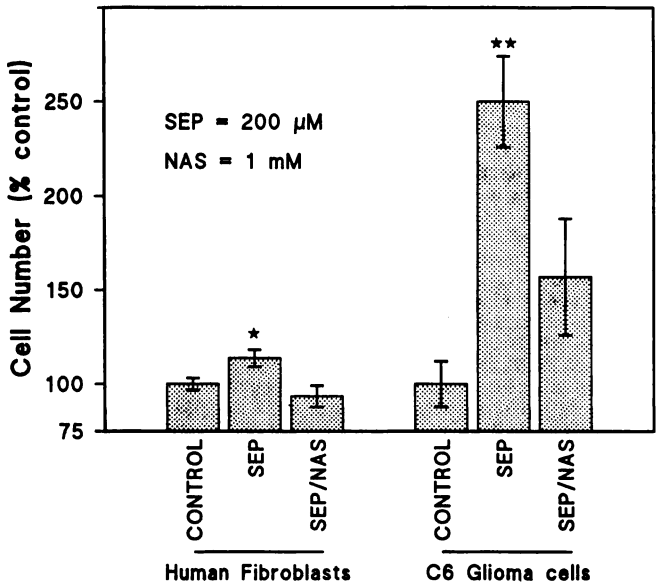

Fig. 6. The effect of sepiapterin alone or in combination with NAS on the proliferation of human fibroblasts and rat C6 glioma cells. Human fibroblasts were plated in 24-well plates (100,000 cells/well) and incubated for 24 hr with sepiapterin (200 μM) alone or in combination with 1 mM NAS. Cells were then harvested by trypsinization and counted using a Coulter counter. C6 glioma cells were plated in 96-well plates similarly to PC12 cells, and cell number was determined by the colorimetric proliferation assay as described in Materials and Methods. *, **, $p < 0.05$, 0.01, respectively; Student's *t* test.

The enhancement of PC12 cell growth with sepiapterin, which is converted intracellularly to BH_4, was greater than that observed with epidermal growth factor (145% of control). In addition, although the normal doubling time of PC12 cells was ~72 hr, incubation with 400 μM sepiapterin reduced it to 24 hr. The growth curves in Fig. 2 indicate that the growth rate of PC12 cells during exponential growth is enhanced in the presence of sepiapterin. Furthermore, PC12 cells reach similar saturation densities in the presence or absence of sepiapterin. Because all experiments were conducted in exponentially growing PC12 cells and the saturation density of PC12 cells was not altered by sepiapterin, changes in saturation density could not account for the effect of BH_4 on cell growth.

In all experiments, 24 hr elapsed after plating to allow cell attachment before the application of test conditions. Although a decrease in cell attachment was observed in cell-counting experiments with BH_4, no similar effect was observed with sepiapterin, suggesting that this was an extracellular effect of BH_4. In all experiments with cell counting or color formation assays, we measured total cell number in the plates and the media, and significant changes in total cell number were detected with elevated intracellular BH_4, by incubation of cells with BH_4 or sepiapterin. Thus, an alteration in plating density or cell attachment cannot explain the higher number of cells observed after elevation of intracellular BH_4 levels.

The increased cell number after elevation of intracellular BH_4 could also be caused by prevention of cell death. However, in cell-counting experiments, the percentage of dead cells (e.g., cells not excluding Trypan blue) both in the media and on the plates was always <5% of the total cells and was not different between control and treated conditions. The morphology of these dead cells under the microscope did not suggest apoptotic cell death (no evidence of cell or nuclear shrinkage). Furthermore, although significant DNA fragmentation was evident when PC12 cells were incubated for 24 hr in the absence of serum, no DNA fragmentation was observed under control culturing conditions (29, 30).[1] Thus, elevation of intracellular BH_4 actually stimulates cell proliferation as opposed to enhancing PC12 cell survival. Our results indicate that BH_4 reduces the doubling time of exponentially growing PC12 cells, resulting in an increased growth rate. Because changes in the doubling time reflect changes in the cell cycle, our results indicate that BH_4 affects the PC12 cell cycle.

PC12 cell growth enhancement was seen after 24 hr of incubation at concentrations of BH_4 near the physiological range (100 μM) found in cultured cells (31) and brain (32). Sepiapterin increased cell proliferation and intracellular BH_4 levels more than equimolar concentrations of either BH_4 or BH_2. Our results suggest that both BH_4 and BH_2 may degrade over time in the medium to a greater extent than sepiapterin. We previously reported that BH_4 enters PC12 cells by passive diffusion, with equilibration between the extracellular and intracellular compartments occurring in

[1] P. Z. Anastasiadis, unpublished observations.

