Antagonistic Effect of N-(3-Aminopropyl)cyclohexylamine on Neurotrophic Action of Spermine in Primary Cultured Rat Hippocampal and Cerebellar Neurons

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ABSTRACT—We previously found that spermine potently promotes the neuronal survival and regeneration of primary cultured brain neurons. N-(3-Aminopropyl)cyclohexylamine (APCHA) was originally developed as a spermine synthase inhibitor. To test if endogenous spermine biosynthesis contributes to neuronal survival and morphogenesis, we examined the effects of APCHA in primary cultured rat hippocampal and cerebellar neurons. APCHA at concentrations up to 10^{-6} M did not affect the neuronal survival, but significantly blocked the survival-promoting effect of spermine (10^{-8} M). APCHA also blocked the spermine-induced promotion of neurite regeneration following axotomy. Unlike APCHA, another cyclohexylamine derivative trans-4-methylcyclohexylamine did not affect the neurotrophic effect of spermine. These results suggest that in primary cultured brain neurons, APCHA works as a spermine antagonist rather than as a spermine synthesis inhibitor.

Keywords: Spermine, N-(3-Aminopropyl)cyclohexylamine, Neuronal survival, Regeneration,
         Primary cultured neuron

Natural polyamines, spermine, spermidine and putrescine, are ubiquitously distributed in eukaryotic tissues and are known to play important roles in proliferation and differentiation of many types of cells (1, 2). Although the polyamines are present at high concentrations in the mammalian brain (3–5), their functions for brain neurons are not well understood. We have recently found that spermine promotes the survival and neurite outgrowth of primary cultured hippocampal neurons (6, 7). Furthermore, spermine promotes the regeneration of injured axons of cultured rat hippocampal neurons (8). These observations suggest that spermine functions as a neurotrophic factor for brain neurons. The neurotrophic activity of spermine is of great interest with respect to the developement of therapeutic drugs for neurodegenerative disorders such as Alzheimer's disease.

Spermine is endogenously formed from spermidine by spermine synthase (1, 2). N-(3-Aminopropyl)cyclohexylamine (APCHA) was originally developed as a potent and selective inhibitor of spermine synthase (9, 10). To test if endogenous spermine biosynthesis contributes to neuronal survival and morphogenesis, we examined the effect of APCHA on cultured brain neurons. However, as a result, we obtained unexpected but very interesting results. APCHA alone did not affect the neuronal survival and neuritogenesis, but very potently blocked the neurotrophic action of spermine. Here we report the possibility that APCHA works as a spermine antagonist for cultured brain neurons.

MATERIALS AND METHODS

Drugs

Spermine was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The hydrochloride forms of APCHA and trans-4-methylcyclohexylamine (4MCHA) were synthetized at the Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Josai University, as described previously (9). Chemical structures of these analogues are shown in Fig. 1.
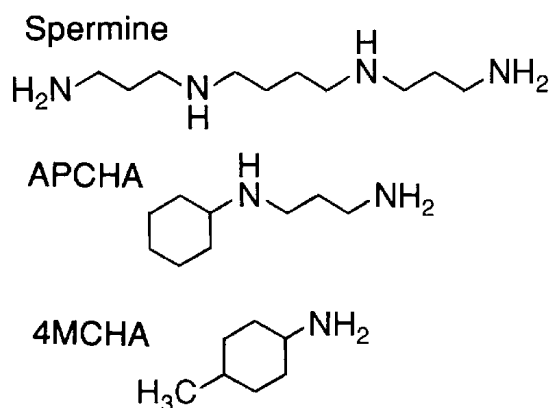
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Fig. 1. Chemical structures of spermine, APCHA and 4MCHA.

Cell dissociation

Procedures for cell dissociation were the same as described in our previous paper (11). Briefly, the hippocampus and cerebellum were dissected from 18-day-old embryos of Wistar rats. The cells were dissociated by incubation with 0.25% trypsin and 0.01% DNase I at 37℃ for 30 min, followed by pipetting, and then they were suspended in modified Eagle's medium supplemented with 10% fetal bovine serum (7).

Evaluation of neuronal survival

The dissociated cells were plated on polylysine-coated 48-well plates (1 cm²/well) at a density of 100,000 cells/ cm². After the plated cells were cultured in the serum-containing medium for 24 hr, the medium was changed to serum-free modified Eagle's medium supplemented with transferrin, insulin and progesterone (7). The drugs were added at this time. After 3 days, the cultures were fixed with 4% paraformaldehyde, and the number of surviving neurons in each well was counted under a microscope. The neuronal cells were visualized by specific immunostaining with monoclonal antibodies to microtubule-associated protein 2.

