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STIMULATORY EFFECT OF 4-ALKYLCATECHOLS AND THEIR DIACETYLATED DERIVATIVES ON THE SYNTHESIS OF NERVE GROWTH FACTOR

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Abstract—A series of 4-alkylcatechols and 1,2-diacetoxy-4-alkylbenzenes (from methyl to butyl) were chemically synthesized for *in vitro* evaluation as stimulators of nerve growth factor (NGF) synthesis. All compounds were proven to be potent in stimulating NGF synthesis in L-M cells (a mouse fibroblast cell line) and mouse astroglial cells. In a series of 4-alkylcatechols, 4-methylcatechol and 4-ethylcatechol severely affected viability and cell adhesive properties. In a series of 1,2-diacetoxy-4-alkylbenzenes, the concentrations required for the maximal effect and the effective ranges of concentrations were higher than those in the 4-alkylcatechol series, and the cell adhesive properties or viabilities were not affected. Evidence is also presented to indicate that the elevation of NGF synthesis by these compounds was not associated with the cell growth.

Nerve growth factor (NGF¶) is a protein that is required for the survival and differentiation of sensory and sympathetic neurons in the peripheral nervous system (PNS) [1] and of cholinergic neurons of the basal forebrain in the central nervous system (CNS) [2]. NGF is concentrated in large amounts in unexpected organs (mouse submaxillary gland and prostate glands of certain species), but these high local concentrations most likely do not play a role in the nervous tissue [1]. The physiological site of NGF synthesis is thought to be the innervated effector organs [1]. We showed that fibroblast cells cultured from mouse organs having sympathetic innervation [3, 4], established cell lines of mouse fibroblast cells [5], and astroglial cells cultured from mouse brain [6] synthesize and secrete NGF indistinguishable from mouse submaxillary gland β-NGF. We have also demonstrated that catecholamines are a potent stimulator of NGF synthesis in fibroblast cells [5, 7] and astroglial cells [8, 9]. For the stimulatory effect, the catechol ring of catecholamines is essential and the aliphatic side chain enhances this effect, but the terminal amino residue on the side chain is not essential [7, 8]. These observations led us to examine the stimulatory effect of 4-alkylcatechols (from methyl to butyl) and their diacetylated derivatives on NGF synthesis.

We report herein the evaluation of such compounds in terms of their effects on NGF synthesis/secretion in mouse fibroblast cells and mouse astroglial cells.

MATERIALS AND METHODS

Chemicals. 4-Methylcatechol (**1**), 3-methylcatechol, and 4-*tert*-butylcatechol were obtained from Tokyo Kasei (Tokyo, Japan); dopamine was obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) and [*methyl*-^{3}H]thymidine (6.9 Ci/mmol) from ICN Radiochemicals (Irvine, CA, U.S.A.).

Other compounds used in this study were synthesized by the routes shown in Fig. 1. Commercially available 3,4-dimethoxystyrene (**3a**) was first converted to 4-ethylveratrole (**4a**) by catalytic hydrogenation (Pd/C). 4-Ethylveratrole (**4a**) was then converted to 4-ethylcatechol (**5a**) by demethylation [10]; demethylation was carried out by heating in a solvent such as acetic acid containing hydrobromic acid. 4-Propylcatechol (**5b**) was obtained in the same way [11] from 1,2-dimethoxy-4-prophenylbenzene (**3b**), which is commercially available. 4-Butyrylveratrole (**8**), which was obtained by Friedel–Craft reaction of veratrole (**7**) and *n*-butylic acid, was converted to 4-*n*-butylveratrole (**9**) by a Wolff–Kishner reduction. And 4-*n*-butylveratrole (**9**) was converted to 4-*n*-butylcatechol (**10**) by demethylation as described above. A series of 1,2-diacetoxy-4-alkylbenzenes (**2, 6a, 6b, 11**) were prepared from their corresponding catechol derivatives (**1, 5a, 5b, 10**) by acetylation with acetic anhydride in the presence of a catalytic amount of an acid such as sulfuric acid.

