Vol. 181, No. 1, 1991
November 27, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 265-271

TROPHIC EFFECT OF β-AMYLOID PRECURSOR PROTEIN ON CEREBRAL CORTICAL NEURONS IN CULTURE

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Received October 14, 1991

SUMMARY We investigated the effect of human β-amyloid precursor protein (APP) on rat primary cerebral cortical neurons cultured in a serum-free medium. Two secretory APP species (APP667 and APP592) with and without the protease inhibitor domain were produced by COS-1 cells transfected with APP cDNAs, which encode the N-terminal portions of APP770 and APP695. Both highly purified APP species, when added to the medium, enhanced neuronal survival and neurite extension in a dose-dependent manner with a maximum effect at approximately 100 nM. These results suggest that secreted forms of APP have trophic activity for cerebral cortical neurons.

The amyloid β-protein is a major constituent of the senile plaque and vascular amyloid in Alzheimer's disease (AD) (1,2) and derived from a larger β-amyloid precursor protein (APP) (3). At least three isoforms of APP (APP695, APP751 and APP770) have been identified, two of which (APP751 and APP770) contain an insert homologous to protease inhibitors of the Kunitz type (KPI) (4). APP mRNAs are expressed in almost every tissue, but the ratio of three isoforms differs among tissues. (5,6). The APPs have a characteristic structure of an integral membrane glycoprotein, with the β-protein region derived from portions of the extracellular and transmembrane domain (3). Many types of cell lines have been shown to secrete APPs lacking the cytoplasmic domain (7-9). Secreted APP possessing the KPI-domain appears to be identical to the protease nexin II which is secreted by fibroblasts (10,11).

0006-291X/91 $1.50

Recent reports using a cell culture system have indicated that APP may play a role in the regulation of the fibroblast growth (12) and in the modulation of cell adhesion (13-15). In addition, β-protein itself and a C-terminal fragment of APP containing the β-protein region have been reported to exert neurotoxic and neurotrophic effects on cultured neurons (16-19). However, little is known as to whether or not secreted forms of APP have any effect on neuronal cells. In the present study, we investigated the effect of secreted APP on rat primary cerebral cortical neurons using APP species obtained from COS-1 cells transfected with cDNAs, which encode the N-terminal portions of APP770 and APP695.

MATERIALS AND METHODS

Cell Culture

The cerebral cortices of 17-day embryonic Wistar rats were dissected in Ca, Mg-free Hanks' balanced salt solution (HBSS), and incubated in 0.1% trypsin containing 10 μg/ml DNase I in Ca, Mg-free HBSS, for 5 min at 37°C. The tissues were washed with 1% BSA in Ca, Mg-free HBSS; and dissociated by pipetting in a serum-free medium (SFM), a modification of the N2 medium as described by Bottenstein and Sato (20). SFM consisted of 1:1 mixture of Dulbecco's modified medium (DMEM) and Ham's F12 supplemented with 0.67 μg/ml vitamin B12, 810 μg/ml glucose, 73 μg/ml glutamine, 50 μg/ml gentamicin and the N2 supplements (20), at pH 7.4. The cell suspension was centrifuged at 1000 rpm for 5 min and resuspended in SFM. After filtration through a double layer of stainless steel mesh (50 μm), cells were again centrifuged and resuspended in SFM. The cells were plated at a density of about 2×10^4 cells/cm^2 on a 24-well plate (Corning) coated with poly-D-lysine, and maintained at 37°C with 5% CO_2.

Production and Purification of APP

APP667 and APP592, which correspond to APP770 and APP695 respectively, were produced by the method reported in a previous study (21) (Fig. 1A). APP667 and APP592 expression plasmids were constructed by the insertion of *Bam*H I-*Hin*d III fragment from pUC18 into *Bgl* II-*Hin*d III-digested pSVMT-APP770 and pSVMT-APP695 (4), respectively, to replace the C-terminal 104 a.a. (amino acids) of the parent APPs with a leucine residue. The plasmids were transfected into COS-1 cells by the method described previously (22). We purified the secreted APP667 from the conditioned medium of the transfected cells by HPLC on a MONO-Q column and the secreted APP592 from the conditioned medium by HPLC on a DEAE-5PW and Heparin-5PW. The purified proteins obtained were confirmed to be homogenous on SDS-PAGE (Fig. 1B). The molecular weights of APP592 and APP667 estimated by SDS-PAGE were about 95,000 and 100,000 respectively.

Determination of APP Effect

APP592 or APP667 was added to the medium at the time of plating (Day 0) at various concentrations. Duplicate wells were treated with each concentration of APP. The cultures were maintained for 3 days, and then fixed in 4% paraformaldehyde and 0.12 M sucrose in PBS at 37°C for 30 min (18). The number of viable neurons and the length of the longest neurite were measured under phase contrast microscopy by the method described in Table 1.