Observation of neurite regeneration following axotomy

We used the ACAS 470 Work Station (Meridian, USA) to memorize the cell position on the microscope stage and to deliver Argon laser beams (488 nm) (8). The dissociated hippocampal cells were plated on polylysine-coated culture dishes at a density of 2,500 cells/cm². The culture dishes were prepared from 35-mm Petri dishes by attaching a special glass coverslip to the inner surface with silicone grease (8). The special glass coverslip was sealed with a film that absorbs the laser beam and coverts the laser beam to heat. When the laser beam was irradiated, the neurites were virtually damaged by the heat. After incubation in the serum-containing medium for 24 hr, the medium was changed to the serum-free medium, and the cells were cultured for a further 24 hr. During this preliminary culture period, hippocampal neurons extended one long process and several short processes. By morphological and immunocytochemical criteria, the long process and several short processes have been identified as axon and dendrites, respectively (12, 13). The term "neurite" is used to include both axons and dendrites. The hippocampal pyramidal neurons that had established axons and dendrites were selected, and the laser beam (output 40 mW, acoust-optic-modulator 35%, 1 sec) was delivered to the axonal growth cone. The cells were photographed immediately after laser irradiation, and then drugs were added to the culture medium. The same cells were photographed 24 and 48 hr after the addition of drugs. Morphological parameters were measured by tracing the photographs on a digitizing tablet.

Most of the selected neurons survived in the control cultures, at least during the 48-hr observation period. Furthermore, the moderate condition of laser irradiation employed in this study did not significantly affect the survival of neurons (8). Therefore, the effects of drugs observed in this condition are independent of their possible effects on neuronal survival. Since most of the neurons began to degenerate at about 3 days after axotomy, we did not examine the effects of drugs on neurite regeneration for more than 48 hr.

RESULTS

Effect of APCHA on neuronal survival

APCHA at concentrations up to 10^{-6} M did not affect the survival of hippocampal and cerebellar neurons, but at 10^{-5} M, it significantly decreased the number of surviving neurons in both hippocampal and cerebellar cultures. The numbers of surviving neurons (cells/cm²) after 3 days were as follows: hippocampus: control, 11220 ± 720 (n=5); 10^{-5} M APCHA, 6890 ± 850 (n=5, P<0.01 vs control, Duncan's test); cerebellum: control, 8460 ± 380 (n=5); 10^{-5} M APCHA, 5970 ± 610 (n=5, P<0.01 vs control, Duncan's test).

Consistent with our previous observation (6, 7), spermine (10^{-8} M) promoted neuronal survival. The survival-promoting effect of spermine was attenuated in the presence of APCHA (Fig. 2). The antagonistic effect of APCHA was concentration-depedent, and the effect of spermine was completely abolished in the presence of 10^{-8} to 10^{-7} M APCHA. Similar results were obtained in both hippocampal and cerebellar cultures. We also examined the effect of another cyclohexylamine derivative, 4MCHA. Unlike APCHA, 4MCHA ($10^{-9} - 10^{-7}$ M) did not affect the survival-promoting effect of spermine (Fig. 3).
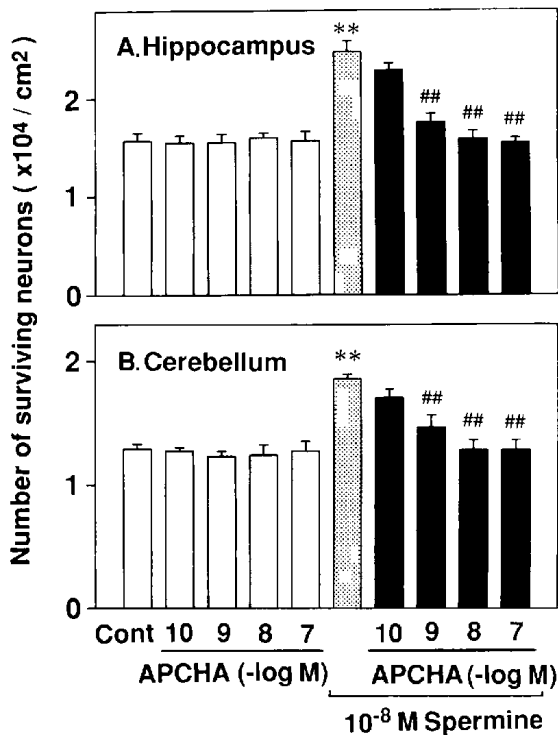
Fig. 2. APCHA blocks the survival-promoting effect of spermine (10^{-8} M) in hippocampal (A) and cerebellar (B) cultures. Ordinates indicate the number of surviving neurons per cm^2 (well). The data are reported as the mean and S.E.M. of 5 observations. Significance of the difference was determined by Duncan's multiple range test: **$P < 0.01$ vs the control group, ##$P < 0.01$ vs the group treated with spermine alone.
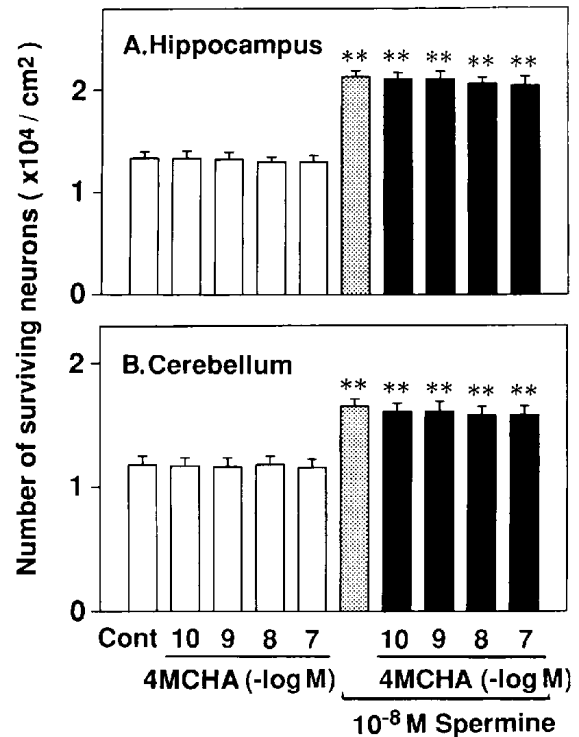