Cells. L-M cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.)

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¶ Abbreviations: NGF, nerve growth factor; PNS, peripheral nervous system; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BSA, bovine serum albumin; CM, conditioned medium; EIA, enzyme immunoassay; SDS, sodium dodecyl sulfate; and TCA, trichloroacetic acid.

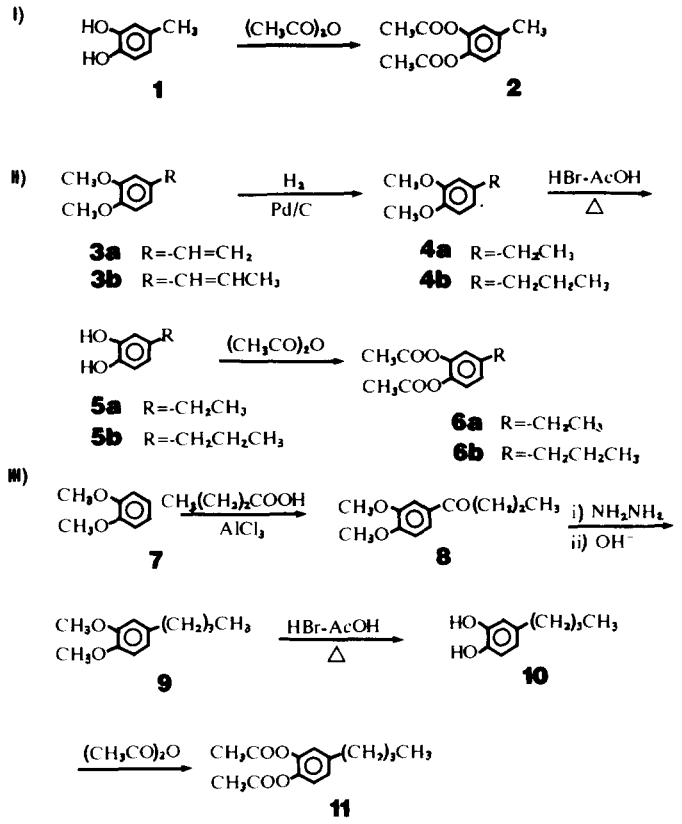

Fig. 1. Structures and steps for synthesis of a series of 4-alkylcatechols and their diacetylated derivatives.

and maintained as monolayer cultures in medium 199 (Flow Laboratories, Inc., McLean, VA, U.S.A.) supplemented with 0.5% peptone (Difco Laboratories, Detroit, MI, U.S.A.) as described before [5]. To study the effect of the compounds, we plated the cells in 96-well plates (Falcon Plastics, Oxnard, CA, U.S.A.) at approximately $1–2 \times 10^4$ cells/cm^2 and cultured them for several days. Before the cells reached confluence, they were treated singly with each of the above compounds for 24 hr.

Astroglial cells were prepared from the whole brains of 8-day-old ICR mice [6] and maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 10% fetal calf serum (FCS) (Hyclone, Sterile Systems Inc., Logan, UT, U.S.A.). The cells were plated in 96-well plates at approximately $1–2 \times 10^4$ cells/cm^2. When the cells had reached a density of $1–2 \times 10^5$ cells/cm^2, they were used as the growing cells. Other cultures, after having reached confluence ($0.5–1 \times 10^6$ cells/cm^2), were cultured for about another 2 weeks in FCS-free medium containing 0.5% bovine serum albumin (BSA) (Armour Pharmaceutical Co., Kankakee, IL, U.S.A.) to bring them to the quiescent phase [12].

Determination of NGF level. For the determination of NGF, the conditioned medium (CM) was applied directly to the enzyme immunoassay (EIA) system [13]. The detection limit of the EIA with submaxillary gland NGF was 1 pg/mL. The EIA system was not affected by the compounds or culture medium used in this work.

