Effect of Tetrahydrobiopterin on Dopamine Release from PC12 Cells

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ABSTRACT
We previously reported that tetrahydrobiopterin (6R-BH₄), a natural cofactor for aromatic amino acid hydroxylases and nitric oxide synthase, stimulates dopamine release from rat striatum independently of its cofactor activity. In the present study, we further investigated the effect of 6R-BH₄ on dopamine release using differentiated PC12 cells. In PC12 cells, 6R-BH₄ (10 μM and 30 μM) stimulated dopamine release in a dose-related manner, which was persisted in the presence of α-methyl-p-tyrosine (1 mM) or L-nitro-arginine methyl ester (100 μM). Dopamine release from PC12 cells was not stimulated by 6S-BH₄, a diastereoisomer of 6R-BH₄, or sepiapterin, a precursor of 6R-BH₄. These results were consistent with the effects of 6R-BH₄ on dopamine release from rat striatum. Dopamine release induced by 6R-BH₄ was inhibited by nicardipine (1 μM), suggesting that 6R-BH₄-induced dopamine release is mediated by Ca²⁺ channel activation. ⁴²Ca²⁺ uptake and intracellular Ca²⁺ concentration in PC12 cells were increased by 6R-BH₄. Membrane potential of PC12 cells was depolarized by 6R-BH₄. These data taken together suggest that 6R-BH₄ stimulates dopamine release from PC12 cells by inducing membrane depolarization which is a trigger for Ca²⁺ channel activation.

Tetrahydrobiopterin (6R-BH₄) is a natural cofactor for phenylalanine hydroxylase (1), tyrosine hydroxylase (11), tryptophan hydroxylase (9) and nitric oxide synthase (7). We previously reported that 6R-BH₄ stimulates dopamine release from rat striatum (2-4). The dopamine releasing action of 6R-BH₄ was independent of its cofactor activity (2, 3). That is, 6R-BH₄ stimulated dopamine release under inhibition of tyrosine hydroxylase or nitric oxide synthase. In dorsal motor nucleus of vagus of rats, 6R-BH₄ activated Ca²⁺ channels (12). These results suggest that 6R-BH₄ stimulated dopamine release by activating Ca²⁺ channels. We previously reported that 6R-BH₄ increased ⁴²Ca²⁺ uptake (5) and intracellular Ca²⁺ concentration in differentiated PC12 cells (6). These data suggest that 6R-BH₄ activates Ca²⁺ channels in differentiated PC12 cells. In the present study, to further investigate the dopamine releasing action of 6R-BH₄, we examined the effect of 6R-BH₄ on dopamine release from differentiated PC12 cells.

We demonstrated that 6R-BH₄ activates Ca²⁺ channels by inducing membrane depolarization in differentiated PC12 cells.

MATERIALS AND METHODS

Drugs
6R-BH₄·HCl is a generous gift from Suntory Biomedical Research Center, Osaka, Japan. 6S-BH₄-sulfate and sepiapterin (SP) were purchased from Wako (Osaka, Japan). Mouse nerve growth factor (NGF, 7.0S) and bis-(1, 3-dimethyl-
thiobarbituric acid)-trimethine oxonol (DiSBaC<sub>2</sub> (3)) were purchased from Funakoshi (Tokyo, Japan). Fura-2 acetoxymethylester (fura-2/AM) was purchased from Dojin (Kumamoto, Japan). Nicardipine, α-methyl-p-tyrosine (α-MT), l-nitro-arginine methyl ester (L-NAME) and dopamine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). <sup>45</sup>Ca<sup>2+</sup> was purchased from New England Nuclear Research Product (Boston, MA, U.S.A.). All other chemicals are of the purest grade available from regular commercial sources.

**PC12 cells**

PC12 cells were obtained from Riken Cell Bank (Tsukuba, Japan), cultured and differentiated by NGF as reported previously (13).

**Dopamine release assay**

For dopamine release assay, PC12 cells were cultured in poly-l-lysine coated 6-well culture dishes. Dopamine release assay was carried out as reported previously (5, 6). In brief, after washing with Krebs-HEPES buffer (NaCl 140 (mM), KCl 4.7, CaCl<sub>2</sub> 1, MgCl<sub>2</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, glucose 11, HEPES 5, pH 7.4), the cells were preincubated with Krebs-HEPES buffer at 37°C for 10 min. During preincubation time, α-MT (1 mM) or l-NAME (1 mM) was added to the incubation medium. Then the cells were incubated with test drugs at 37°C for 10 min. Both dopamine levels in the incubation medium and in the cells were measured by HPLC with electrochemical detection. Dopamine release was expressed as (released dopamine/released dopamine and cellular dopamine) × 100 (%).

**<sup>45</sup>Ca<sup>2+</sup> uptake assay**

<sup>45</sup>Ca<sup>2+</sup> uptake into PC12 cells were measured as reported previously (13).

**Intracellular Ca<sup>2+</sup> concentration assay**

Intracellular Ca<sup>2+</sup> concentrations in PC12 cells were measured using fura-2 as reported previously (10).