Fig. 3. 4MCHA does not affect the survival-promoting effect of spermine (10^{-8} M) in hippocampal (A) and cerebellar (B) cultures. The data are reported as the mean and S.E.M. of 5 observations. **$P < 0.01$ vs the control group, Duncan's multiple range test.

Effect of APCHA on neurite regeneration following axotomy

First, the influence of laser irradiation was checked in control cultures. Following laser irradiation, the axonal elongation ceased, but there were no changes in the growth of branches at the proximal part of the axon, the number of neurites per soma and the dendritic growth, consistent with our previous observation (8).

Since the distance of the injured site from the soma may influence the regenerative response of the neurons, we confirmed that the axon length from the soma to the injured site was not significantly different among the tested groups (Fig. 4A). APCHA ($10^{-9} - 10^{-7}$ M) alone did not significantly affect the axonal re-elongation from the injured site (Fig. 4B) nor the total length of branches at the proximal part of the injured axon (Fig. 4C) nor the total length of uninjured dendrites (Fig. 4D). Spermine (10^{-8} M) promoted the axonal re-elongation from the injured site in a time-dependent manner (Fig. 4B), but did not significantly affect the other morphological parameters (Fig. 4: C and D). The regeneration-promoting effect of spermine (10^{-8} M) was attenuated by

APCHA in a concentration-dependent manner (Fig. 4B). However, it should be noted that the survival-promoting effect of spermine was completely abolished by 10^{-8} M APCHA (Fig. 2), while the regeneration-promoting effect of spermine was not completely blocked even in the presence of 10^{-7} M APCHA (Fig. 4B). Unlike APCHA, 4MCHA ($10^{-9} - 10^{-7}$ M) did not block the regeneration-promoting effect of spermine (data not shown, n = 30 cells).

DISCUSSION

The major finding of this study was that APCHA blocked the neurotrophic action of spermine. 4MCHA, another cyclohexylamine derivative, showed no influence on the action of spermine, suggesting the specificity of the effect of APCHA. It can be concluded that APCHA works as a spermine antagonist for brain neurons.