Determination of DNA synthesis. Cells grown in 24-well plates were cultured in the presence of 0.1 mM 4-methylcatechol (0.5 mL) for 24 hr and the CM was removed. The cells were then cultured in fresh medium (0.2 mL) containing 0.1 mM 4-methylcatechol and [^{3}H]thymidine (2.5 μCi/mL) for 2 hr.

After the medium was removed, the cells were dissolved in 1 mL of 0.5% sodium dodecyl sulfate (SDS). The acid-insoluble materials precipitated with 0.1 mL of 100% trichloroacetic acid (TCA) were collected on glass fiber filters (Whatman GF/C). After extensive washing of the precipitates with 5% TCA and 95% ethanol, the ^{3}H-radioactivity on the filters was determined in a liquid scintillation counter.

Protein determination. Total cellular protein was determined in 0.05-mL aliquot of the SDS extract by the method of Lowry *et al.* [14] with BSA as standard.

RESULTS AND DISCUSSION

4-Methylcatechol (homocatecol), which is the simplest compound in the first series with one carbon on the side chain, is soluble in water. 4-Ethylcatechol, which is the deaminated derivative of dopamine, is

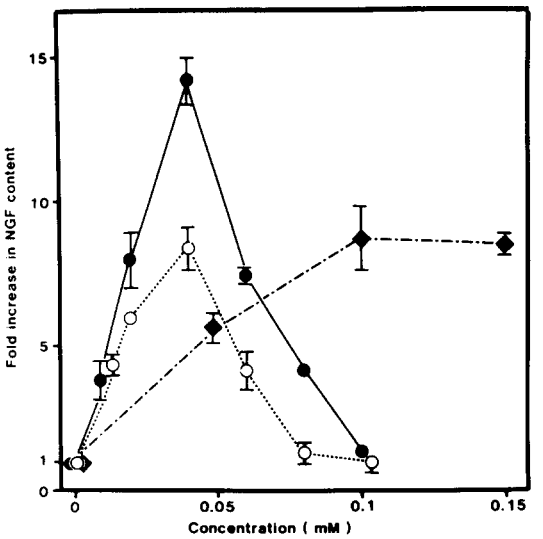

Fig. 2. Effects of 3- or 4-methylcatechol on the NGF content in the medium of mouse L-M cells. Cells were incubated for 24 hr with various concentrations of 4-methylcatechol (●), 3-methylcatechol (○), or dopamine (◆). The NGF content in the medium was determined by EIA and expressed as fold increase over that in the absence of a given drug (41.2 ± 4.78 pg/well/day). Each point is the mean ± SEM of four determinations.

insoluble in water, as the deamination of catecholamine increases its lipophilicity [15]. 4-Propylcatechol, 4-*n*-butylcatechol and 4-*tert*-butylcatechol are also insoluble in water. The compounds in the second series with esterified hydroxy groups are more lipophilic. For the estimation of their effect on NGF synthesis/secretion, compounds other than 4-methylcatechol were dissolved in ethanol and then added to the medium. Ethanol at a concentration of less than 1% did not affect the viability or the ability for NGF synthesis of fibroblast cells and astroglial cells. Since NGF synthesized by fibroblast cells and astroglial cells is secreted rapidly into the medium and its cellular content is constant, the measurement of NGF content in the CM was considered to reflect the amount of NGF synthesized [3, 6]. Figure 2 shows the NGF contents in the CM when L-M cells were cultured for 24 hr in the presence of 4-methylcatechol. 4-Methylcatechol obviously increased the amount of NGF secreted by L-M cells. Its concentration for the maximal effect was lower and its maximal effect was greater than those of dopamine, one of the catecholamines. We previously showed by Northern blot analysis that 4-methylcatechol (as well as catecholamines) stimulates NGF synthesis by increasing the cellular content of NGF mRNA [8]. The rapid decrease in the amount of NGF synthesis/secretion observed at 4-methylcatechol concentrations over 0.05 mM was likely caused by cytotoxicity. Catecholamines including dopamine do not affect cell viability even at concentrations of more than 0.2 mM, whereas dead cells appeared at a concentration of 0.06 mM 4-methylcatechol, and all cells died at a concentration over 0.1 mM.

