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A Cyclic AMP Analog and High Potassium Prevent the Death of Cultured Septal Cholinergic Neurons after Nerve Growth Factor Withdrawal

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Survival, following the addition of a cAMP analog and high K^+ to the medium, of cultured fetal septal cholinergic neurons was examined after nerve growth factor (NGF) deprivation. The number of acetylcholinesterase positive cells, which had progressively grown during 11—13 d of culture with NGF (50 ng/ml), was greatly reduced following 5 d of extended culture without NGF (55% of that with NGF). The degeneration of the cholinergic neurons was markedly reduced by addition of dibutyryl cAMP (dbcAMP, 1 mM), forskolin (10 μM) or KCl (15 mM) to the medium. K252b, which blocks the survival of NGF, had no effect on the action of dbcAMP. H-8 and nifedipine inhibited the survival of dbcAMP and high KCl, respectively. These results suggest that NGF, dbcAMP and high K^+ promote the survival of septal cholinergic neurons acting *via* the receptor tyrosine kinase, protein kinase A and a Ca^{2+}-dependent mechanism, respectively.

Key words nerve growth factor; cAMP; high potassium ion; septal cholinergic neuron; survival

Regulation of neuronal development and survival is achieved by the complex interplay of effects such as synaptic input and trophic factors released from target tissue. The best characterized neurotrophic molecule is nerve growth factor (NGF), which supports the survival of peripheral sympathetic and sensory neurons and central cholinergic neurons. It has been proposed that the projections of forebrain cholinergic neurons to the hippocampus and cortex are supported by a target-derived neurotrophic factor.[1] It has also been shown in *in vitro* experiments that NGF is essential for the development and maintenance of septal cholinergic neurons.[2,3]

It has been reported that vasoactive intestinal peptide, which increases intracellular cAMP, is an endogenous factor that regulates the survival of the spinal cord[4] and retinal ganglion neurons.[5] The survival of mesencephalic dopaminergic neurons,[6] central noradrenergic neurons[7] and sympathetic neurons,[8] but not that of hippocampal neurons,[9] in culture is supported by cAMP analogs. Chronic depolarization of the plasma membrane by elevated K^+ also prevents the death of developing sympathetic neurons after acute withdrawal of NGF.[10] High K^+ or subtoxic concentrations of excitatory amino acids prevent the cell death of cultured cerebellar granule neurons.[11]

The aim of our study is to investigate if cAMP and high potassium can promote the survival of septal cholinergic neurons. In this report, we have demonstrated using fetal septal cholinergic neurons that the cell death of these neurons, following NGF withdrawal after 12—13 d of culture with NGF, is prevented by the addition of a cAMP analog or high K^+ in the extended culture.

MATERIALS AND METHODS

Preparation of Cell Cultures Under a stereomicroscope, the septal region was dissected from fetal rats of embryonic age E17-18.[2,12] Tissue pieces were transferred to Leibowitz L-15 medium and incubated in 0.25% trypsin and 0.01% deoxyribonuclease (DNase) I in phosphate buffered saline (PBS) for 15 min at 37 °C. Tissue fragments were spun down in a centrifuge at $1000 \times g$ (3 min) following the addition of 3 ml heat-inactivated horse serum. The pellet was resuspended in the serum-containing medium which consisted of 90% 1:1 mixture of Dulbecco's modified Eagle's and Ham's F12 media containing 15 mM HEPES buffer (pH 7.2), 30 mM selenium, 1.9 mg/ml sodium bicarbonate and 100 μg/ml kanamycin sulfate, 5% (v/v) precolostrum newborn calf serum and 5% (v/v) heat-inactivated horse serum. Cells were then dissociated by gentle trituration with a fire-polished Pasteur pipet. After trituration, cells were filtered using lens cleaning paper. The dissociated cells in the presence of 50—100 ng/ml NGF were plated at a density of 2×10^5 cells/cm^2 in 48-well plates (Costar) coated with polyethylenimine and cultured for 11—13 d, as specified in the Results, without medium change. After washing the cell surface with medium, fresh culture medium containing various agents such as NGF and dbcAMP was added to the cells and cultured for an additional 5 d as specified in the Results.