<10 min (33). Our current results also confirm that BH_4 efflux is quite rapid, most likely due to the degradation of BH_4 in the medium to other pterin and nonpterin metabolites. Because BH_4 efflux from PC12 cells after 200 μM exogenous BH_4 occurs predominantly within the first 3 hr, the sustained levels of BH_4 after 24 hr in the presence of 200 μM sepiapterin suggest that sepiapterin may enter cells more effectively and is more resistant to degradation than BH_4. Thus, even as the BH_4 formed from sepiapterin may be degrading, there is a continuous supply of sepiapterin for maintenance of the intracellular BH_4 pool over the 24-hr period. It is also possible that the removal of intracellular sepiapterin through its conversion to BH_4 provides a continuous concentration gradient that allows more total sepiapterin to enter by passive diffusion. Because sepiapterin is converted enzymatically to BH_4 intracellularly, this is consistent with BH_4 enhancing cellular proliferation through an intracellular mechanism rather than via a plasma membrane receptor.

Our results indicate that enhancement of PC12 cell proliferation by elevated intracellular BH_4 content is proportional to the duration of BH_4 elevation. When PC12 cells were in the presence of sepiapterin for only the initial 6 of 24 hr, the number of cells dropped from the 180% of control value at 24-hr sepiapterin exposure to 130% of control. This value of 130% is essentially the same as the increase in proliferation seen after 24-hr incubation with BH_4. It is likely that the 6-hr sepiapterin is equivalent to the 24-hr BH_4 because BH_4 levels are not maintained on prolonged incubation (intracellular BH_4 was much lower at 3 and 6 hr after incubation with BH_4 than with sepiapterin). Furthermore, methotrexate did not prevent exogenous BH_4 from entering cells, yet it lowered the PC12 cell level of BH_4 to less than control at 6 and 24 hr (Fig. 4) without altering the BH_4-induced enhancement of proliferation. Thus, prolonged elevation of intracellular BH_4 levels above a certain threshold level allows maximal stimulation of PC12 cell proliferation. However, because the PC12 cells are not synchronized with respect to the initiation of cell division, we cannot determine whether dividing cells must be in the presence of BH_4 continuously to obtain maximal stimulation or if BH_4 serves as a short term stimulus to enhance proliferation.

Neither NAS nor methotrexate had any effect on basal cell proliferation rates even though intracellular BH_4 levels were reduced to below detectable levels. Because PC12 cells were grown in a serum-containing medium, it is possible that factors in the serum contribute to the maintenance of a basal proliferation rate even under conditions of reduced intracellular BH_4. It is also possible that only small amounts of intracellular BH_4 at particular sites of action are required to maintain proliferation or that the basal proliferation rate of PC12 cells is independent of control levels of BH_4.

Several cell types in culture enhance their basal proliferation rate in response to elevations in intracellular cAMP levels (34–36). BH_4 apparently does not enhance PC12 cell proliferation via a short term elevation of cAMP levels (up to 40 min). BH_4 also does not appear to act through enhancing catecholamine or nitric oxide biosynthesis as inhibitors of tyrosine hydroxylase or nitric oxide synthase activities did not affect the BH_4-induced increase in proliferation.

We have shown for the first time that BH_4-induced enhancement of proliferation is not restricted to murine erythroleukemia cells (15, 17). BH_4 also enhances the proliferation of rat PC12 cells, rat C6 glioma, and human fibroblasts. The extent to which BH_4 is involved in the proliferation of all dividing cells and whether the BH_4 requirement for proliferation can be overridden by other factors are unknown. Prior inconsistent results on the effects of BH_4 on cell proliferation (17, 21) might be due to mutations that are introduced either during malignant transformation of cells or by spontaneous mutations in cultured cells that can override modulation of cell proliferation by BH_4. Cultured cells carrying such spontaneous mutations would be selected over slower growing cells because of their higher replication rates. Also, serum contains several mitogens and growth factors that might mask the BH_4-induced enhancement of cell proliferation. Differences in the magnitude of the sepiapterin effect on the growth of PC12 cells, C6 gliomas, and human fibroblasts may also reflect changes in the efficacy of the salvage BH_4 biosynthetic pathway in these cells.

Our results suggest that the salvage pathway is necessary for the maintenance of BH_4 levels in cultured cells, possibly due to the increased production of BH_2 in the media after oxidative degradation of BH_4. However, the salvage pathway is not required for the maintenance of BH_4 levels *in vivo* (37, 38), and, thus, methotrexate may not be effective in inhibiting the growth of a BH_4 responsive tumor. The significance of the BH_4 effect in normal and malignant cell growth and development, as well as its mechanism of action, needs further investigation. Preliminary data suggest that the mitogenic effect of epidermal growth factor in PC12 cells is mediated through the elevation of intracellular BH_4. The mechanism by which BH_4 affects the cell cycle and reduces doubling times, as well as the BH_4 mediation of growth factor-induced mitogenic responses, are under investigation in our laboratory.

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