Normal L-M cells are rounded and adhere firmly to the culture wells. When L-M cells were cultured in the presence of catecholamines, cells became spindle shaped, and none of them floated. However, when L-M cells were cultured with 4-methylcatechol at a concentration of more than 0.04 mM, some of the cells became detached from the surface of the culture vessel. At a concentration of 0.08 mM, most cells floated in the medium. 4-Methylcatechol inhibited cell adhesion to both other cell surfaces and the culture vessel surface. It was most likely that the cytotoxicity was related to a decrease in cell adhesiveness. We have not yet investigated the relationship between the increase in NGF synthesis and the decrease in cell adhesion.

Figure 2 also shows that the concentration for the maximal effect of 3-methylcatechol was the same as that of 4-methylcatechol. However, the magnitude of its maximal effect was lower than that of 4-methylcatechol, and the cytotoxicity of 3-methylcatechol was more severe than that of 4-methylcatechol. These results indicate that the 4-position of the side chain is preferable for the maximal stimulation of NGF synthesis.

Figure 3a shows the fold increase of NGF content in the CM when L-M cells were cultured in the presence of a series of 4-alkylcatechols. 4-Ethylcatechol and 4-propylcatechol showed bell-shaped concentration–response curves in a manner similar to 4-methylcatechol, probably because the cells died at higher concentrations of these catechols. Cytotoxicity was not observed with 4-*n*-butylcatechol or 4-*tert*-butylcatechol. The concentrations required for the maximal effect were ranked as follows: methyl < ethyl, propyl < *n*-butyl, *tert*-butyl. The rank order of their maximal effect was methyl > ethyl, propyl > *n*-butyl, *tert*-butyl, and that of the range of effective concentrations of these compounds was methyl < ethyl, propyl < *n*-butyl, *tert*-butyl. The order of effect on cell adhesion was methyl > ethyl ≫ propyl > *n*-butyl, *tert*-butyl. These results indicate that the compounds with long alkyl chains are less active and less toxic, that is to say milder, than those with short alkyl chains.

Figure 3B shows the NGF content in the CM when L-M cells were cultured in the presence of a series of 1,2-diacetoxy-4-alkylbenzenes. In this series, the concentrations required for the maximal effect and the effective ranges of concentrations were higher than those in the first series. Some cells in the presence of 1,2-diacetoxy-4-methylbenzene had a round shape, whereas no compounds with ethyl, propyl, or butyl chains evoked any morphological change. Also, no compounds in the second series exhibited cytotoxicity.

As two phenolic hydroxy groups are essential for the stimulatory effect on NGF synthesis [7], the compounds in the second series are most likely deacetylated within the cells and act there as 4-alkylcatechol. When L-M cells were cultured in the presence of 4-methylcatechol or 1,2-diacetoxy-4-methylbenzene and the amount of NGF in the CM was measured at the culture times of 2, 4, 6, and 8 hr, NGF contents started to increase over the basal level after a lag time of 4 hr in both cases (data not

