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STIMULATION OF NERVE GROWTH FACTOR SYNTHESIS/SECRETION BY PROPENTOFYLLINE IN CULTURED MOUSE ASTROGLIAL CELLS

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Abstract—The xanthine derivative propentofylline has been reported to exhibit protective effects on neuronal cells in the brain, which include improvement of impaired learning and memory and protection against delayed ischemically-induced neuronal cell death in the hippocampal CA1 subfield. To investigate the mechanism of action of this drug, we examined its effect on nerve growth factor (NGF) synthesis in cultured mouse astroglial cells because NGF is known to function as a neurotrophic molecule that is required to maintain neuronal activities. The drug was found to increase the NGF content in the conditioned medium to a level over ten times that of the control. This result suggests that the action of propentofylline in the brain includes stimulation of synthesis/secretion of NGF in astroglial cells.

Propentofylline or 3,7-dihydro-3-methyl-1-(5-oxohexyl)-7-propyl-1*H*-purine-2,6-dione (Fig. 1) is a xanthine derivative which exhibits several effects in the brain, e.g. prevention of cerebral metabolic disorder during anoxia [1] and improvement of cerebral edema [2]. Besides these properties, two additional pharmacological effects of propentofylline have been reported recently. Goto *et al.* showed that the drug improves the decreased learning ability of aged spontaneously hypertensive rats [3], and DeLeo *et al.* demonstrated that propentofylline protects against delayed ischemically-induced neuronal cell death in the hippocampus CA1 subfield of the mongolian gerbil [4, 5]. Since peripherally administered propentofylline has been proven to move into the brain [6], its effects on brain functions are suggested to be mediated by its direct action on neurons and/or glial cells.

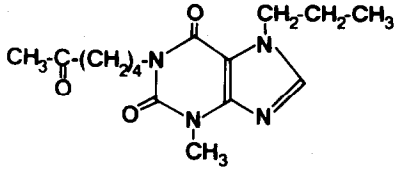

Fig. 1. Chemical structure of propentofylline.

Nerve growth factor (NGF†) is a protein composed of 118 amino acid residues, and it is known to be required for the development and maintenance of peripheral sympathetic and sensory neurons [7]. In the brain, it was found recently that NGF functions as a neurotrophic molecule for the magnocellular cholinergic neurons in basal forebrain nuclei thought to be probably involved in memory processes [8–10]. Also, we have shown that astrological cells cultured from mouse brain synthesize and secrete NGF [11, 12]. Since astroglial NGF synthesis is considered to be related to protection of neuronal activities, the protective effect of propentofylline in the brain may be mediated by this growth factor. Accordingly, we examined whether propentofylline modulates NGF synthesis in cultured mouse astroglial cells.

MATERIALS AND METHODS

Materials. Mouse submaxillary gland β-NGF and anti-β-NGF antiserum were prepared as described previously [13]. Other materials and their sources were as follows: propentofylline, from Hoechst Japan Ltd (Tokyo, Japan); DMEM and FCS, from GIBCO (Grand Island, NY, U.S.A.); cell culture vessels, from Falcon (Becton Dickinson & Co., NJ, U.S.A.); BSA, from the Armour Pharmaceutical Co. (Kankakee, WI, U.S.A.); standard proteins for molecular weight determination, from Pharmacia Fine Chemicals (Uppsala, Sweden); and anti-GFAP antibodies, from BioGenex Lab. (Dublin, CA, U.S.A.). All other reagents were from Nacalai Tesque Inc. (Kyoto, Japan).

Cell culture. Astroglial cells were cultured from the whole brains of ICR mice that were a few days old and maintained in DMEM containing 10% FCS as described before [11]. Over 98% of the cells contained glial fibrillary acidic protein (GFAP) as judged by biotin-streptoavidin immunostaining using anti-GFAP rabbit antibodies. Because cells in the mature brain have finished proliferating, we decided to use quiescent astroglial cells for our study. Preparation of quiescent cells was performed according to the procedures previously reported [14]. Namely, astroglial cells were inoculated into 96-well plates (well surface, 0.28 cm^2) and cultured in FCS-containing DMEM until confluence was reached (about

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† Abbreviations: NGF, nerve growth factor; EIA, enzyme immunoassay; BSA, bovine serum albumin; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; CM, conditioned medium; CA, cornu ammonis; and GFAP, glial fibrillary acidic protein.