Histochemistry To count the number of the acetylcholinesterase (AChE)-positive neurons, the method described by Hartikka and Hefti[2] was used. Cultures were washed 3 times with PBS and fixed for 30 min in 4% formaldehyde (pH 7.4) at 20 °C. They were then incubated for 3 d at 4 °C in 50 mM acetate buffer, pH 5.0, containing 4 mM acetylthiocholine iodine, 2 mM copper sulfate, 10 mM glycine, and 10 mg/ml gelatin. Nonspecific cholinesterases were inhibited by inclusion of 0.2 mM ethopropazine in the incubation medium. After incubation, gelatin was dissolved by incubating the cultures briefly at 37 °C. They were then rinsed with distilled water, exposed for 1 min to 1.25% Na_2S, washed with distilled water, and exposed for 1 min to 1% $AgNO_3$. The cultures were then washed with water and embedded in glycerol gelatin. The number of stained cells in the total area of each well (1 cm^2/well) was counted. Each value represents the mean $\pm$ S.E.M. of the number of stained cells (4—6 wells). Statistical analysis was performed by one-way ANOVA and Scheffe's F-test.

Chemicals 7S NGF is a complex of three different molecular components which have been termed α, β and

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γ. Only the β component of 7S NGF (named 2.5S NGF) is biologically active.[13] 7S NGF from mouse submaxillary glands and forskolin were purchased from Sigma. Dibutyryl cAMP (dbcAMP) was from Boehringer Mannheim Co., Ltd. H-8 was from Seikagaku Co., Ltd. (Japan) and K252b was obtained from Funakoshi Co., Ltd. (Japan).

RESULTS

We used E17-18 fetuses as the source of brain tissue because most neurons in the septal region are considered to complete their final cell division by this stage.[12] At the time of plating, most of the cells collected from the septum had appearance of neurons under the microscope, showing phase-brightness and halos and thin processes around the cell border. The neuronal processes increased in length and number on culturing the cells with 50 ng/ml NGF. Addition of NGF to the medium significantly promoted the rate of surviving AChE-positive neurons, more than 3 times as high as that of controls, in 12 d of culture (data not shown). After culturing the fetal septal neurons in the presence of NGF for 12 d, they were maintained in the presence or absence of NGF (50 ng/ml) for an additional 3 or 5 d in order to examine the dependence on NGF of the survival of the cholinergic neurons. The number of AChE-positive neurons decreased by 30—55% on average

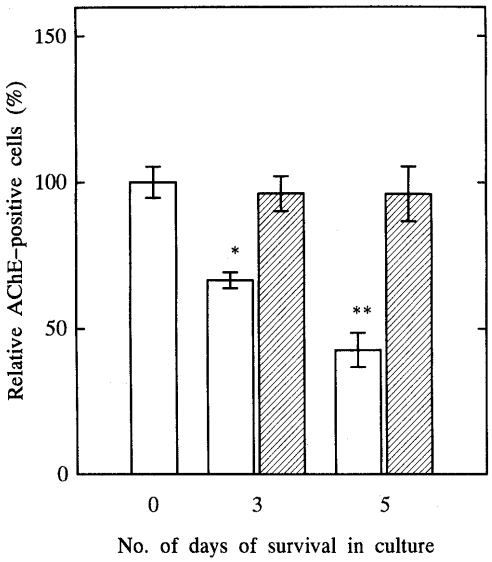

Fig. 1. Degeneration of the Septal Cholinergic Neurons after NGF Deprivation

The septal neurons were cultured with NGF (50 ng/ml at start) for 12 d. Then they were deprived of NGF and cultured with (hatched bar) or without (open bar) NGF (50 ng/ml) for an additional 3 or 5 d. Each value represents the mean ± S.E.M. of the number of stained cells (6 wells) relative to day 0. * Different from day 0 at $p<0.05$. ** Different from day 0 at $p<0.01$.