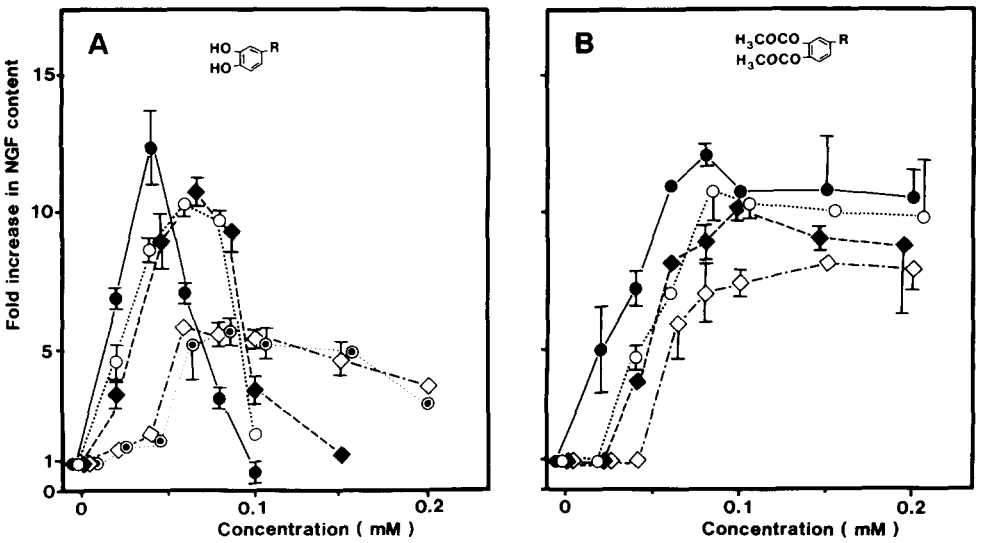

Fig. 3. Effects of 4-alkylcatechols (A) and 1,2-diacetoxy-4-alkylbenzenes (B) on NGF synthesis in mouse L-M cells. Experimental conditions were the same as those described in the legend of Fig. 2. The structures of their side chains are —CH_3 (●), —CH_2CH_3 (○), —$(CH_2)_2CH_3$ (◆), —$(CH_2)_3CH_3$ (◇), and —$C(CH_3)_3$ (◉).

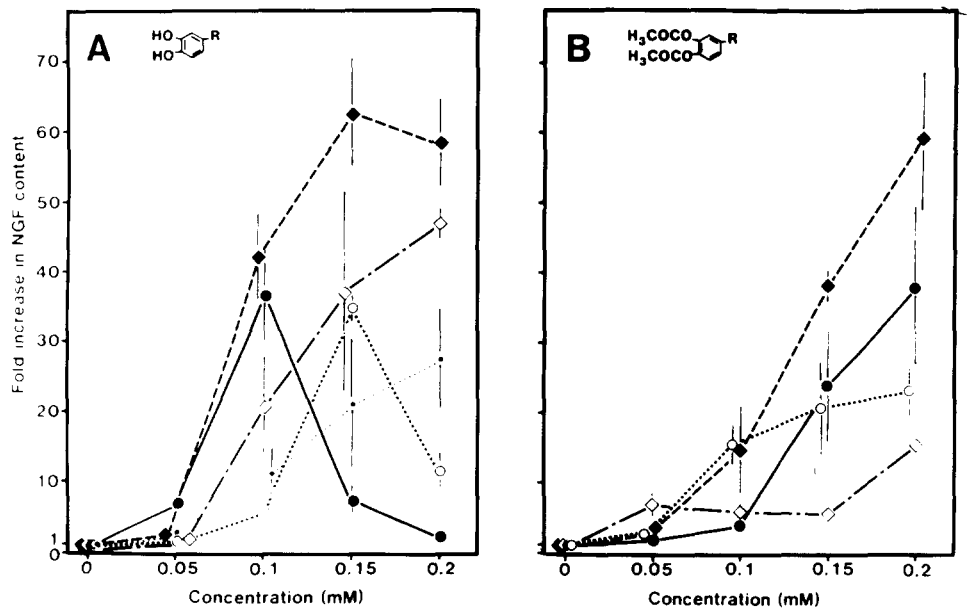

Fig. 4. Effects of 4-alkylcatechols (A) and 1,2-diacetoxy-4-alkylbenzenes (B) on the NGF content in the medium of mouse astroglial cells in quiescent phase. Cells were incubated for 24 hr with various concentrations of the compounds dissolved in DMEM–0.5% BSA. The structures of their side chains are the same as those described in the legend of Fig. 3. The NGF content in the medium is expressed as fold increase over that in the absence of a given compound (0.61 ± 0.08 pg/well/day).

shown). This result suggests that deacetylation of 1,2-diacetoxy-4-alkylbenzene rapidly occurs in cells.