APCHA completely inhibits the spermine synthase activity at less than 1 μM (9, 10). The decreased neuronal survival in the presence of 10^{-5} M APCHA may be due to some toxic effect, but not due to inhibition of spermine biosynthesis. The fact that APCHA at 10^{-6} M or less does not affect the neuronal survival suggests that the en-
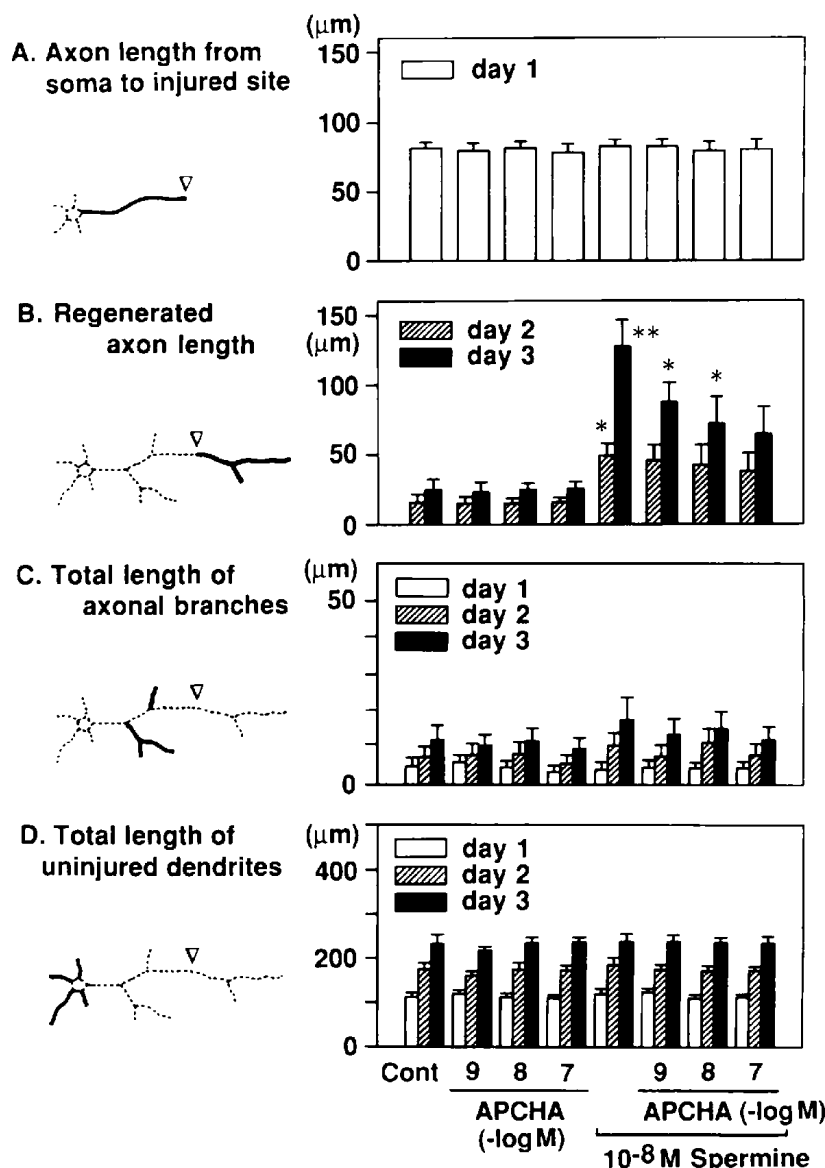
Fig. 4. APCHA blocks the regeneration-promoting effect of spermine (10^{-8} M) in hippocampal cultures. Several morphological parameters were measured immediately following axotomy (white columns) and 24 (shaded columns) or 48 hr (solid black columns) after axotomy. The definition of each morphological parameter is illustrated on the left side of the graphs. First (A) the axon length from the soma to the injured site was measured from the photographs taken immediately after laser irradiation, and it was confirmed that this value was not different among the tested groups. (B) Regenerated axon length from injured site, (C) total length of axonal branches and (D) total length of uninjured dendrites correspond to the lengths of lines drawn in bold strokes. The data are reported as the mean and S.E.M. (n=30 cells). *P<0.05, **P<0.01 vs the control group (Cont), Duncan's multiple range test.

dogenous spermine biosynthesis is not important for the neuronal survival or that the rate of spermine biosynthesis in cultured neurons is too slow to be influenced by APCHA treatment for 3 days.

We have previously observed that spermine, but not spermidine and putrescine, promoted neuronal survival (6, 7), while all three polyamines similarly promoted neurite regeneration (8). In addition, APCHA antagonized

the survival-promoting effect of spermine more potently than the regeneration-promoting effect of spermine (Figs. 2 and 4). These observations suggest that spermine exerts the survival-promoting effect and the regeneration-promoting effect through different molecular mechanisms.

It remains to be investigated how APCHA antagonizes the neurotrophic action of spermine. APCHA is supposed to work as a spermine synthase inhibitor by com-

peting with spermidine at the active site of the enzyme (9). Because of the structural similarity between APCHA and spermine, APCHA may compete with spermine on the molecule that mediates the neurotrophic action of spermine. APCHA may be useful as a pharmacological tool for studying the mechanisms of spermine action in the central nervous system.

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