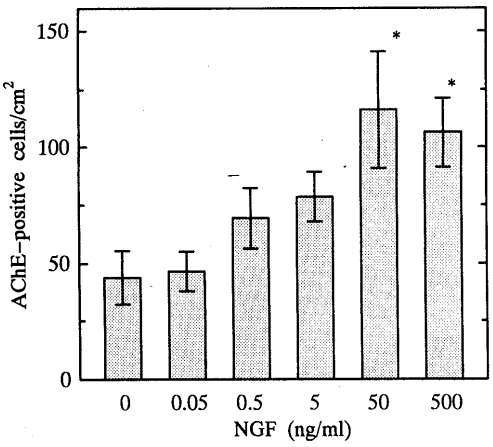

Fig. 2. Dose-Dependence of the Survival Effect of NGF on the Septal Cholinergic Neurons

The septal neurons were cultured with NGF (50 ng/ml at start) for 11 d. Then they were washed with culture medium to remove NGF and cultured with or without NGF (0.05—50 ng/ml) for an additional 5 d. Each value represents the mean ± S.E.M. of the number of stained cells (4 wells). * Different from 0 ng/ml at $p<0.05$.

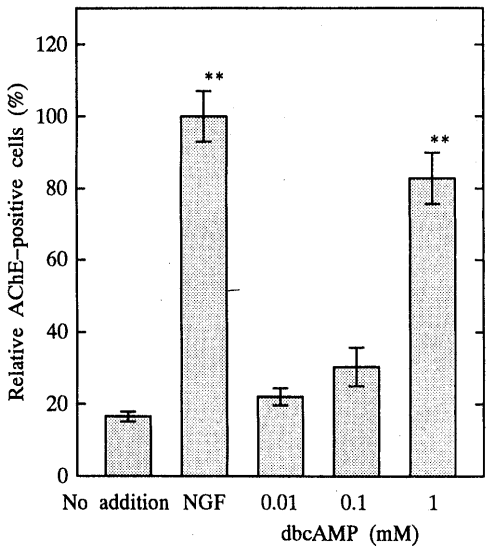

Fig. 3. Dose-Dependence of the Effect of dbcAMP on the Survival of the Septal Neurons

The neurons were cultured with NGF (50 ng/ml at start) for 11 d. Then they were deprived of NGF and cultured with either NGF (50 ng/ml) or dbcAMP (0.01—1 mM) or either for an additional 5 d. Each value represents the mean ± S.E.M. of the number of stained cells (4 wells) relative to NGF. ** Different from no addition at $p<0.01$.

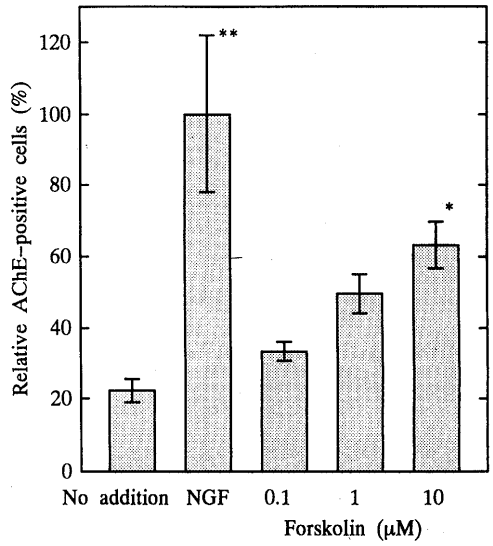

Fig. 4. Dose-Dependence of the Effect of Forskolin on the Survival of the Septal Neurons

The neurons were cultured with NGF (50 ng/ml at start) for 11 d. Then they were deprived of NGF and cultured with either NGF (50 ng/ml) or forskolin (0.1—10 μM) or either for an additional 5 d. Each value represents the mean ± S.E.M. of the number of stained cells (4 wells) relative to NGF. * Different from no addition at $p<0.05$. ** Different from no addition at $p<0.01$.

after withdrawal of NGF, whereas it was unchanged in the cultures containing NGF throughout the experiment (Fig.1). The survival effect of NGF was concentration-dependent up to 50 ng/ml NGF during 5 d of culture (Fig. 2).