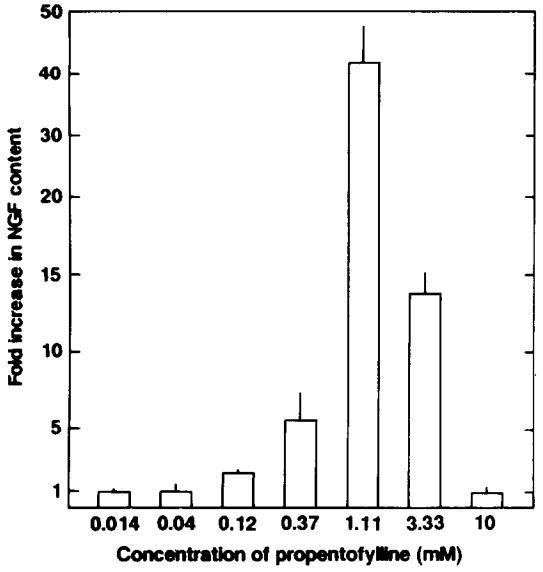

Fig. 2. Effect of propentofylline on NGF synthesis in cultured mouse astroglial cells. Astroglial cells were cultured with various concentrations of propentofylline (0.1 mL) in 96-well plates for 24 hr. Then the medium was collected. The NGF content in the medium was determined by enzyme immunoassay (EIA) and expressed as fold increase over that in the absence of the drug. Each value is the mean ± SE of three determinations.

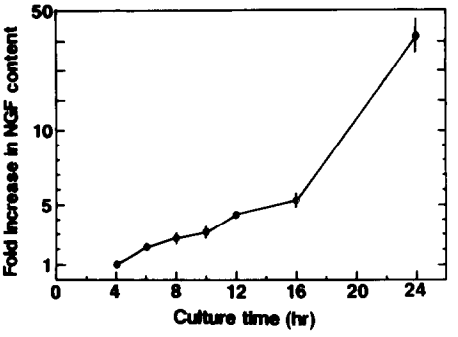

Fig. 3. Time course of NGF content in conditioned medium (CM) of astroglial cells treated with 1.11 mM propentofylline. Astroglial cells were cultured with 1.11 mM propentofylline (0.1 mL) in 96-well plates, and the amounts of NGF in CM were measured at 2, 4, 6, 8, 10, 12, 16, and 24 hr. The NGF content is expressed as fold increase over that in the absence of the drug at the same culture time. Each point is the mean ± SE of three determinations.

1.5×10^5 cells/cm^2). Then, they were cultured for an additional 1 week in FCS-free DMEM containing 0.5% BSA, with medium changes every 3 days. Most of the cells were arrested in the quiescent phase, because the cells never proliferated in the FCS-free medium, and the incorporation of [*methyl*-^{3}H]thymidine into trichloroacetic acid-insoluble material was below 2% of that found in the growing cells. Then, the medium was changed to DMEM containing 0.5% BSA with or without any drugs, and the cells were cultured for 24 hr.

Measurement of NGF contents in CM. This was performed by a two-site EIA system for mouse submaxillary gland β-NGF which was developed in our laboratory. The specificity and reliability of the EIA system have been confirmed in another investigation of ours [13]. The EIA system was not affected by the test samples assayed in this study.