**Membrane potential assay**

Membrane depolarization was estimated by using a voltage-sensitive fluorescent dye, DiSBaC<sub>2</sub> (3) (8). The cells were collected and suspended in Krebs-HEPES buffer at 10<sup>6</sup> cells/mL. Aliquots of 2 mL of the cells were placed in a cuvette of spectrofluorophotometer (Shimadzu RF-5000, Kyoto, Japan) for measurement of fluorescence (excitation at 535 nm and emission at 559 nm). During the measurement, cell suspension was stirred with magnetic stirrer. After 30-60 sec, 50 μL of 6 μM DiSBaC<sub>2</sub> (3) was added into the cuvette by a microsyringe. After a stable baseline was attained, EPO was added to the cell suspension. Administered drugs were present in the incubation mixture until the end of the experiments. At the end of each experiment, cells were depolarized with 50 mM KCl to estimate the cell viability.

**RESULTS**

When 6R-BH<sub>4</sub> was added to the incubation medium at 10 or 30 μM, dopamine release increased in a dose-related manner. The dopamine release induced by 6R-BH<sub>4</sub> was inhibited by 1 μM nicardipine, a Ca<sup>2+</sup> channel blocker. In contrast, 6R-BH<sub>4</sub>-induced increase in dopamine release was persistent in the presence of α-MT, an inhibitor for tyrosine hydroxylase or l-NAME, an inhibitor for nitric oxide synthase (Fig. 1A). The dopamine release from PC12 cells was not stimulated by 6S-BH<sub>4</sub>, a diastereoisomer of 6R-BH<sub>4</sub> or sepiapterin, a precursor of 6R-BH<sub>4</sub> (Fig. 1B). <sup>45</sup>Ca<sup>2+</sup> uptake as an index for Ca<sup>2+</sup>

<table>
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<th>Table 1 Effect of 6R-BH&lt;sub&gt;4&lt;/sub&gt; on &lt;sup&gt;45&lt;/sup&gt;Ca&lt;sup&gt;2+&lt;/sup&gt; uptake into PC12 cells</th>
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<td><strong>&lt;sup&gt;45&lt;/sup&gt;Ca&lt;sup&gt;2+&lt;/sup&gt; uptake (nmol/mg protein/min)</strong></td>
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<td>control</td>
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<td>KCl 50 mM + nicardipine 1 μM</td>
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<td>6R-BH&lt;sub&gt;4&lt;/sub&gt; 10 μM</td>
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<td>6R-BH&lt;sub&gt;4&lt;/sub&gt; 30 μM</td>
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<tr>
<td>6R-BH&lt;sub&gt;4&lt;/sub&gt; 30 μM + nicardipine 1 μM</td>
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Each value is the mean ± SEM of five determinations. *, P<0.05 vs. the control group.
6R-BH₄ stimulates DA release from PC12 cells

Fig. 1 Effect of 6R-BH₄ (A) and its related agents (B) on dopamine release from PC12 cells. Each bar indicates mean ± SEM of six experiments. *, P<0.05 vs. control. #, P<0.05 vs 6R-BH₄ 30 µM

Fig. 2 Effects of 6R-BH₄ on intracellular Ca²⁺ concentrations (A) and membrane potential (B) in PC12 cells. Each trace was representative result of ten different experiments.
channel activity was also increased by 6R-BH₄ in a dose-related manner. The increase in ⁴²Ca²⁺ uptake induced by 6R-BH₄ was abolished by 1 μM nicardipine (Table 1). Intracellular Ca²⁺ concentration in PC12 cells was increased following administration of 6R-BH₄ (Fig. 2A). Membrane potential of PC12 cells was depolarized by 6R-BH₄ (Fig. 2B).

DISCUSSION

Dopamine release from PC12 cells was stimulated by 6R-BH₄ as observed in the rat striatum in vivo (2-4). The dopamine release induced by 6R-BH₄ persisted under an inhibition of tyrosine hydroxylase activity or nitric oxide synthase activity, suggesting that the effect of 6R-BH₄ on dopamine release is independent of its cofactor activities. The dopamine release was not stimulated by 6S-BH₄ or SP. These data were consistent with the effect of 6R-BH₄ on dopamine release from rat striatum. The dopamine release induced by 6R-BH₄ was inhibited by nicardipine, suggesting that the effect of 6R-BH₄ is mediated by Ca²⁺ channels. We observed that Ca²⁺ channels were activated by 6R-BH₄ in the rat brain (12). In the present study, we found that ⁴²Ca²⁺ uptake into PC12 cells and intracellular Ca²⁺ concentrations were increased by 6R-BH₄. Thus it is suggested that 6R-BH₄ activated Ca²⁺ channels in PC12 cells. Since membrane potential was depolarized by 6R-BH₄ in PC12 cells, it is assumed that Ca²⁺ channels in PC12 cells were activated due to membrane depolarization induced by 6R-BH₄. It remains to be clarified whether 6R-BH₄ modulates kinetics of Ca²⁺ channels in PC12 cells. In the present study, we observed that the dopamine releasing action of 6R-BH₄ in PC12 cells is independent of its cofactor activity as well as dopamine releasing action in the rat striatum. In PC12 cells, 6R-BH₄ acted as a membrane depolarizing agent. In our preliminary experiments, we observed that 6R-BH₄ induced depolarization in the neurons of dorsal motor nucleus of vagus in rats. It is possible, therefore, that 6R-BH₄ induces depolarization in dopaminergic nerve terminals in striatum.

REFERENCES