Addition of dbcAMP or forskolin to the incubation medium has been reported to promote the survival of PC12 cells[14] and rat sympathetic and sensory neurons.[9] We showed that both dbcAMP and forskolin, an adenyl cyclase activator, significantly increased the survival of septal cholinergic neurons at 1 mM and 10 μM, respectively, during 5 d of cultures after withdrawal of NGF (Figs. 3 and 4), although their effect did not exceed that of NGF. Addition of a protein kinase A inhibitor, H-8,[15] abolished the survival effect of dbcAMP (Fig. 5).

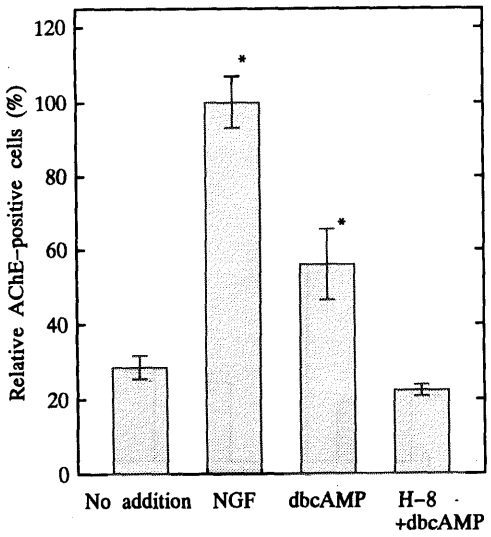

Fig. 5. Effect of H-8 on dbcAMP-Promoted Survival in Septal Neurons

The neurons were cultured with NGF (50 ng/ml at start) for 12 d. Then they were deprived of NGF and cultured with NGF (50 ng/ml) or dbcAMP (1 mM) in the presence or absence of H-8 (10 μM) for an additional 5 d. Each value represents the mean ± S.E.M. of the number of stained cells (4 wells) relative to NGF. * Different from no addition at $p < 0.05$.

The neurotrophic effects of NGF are specifically inhibited by K252a and K252b due to inhibition of the receptor protein tyrosine kinase.[16,17] The latter compound is more hydrophilic than the former and so possibly acts on the extracellular part of the NGF receptor.[18,19] The addition of K252b to the medium of surviving cultures completely canceled the effect of NGF, but had no effect on the action of dbcAMP (Figs. 6 and 7). Therefore, the survival effect of dbcAMP may not involve the NGF receptor in the cholinergic neuron.

Addition of KCl (15 mM) to the medium had a positive survival effect on AChE-positive cells, which was blocked by the presence of nifedipine (5 μM, Fig. 8). Nifedipine does not inhibit the effect of NGF and, therefore, the voltage-dependent calcium channel is assumed to be essential for the survival effect of high KCl concentrations, but not for that of NGF.

DISCUSSION

Recently Nakamura *et al.*[20] reported that the addition of NGF, a cAMP analog or high K^+ for 3—5 d was essential for the survival of cultured basal forebrain cholinergic neurons from postnatal 2-week old rats. In the present work we observed a similar dependence of cultured fetal septal cholinergic neurons on NGF, a cAMP analog and high K^+ after 12—13 d culture with NGF. The basal forebrain neurons in cultures from postnatal rats have probably developed to maturity *in vivo*, to be indispensable for NGF, while the fetal septal neurons in our experiments have grown to maturity *in vitro* on culture with NGF for some 12 d. These results all suggest that the cAMP analog and depolarization with high K^+ are capable of preventing the cell death of basal forebrain cholinergic neurons due to deprivation of NGF until synapse formation ends.