RESULTS

Astroglial cells were cultured for 24 hr with various concentrations of propentofylline, and the amounts of NGF in the CM were measured. The NGF content in the medium is considered to be the amount of NGF synthesized during culture, because NGF is secreted rapidly and the intracellular content of NGF is low [15]. As shown in Fig. 2, propentofylline clearly increased the NGF content in the CM at concentrations between 0.12 and 3.33 mM. The maximum response was observed at 1.11 mM. Above this concentration propentofylline appeared to cause cell damage, for the increase was markedly less at 3.33 mM and nonexistent at 10 mM. This effect of propentofylline was not due to the suppression of NGF degradation, because the addition of cell extracts from propentofylline-treated cells to the CM of control cells did not affect the NGF content (data not shown).

The effect of 1.11 mM propentofylline was followed with time of incubation, as shown in Fig. 3. NGF in the CM was below the detection limit at the culture time of 2 hr and was first detected at 4 hr. At 24 hr the level of NGF secreted (140 ± 19 pg/mL) was 42 times higher than that of the control (3.3 ± 0.3 pg/mL).

The molecular size of NGF synthesized and secreted by astroglial cells stimulated with propentofylline was compared with that of control astroglial cell NGF by gel-filtration with a Bio-Gel P-100 column. Figure 4 illustrates the elution profiles of NGF in both CMs. Both NGFs were eluted from the column at the same position, corresponding to a molecular weight of about 23,000. Therefore, the molecule whose synthesis was stimulated by propentofylline appears to be the same as NGF normally secreted by astroglial cells.

Earlier, we showed that catecholamines are potent stimulators of NGF synthesis in cultured astroglial cells [14]. Therefore, the effect of propentofylline on NGF synthesis was assessed in the presence of epinephrine. The results of Fig. 5 show that propentofylline and epinephrine acted additively in increasing the NGF content.

DISCUSSION

We have shown by the above experiments that propentofylline has the ability to stimulate NGF synthesis in cultured mouse astroglial cells. This effect of propentofylline was exhibited at drug concentrations ranging from 0.12 to 3.33 mM, with the maximum at 1.11 mM. The time–course study showed a lag period of 6 hr before the stimulatory effect of propentofylline was evident, even when

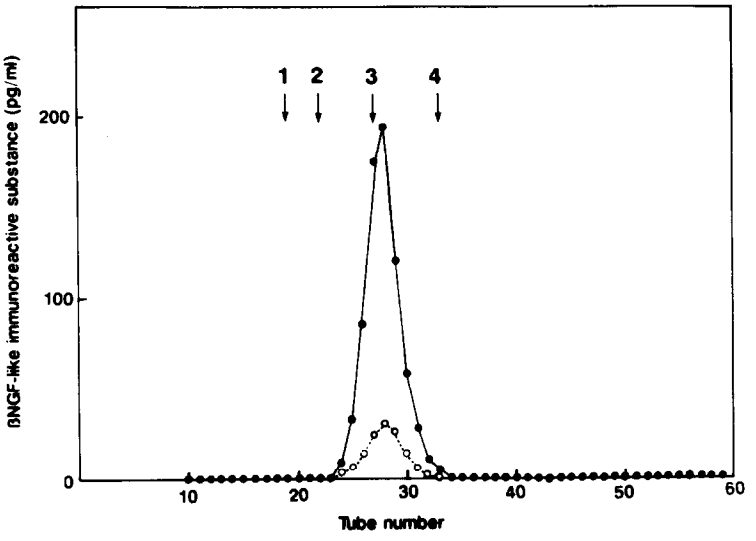

Fig. 4. Bio-Gel P-100 gel filtration of CM of astroglial cells treated with propentofylline. Astroglial cells were cultured with or without 1 mM propentofylline (10 mL) in a culture dish (surface, 55 cm^2) for 24 hr. The NGF contents in drug-treated and control medium were 80 and 8 pg/mL respectively. Each medium was lyophilized. The dry residue was redissolved in 1 mL of 0.1 M Tris–HCl buffer, pH 7.6, containing 1.0 M NaCl and applied to a Bio-Gel P-100 column (0.9 × 62 cm) equilibrated with 0.1 M Tris–HCl buffer, pH 7.6, containing 1.0 M NaCl, 0.1% NaN_3 and 0.1% BSA. Fractions of 0.7 mL were collected at a flow rate of 2.6 mL/hr. Each point is the mean of three determinations by EIA. Key: (○) control culture medium; and (●) profile from drug-treated cultures. Calibration of the column was performed using (1) BSA, (2) horseradish peroxidase, (3) purified submaxillary gland β-NGF, and (4) cytochrome *c*.