We demonstrated earlier that the synthesis/secretion of NGF in cultured mouse astroglial cells is regulated in a growth-dependent manner [12], with catecholamine stimulation of NGF synthesis/secretion in quiescent cells and suppression in growing cells [9]. The astroglial cells in post-developmental brain are in the quiescent phase, and mitotic cells appear only during development or after brain injury [12]. At first, we investigated the effect of the 4-alkylcatechol series on the quiescent astroglial cells (Fig. 4A). Both 4-methylcatechol and 4-ethylcatechol showed a bell-shaped concentration–curve, because the cells died at high concentrations. 4-Propylcatechol was the most effective in increasing the amount of NGF synthesis/secretion; the amount was 60- to 70-fold greater than that of the control at

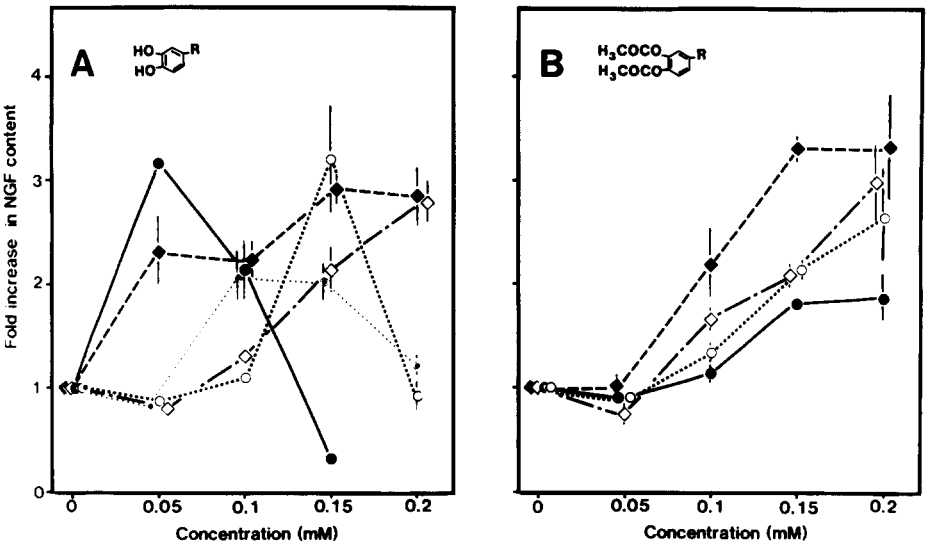

Fig. 5. Effects of 4-alkylcatechols (A) and 1,2-diacetoxy-4-alkylbenzenes (B) on the NGF content in the medium of mouse astroglial cells in growing phase. The NGF content in the CM of control cells was 14.2 ± 1.03 pg/well/day. The compounds were dissolved in DMEM–10% FCS. The structures of their side chains are the same as those described in the legend of Fig. 3.

a concentration of 0.15 mM. 4-*n*-Butylcatechol and 4-*tert*-butylcatechol were less effective than 4-propylcatechol. In the second series, 1,2-diacetoxy-4-propylbenzene was the most effective, the NGF content being increased about 60-fold at 0.2 mM (Fig. 4B). None of the compounds in the second series affected viability or the cell adhesive property of the astroglial cells at the concentrations tested.