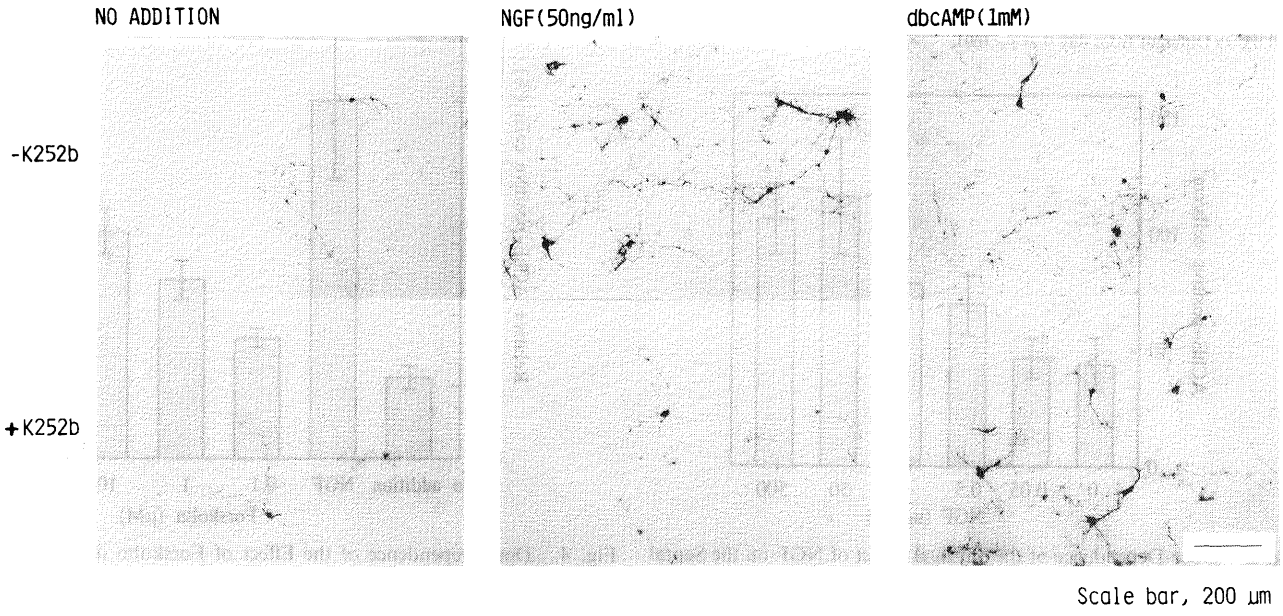

Fig. 6. Effects of K252b on NGF- and dbcAMP-Promoted Survival in Septal Neurons

The neurons were cultured with NGF (50 ng/ml at start) for 11 d. Then they were deprived of NGF and cultured with NGF (50 ng/ml) or dbcAMP (1 mM) in the presence or absence of K252b (2 μM) for an additional 5 d. Phase contrast photomicrographs were taken at the end of the experiment.

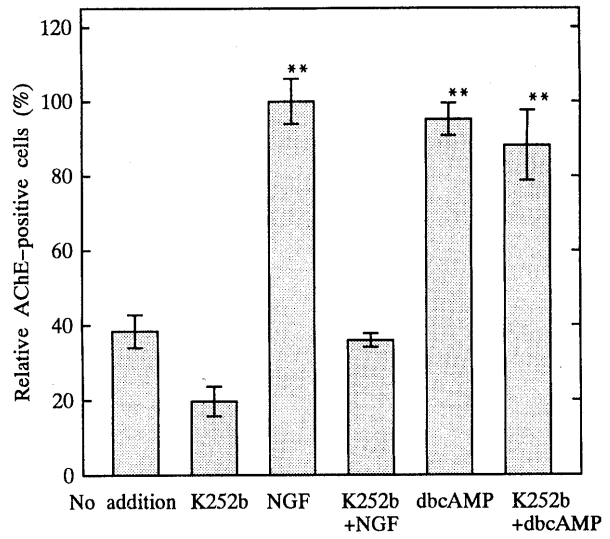

Fig. 7. Effects of K252b on NGF- and dbcAMP-Promoted Survival in Septal Neurons

The neurons were cultured as described in the legend to Fig. 6. Each value represents the mean ± S.E.M. of the number of stained cells (4 wells) relative to NGF. ** Different from no addition at $p<0.01$.

