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Increase in Tetrahydrobiopterin Release from PC12 Cells under Hypotonic Culture Conditions is Inhibited by HgCl₂

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Abstract

Tetrahydrobiopterin (BH₄) was released from PC12 cells to the extracellular fluid. We found that the BH₄ outward flow from the cells placed in Hanks medium was increased when NaCl concentration of the medium was decreased. Increase in the BH₄ out flow was not observed when NaCl in the Hanks medium was substituted with sodium glutamate (0.14 M), choline chloride (0.14 M) or sucrose (0.25 M). HgCl₂, an inhibitor of water channel, aquaporin, prevented the increase in BH₄ out flow caused by the hypotonic medium. The results suggested the presence of a site in BH₄ transport which was stimulated by osmotic pressure and/or water influx.

Key words: tetrahydrobiopterin, water transport, aquaporin inhibitor, PC12 cells

Introduction

Intracellular tetrahydrobiopterin (BH₄) serves as an essential cofactor for aromatic amino acid hydroxylases (1-3) and nitric oxide synthase (4). BH₄ in the extracellular fluid was reported to induce exocytotic release of monoamines (5), and to be taken up by the cells possessing BH₄-dependent enzymes resulting in an elevation of cellular BH₄ level though the precise uptake mechanism is not definitely clear yet (6). Since BH₄ is synthesized inside the cells, BH₄ transport through plasma membrane seems to be important in regulating the intra- and extra-cellular BH₄ levels and BH₄-dependent functions. We previously reported that BH₄ outward flow from PC12 cells was inhibited by a membrane-permeable cAMP analog (7,8) and suggested the presence of regulatory processes for BH₄ transport by signaling molecules. In the present study, we found that BH₄ out flow from PC12 cells was increased under hypotonic conditions and this increase was inhibited by HgCl₂, an inhibitor of water channels, aquaporins (AQPs) (9,10).

Materials and Methods

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% fetal bovine serum, 7% horse serum, 100 units/ml penicillin and 100 mg/ml streptomycin in a CO₂ incubator at 37°C (11). For BH₄ outward flow experiment, PC12 cells were seeded on a 12-well plate and used 3 or 4 days after seeding. Prior to the experiment, cells were pre-cultured for 90-100 min in the culture medium containing 5 mM sepiapterin, a precursor of BH₄ via salvage pathway, to increase the intracellular BH₄ level so that BH₄ released to the medium was easily detected. Then the medium was changed for that used for BH₄ outward flow assay (200 µl/well). The cells were incubated for 10 min or 20 min in a CO₂ incubator and 100 µl aliquot of the medium was taken to a micro-centrifuge tube from each well and mixed with 5 µl of 4 M perchloric acid (PCA). Remaining medium was removed and 200 µl of 0.2 M PCA was added to extract biopterin and pterin from the cells. Biopterin and pterin in the medium and cells were determined by the method of Fukushima and Nixon (12).

Cell respiration was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT)

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Figure 1. BH_4 outward flow from PC12 cells in Hanks medium of various NaCl concentrations. A: PC12 cells in a 12-well plate were incubated in normal Hanks medium or low NaCl-Hanks medium (40 mM NaCl) for 10 min in a CO_2 incubator at 37°C and then biopterin (BP) and pterin (Ptn) contents in the medium and cells were measured as described in Materials and Methods. (closed bar: BP, open bar: Ptn, hatched bar: sum amount of medium [BP+Ptn] plus cell [BP+Ptn]). Values are the means $\pm$ SD ($n=4$). ** BH_4 outward flow ([BP+Ptn] content in the medium) and cellular [BP+Ptn] content with the low NaCl-Hanks medium were different from those with the normal Hanks medium at $p<0.001$. B: Effect of NaCl concentrations in Hanks medium on the BH_4 outward flow ([BP+Ptn] content in the medium). BH_4 outward flow from the cells was analyzed in the normal Hanks medium (138 mM NaCl) and Hanks medium of various NaCl concentrations for 20 min in a CO_2 incubator at 37°C . Values are the means $\pm$ SD ($n=4$). * $p<0.05$ and ** $p<0.001$ vs. the value obtained with normal Hanks medium (138 mM NaCl).

Figure 2A. Effects of sodium glutamate, choline chloride, and various metal chloride salts on the BH₄ outflow from PC12 cells. (closed bar: [BP+Ptn] in medium, open bar: sum amount of medium [BP+Ptn] plus cell [BP+Ptn]). In the left side panel, PC12 cells in a 12-well plate were incubated in normal Hanks medium (Nor, 138mM NaCl) and low-NaCl Hanks medium (Low; 20 mM NaCl) in the presence and absence of 0.14 M sodium glutamate (Na-G) or 0.14 M choline chloride (Ch-Cl). Values are the means $\pm$ SD (n=3). **p<0.001 vs. the value obtained with low NaCl Hanks medium (Low). In the right side panel, cells were incubated with normal and hypotonic Hanks medium (30 mM NaCl) in the presence and absence of sucrose (0.25 M) for 10 min in a CO₂ incubator at 37°C. Values are the means of duplicate experiments.