We have already indicated that catecholamines decrease the amount of NGF synthesized by growing astroglial cells [9]. The reduction in the growth rate of cells by catecholamines was considered to be one of the likely reasons for the suppressive effect of catecholamines on NGF synthesis/secretion in growing astroglial cells [9]. All compounds in the first and second series increased the amount of NGF synthesized by growing astroglial cells (Fig. 5). 4-Alkylcatechols and 1,2-diacetoxy-4-alkylbenzenes are likely not to suppress the cell growth. To clarify this possibility, quiescent or growing cells cultured in medium containing 0.1 mM 4-methylcatechol were pulse-labeled with [^{3}H]thymidine. Table 1 shows that 4-methylcatechol did not stimulate the incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material in either quiescent cells or growing cells, in contrast to the obvious effect on the NGF content. Table 1 also shows that 4-methylcatechol had no effect on protein content of the cells. Our previous studies have shown that 4-methylcatechol increases NGF mRNA content in both mouse astroglial cells and L-M cells [8]. These results suggested that 4-alkylcatechols and 1,2-diacetoxy-4-alkylbenzenes affected specifically the synthesis of NGF protein.

The results of our previous studies suggested that the stimulatory effect of catecholamines on NGF synthesis is not mediated by α- or β-adrenergic receptors, for neither α-antagonist (phentolamine) nor β-antagonists (propranolol and dichloroisoproterenol) blocked the effect of catecholamines [5]. Further, neither an α-agonist (phenylephrine) nor a β-agonist (salbutamol), which do not have the catechol ring, was effective in stimulating NGF synthesis [5]. The

Table 1. Effect of 4-methylcatechol (0.1 mM) on DNA synthesis and NGF synthesis/secretion by astroglial cells

Treatment	[^{3}H]Thymidine incorporation (dpm/well/2 hr)	NGF synthesis (pg/well/day)	Cell protein (pg/well)
A. Quiescent cells			
None (0.5% BSA)	317 ± 45.9	9.60 ± 1.59	161 ± 12.2
0.5% BSA + 4-methylcatechol	333 ± 26.9	44.9 ± 7.32	162 ± 39.5
10% FCS	57,334 ± 9,544	80.0 ± 14.3	269 ± 39.3
B. Growing cells			
None (10% FCS)	253,688 ± 23,010	115 ± 22.8	200 ± 48.7
10% FCS + 4-methylcatechol	236,977 ± 27,045	267 ± 37.2	207 ± 29.5
0.5% BSA	60,650 ± 7,769	51.7 ± 7.20	184 ± 37.0

After quiescent astroglial cells (A) and growing astroglial cells (B) were incubated for 24 hr in the indicated medium, the amounts of [^{3}H]thymidine incorporation into TCA-insoluble materials, NGF secreted into medium, and total cell protein were determined. Values are the means ± SE of four determinations.

dopaminergic agonist (apomorphine) was also ineffective in stimulating NGF synthesis (data not shown). The present data showed that non-amine derivatives stimulate NGF synthesis, and support our previous suggestion that the stimulatory effect of catecholamines on NGF synthesis is not mediated by catecholaminergic receptors [5]. Catecholamines and 4-alkylcatechols may trigger some yet undefined intracellular reaction(s) after their incorporation into cells [5]. We are now expanding our study to clarify the mechanism by which catecholamines and 4-alkylcatechols stimulate NGF synthesis.

The results of the present study have additional significance. Several recent findings have indicated that intraventricular or intracerebral injection of NGF prevents neuronal death in rat brain lesioned mechanically or chemically [16–20], which opens up the interesting possibility that NGF may be useful as a therapeutic agent for Alzheimer's disease which was suggested to be a disorder of the cholinergic forebrain system and/or any diseases of the CNS. But intracerebral injection of NGF, even if recombinant human NGF becomes available in the near future, would be hardly practical. The most realizable medical procedure might be to regulate the synthesis of endogenous NGF by peripheral administration of certain drugs. As the compounds used in this study are lipophilic, the possibility exists that these compounds may be used as inducers of NGF synthesis *in vivo*, especially for the brain. Also they may be expected not to provoke adrenergic effects as catecholamines. In our preliminary experiments, the NGF content in some regions of brain increased after the intraperitoneally administration of these compounds. We will examine whether they provoke side effects as an adrenergic reagent or not.

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