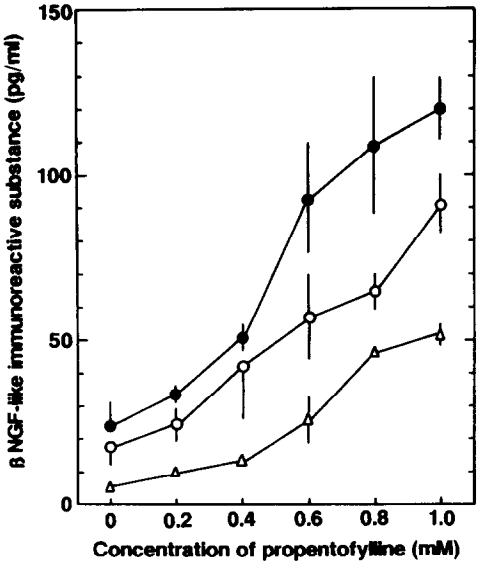

Fig. 5. Effect of propentofylline on astroglial NGF synthesis in the presence of epinephrine. Astroglial cells were cultured with various concentrations of propentofylline in the absence (△) or in the presence of 0.1 mM (○) and 0.2 mM (●) epinephrine in 96-well plates for 24 hr (culture medium, 0.1 mL). Then, the medium was collected. The NEG content in CM was determined by EIA. Each point is the mean ± SE of four determinations.

the drug was added at the optimal concentration of 1.11 mM.

The results of gel filtration coincided with previous work showing that there exist no high molecular weight types of NGF in the CM of astroglial cells and also indicated that the NGF induced by propentofylline has the same molecular weight as control NGF.

In our previous investigation concerning the regulation of synthesis/secretion of NGF by catecholamines in cultured mouse astroglial cells, we demonstrated that catecholamines uniquely accelerate synthesis of NGF-mRNA, resulting in an increase in NGF content, and that the accumulation of NGF-mRNA takes place within 4 hr after catecholamine treatment and then the translated NGF protein is detected [16]. Since the time course of NGF synthesis in astroglial cells treated with propentofylline was similar to that in the case of catecholamines and an additive effect on NGF synthesis was observed in the combination of propentofylline and epinephrine, we suggest that the observed stimulation of NGF synthesis by propentofylline is the result of newly synthesized and accumulated NGF-mRNA.

A recent pharmacological study of NGF showed that continuous intracerebral infusion of NGF could improve retention of a spatial memory task in behaviourly impaired aged rats [17]. According to the report by Goto *et al.* [3], successive administration for a period of 10 days was required to reveal the effect of propentofylline on improvement of decreased learning and memory in aged rats. Judging from this delayed effect of propentofylline, we speculate that the effect is caused in part by the action of NGF induced from astroglial cells by this drug. Also,

it is not unreasonable to extrapolate that the protective effect of propentofylline against delayed ischemically-induced neuronal cell death in hippocampus CA1 subfield is related to the induced NGF, although there is no evidence that NGF acts on the hippocampal neurons.

Astroglial cells in the brain have long been thought to be involved in the regulation of critical neuronal behavior both during development and in the mature brain [18, 19]. From this standpoint, we suggest that the synthesis/secretion of NGF is one of the physiological roles of astroglial cells for the development and maintenance of neurons in the brain. Therefore, we consider that the search for compounds having the ability to stimulate NGF synthesis in astroglial cells seems to be indispensable not only for the elucidation of the mechanism of regulation of NGF in the brain but also for the development of drugs that improve the function of impaired neurons as seen in the aged brain and/or in the brain of patients with Alzheimer's disease. Our present finding will be helpful to attain this goal.

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