Figure 2B. Effect of various metal chloride salts on the BH₄ outflow increased by hypotonic Hanks medium. PC12 cells were incubated in normal Hanks medium (138mM NaCl) and hypotonic Hanks medium (50 mM NaCl) in the presence of various metal chloride salts (20 μ M each) for 10 min in a CO₂ incubator at 37°C. Values are the means $\pm$ SD (n=4). **p<0.001 vs. the value obtained with the hypotonic Hanks medium.

Figure 2C. Effect of HgCl₂ concentrations on the BH₄ outflow increased by the hypotonic Hanks medium. PC12 cells were incubated in the normal Hanks medium (138 mM NaCl) and hypotonic Hanks medium (50 mM NaCl) in the presence of various concentrations of HgCl₂ for 10 min in a CO₂ incubator at 37°C. Values are the means $\pm$ SD (n=4). *p<0.01 and **p<0.001 vs. the value obtained with hypotonic Hanks without HgCl₂.

method (13) to test cell viability during the incubation in the hypotonic medium. After the cells in 96-well plate were incubated for 10 min or 20 min in the hypotonic Hanks medium in a CO₂ incubator, the medium was changed for the culture medium containing 0.2 mg/ml MTT and incubated for further 60 min. Then the medium was removed by aspiration and the cells were solubilized in 100 μ l of dimethylsulfoxide. Formazan formed from MTT was photometrically measured by a micro-plate reader (Multiskan Bichromatic, Labsystems) with a 540 nm filter.

Results and Discussion

BH₄ synthesized in PC12 cells via either de novo or salvage pathway was continuously released to the extracellular fluid. When the de novo BH₄ synthesis in PC12 cells was completely blocked by 2,4-diamino-6-hydroxypridine, a GTP cyclohydrolase I inhibitor, cellular BH₄ level was decreased with a half life of 100-110 min under the usual culture conditions (7). BH₄ in the extracellular fluid (culture medium) was oxidized to 7,8-dihydrobiopterin (BH₂)/biopterin or converted to pterin. Therefore, during the short time period of incubation (e.g., ~30 min) total amount of biopterin species (BH₄, BH₂, oxidized form of biopterin) plus pterin in the medium was same as that of total BH₄ released from the cells (Fig. 1). The rate of the BH₄ outward flow from the cells was essential-

ly the same either in the culture medium, DMEM, or Hanks medium (data not shown). However, BH₄ outflow was increased when NaCl concentration in Hanks medium was decreased (Fig. 1). Cell respiration in the low NaCl-Hanks medium (containing 50 mM NaCl) was slightly inhibited by 10 min incubation and significantly inhibited by 20 min incubation: formazan production estimated from the absorbance at 540 nm was 91.6% that of the control cells incubated in the normal Hanks medium for 20 min.

The increase in BH₄ outward flow was not observed when NaCl in Hanks medium was substituted by either sodium glutamate (0.14 M) or choline chloride (0.14 M), suggesting Na⁺ or Cl⁻ ion itself was not specifically required for controlling BH₄ outflow (Fig. 2A). Sucrose (0.25 M) also blocked the increase in BH₄ outward flow caused by low NaCl concentration in Hanks medium, indicating that the BH₄ outflow was affected by osmotic pressure and/or water influx (Fig. 2A). Then effects of various metal chloride salts including HgCl₂ which inhibits AQPs (9,10), were examined (Fig. 2B). Addition of extra MgCl₂ or CaCl₂ (20 μ M each) did not give significant effect on the BH₄ outflow increased by the hypotonic Hanks medium containing 50 mM NaCl (not shown). Among the salts tested only HgCl₂ inhibited the increase in BH₄ outflow caused by the hypotonic conditions (Fig. 2C). The effect of HgCl₂ was concentration dependent and became maximal at around 50 μ M. At higher concentration (e.g., 100 μ M), HgCl₂ increased the BH₄ out-

flow from PC12 cells (Fig. 2C) even in the normal Hanks medium or in DMEM (not shown), probably due to its cytotoxic effect. These results suggest that water influx stimulated some process of the BH₄ outward flow from the cells though the underlying mechanism is remained to be elucidated.

Na⁺/K⁺-ATPase has a crucial role in regulating osmotic balance, thereby regulating cell volume (14) and maintaining homeostasis of epithelial fluid and electrolyte secretion (15). Na⁺-K⁺-2Cl⁻ cotransporters (NKCCs) and AQPs are also important in regulating water transport not only in fluid-secreting and -absorbing cells but in various cell types. It would be of interest to note that Na⁺/K⁺-ATPase (15), NKCCs (16), and AQPs (9) were reported to be regulated via cAMP/protein kinase A pathway, and that an AQP subtype, AQP1, in secretory vesicle membranes of pancreas was demonstrated to participate in water gating and vesicle swelling which was implicated in exocytosis (10).

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