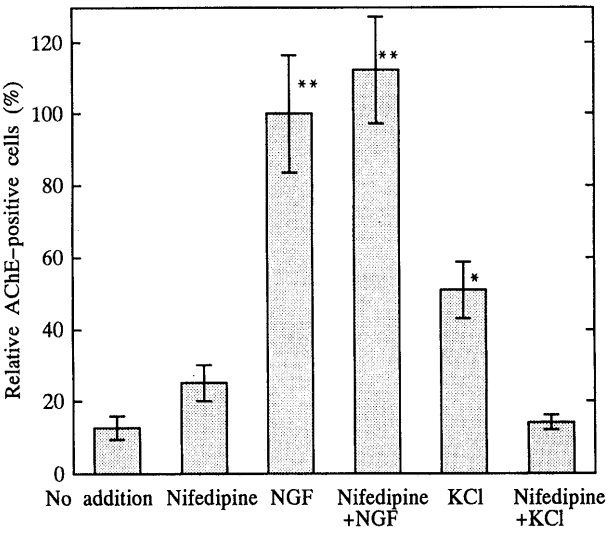

Fig. 8. Effect of High K^+ on the Survival of Septal Neurons

The neurons were cultured with NGF (50 ng/ml at start) for 13 d. Then they were deprived of NGF and cultured with either NGF (50 ng/ml) or KCl (15 mM) or either in the presence or absence of nifedipine (5 μM) for an additional 5 d. Each value represents the mean ± S.E.M. of the number of stained cells (4 wells) relative to NGF. * Different from no addition at $p<0.05$. ** Different from no addition at $p<0.01$.

Svendsen *et al.*[21] demonstrated prevention by inhibition of protein synthesis, of the cell death of septal cholinergic neurons after NGF withdrawal following 14 d of culture with NGF. However, we could not show prevention of the cell death of septal neurons by addition of cycloheximide (0.1—1 μg/ml, data not shown). Nakamura *et al.*[20] discussed the mechanism of the death of cultured basal forebrain neurons associated with deprivation of NGF and stated that cycloheximide had a limited effect on the survival of fetal basal forebrain cholinergic neurons. It seems that the mechanism of cell death in the central nervous system after NGF withdrawal is not the same for different cells and culture conditions.

The mechanism of the survival effect of NGF, the cAMP analog and high K^+ on the septal cholinergic neurons is not yet clear. Our present results suggest that the effect of dbcAMP on survival need not involve the receptor protein tyrosine kinase (Fig. 7) and that of NGF is not dependent on the voltage-dependent calcium channel (Fig. 8). We observed in PC12 cells that the cell death following serum withdrawal from the medium was prevented by the addition of NGF or dbcAMP and the protective effect was abolished by K252b or a protein kinase A inhibitor, respectively. In these cells NGF did not increase either the cellular content of cAMP or the cellular level of free calcium, while forskolin increased cAMP 7-fold and the high K^+ increased the calcium level (unpublished results). Therefore, it is assumed that the mechanism of enhanced survival of septal cholinergic neurons is different in at least the initial pathway for NGF, cAMP and high K^+.

NGF binds to the proto-oncogene product TrkA which activates MAP kinases through several steps including Ras and Raf-1 kinase.[22] It is reported that transfection of the Ras or Raf-1 kinase gene to PC12 cells and the myeloid cell line 32Dcl.3, respectively, prevents the cell death induced by trophic factor withdrawal.[14,23] The MAP kinase cascade is possibly involved in the survival effect of NGF. Recent reports suggest that cAMP and a high potassium, through Ca^{2+} influx, could activate the MAP kinase cascade.[24-26] Therefore, common signalling pathways such as the MAP kinase cascade downstream of these independent initial pathways may promote survival of septal cholinergic neuron.

In conclusion, NGF, a cAMP analog and high K^+, respectively promote survival of septal cholinergic neurons *via* activation of the receptor tyrosine kinase, protein kinase A and some Ca^{2+}-dependent signalling mechanism.

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