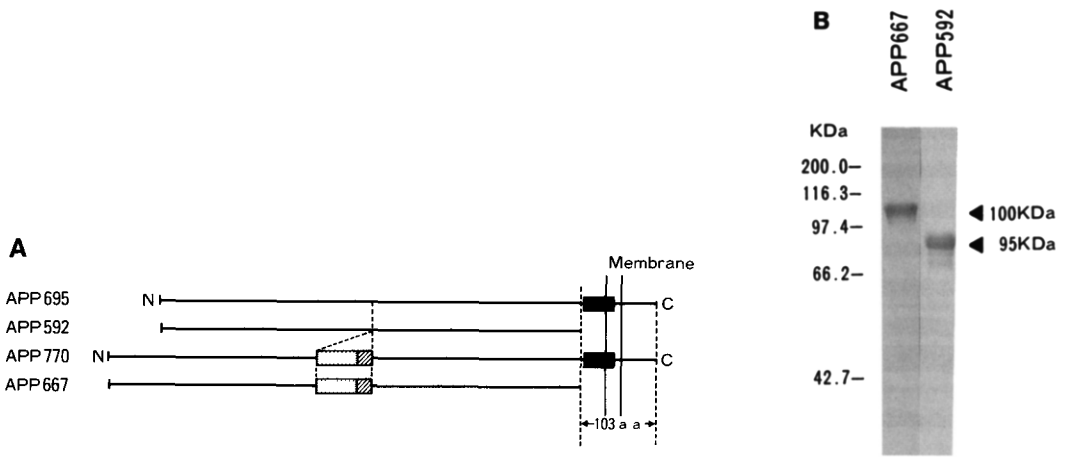

Fig. 1. (A) Schematic representation of APP695, APP592, APP770 and APP667. The closed box represents β-protein, dotted box the Kunitz-type protease inhibitor domain and hatched box the additional 19 a.a. insert.
(B) SDS-PAGE of APP667 and APP592. The purified APP667 (left) and APP592 (right) were electrophoresed in 7.5% polyacrylamide gel containing 0.1% SDS and stained with Coomassie Brilliant Blue.

Table 1. The effects of APP592 and APP667 on neuronal survival and the length of the longest neurite of cultured cortical neurons

APP concentration (nM)	Neuronal survival (cells/mm²)		Length of longest neurite (μm)	
	APP592	APP667	APP592	APP667
Experiment 1				
0 (Control)	35.7 ± 2.5		91 ± 4.5	
1	n.t.	40.4 ± 0.1	n.t.	90 ± 5.5
10	38.8 ± 2.4	38.8 ± 4.5	111 ± 7.7	110 ± 8.5
40	44.2 ± 1.4	43.0 ± 4.8	126 ± 6.8 **	126 ± 6.5 **
100	51.6 ± 3.6	51.0 ± 3.4	148 ± 7.6 **	132 ± 8.5 **
200	n.t.	40.8 ± 4.0	n.t.	139 ± 7.3 **
Experiment 2				
0 (Control)	7.6 ± 2.6		71 ± 3.1	
1	5.8 ± 2.0	6.1 ± 3.3	70 ± 3.0	68 ± 3.5
10	28.0 ± 3.8 *	21.8 ± 0 *	77 ± 4.3	73 ± 4.3
40	48.4 ± 4.8 *	43.0 ± 1.4 **	107 ± 5.8 **	105 ± 6.1 **
100	57.0 ± 4.6 *	55.4 ± 1.6 **	134 ± 8.2 **	135 ± 7.8 **
200	37.0 ± 1.4 **	38.3 ± 1.4 **	144 ± 7.3 **	142 ± 8.5 **

Primary cerebral cortical neurons were cultured with or without APP592 or APP667 for 3 days, and fixed as described in Materials and Methods. We counted the number of surviving cells with distinct neurites in 20 fields (0.25 mm² each) per well under phase-contrast microscopy, scoring 2 wells for each treatment. Each value for neuronal survival represents the mean cell number ± S.E.M. of duplicate wells. We estimated the length of the longest neurite using an ocular micrometer in 30 isolated neurons in 2 wells for each treatment. Each value for the neurite length represents the mean ± S.E.M. The results of two independent experiments are presented. n.t.: not tested.
*$p < 0.05$, **$p < 0.01$, by the Student's t test, compared to the control.

RESULTS AND DISCUSSION

The cells obtained in our culture exhibited neuronal profiles with several thin neurites (Fig. 2). They were further identified as neurons by immunostaining with antibodies against neurofilament protein and microtubule-associated protein 2 (data not shown). In addition, they did not stain positively for glial fibrillary acidic protein (GFAP) (data not shown). We tested the effect of APP on the neurons by adding highly purified APP (APP592 and APP667) to the medium. As shown in Fig. 2, both the survival of neurons and neurite outgrowth were enhanced by the addition of APP592 or APP667. We analyzed these effects quantitatively by measuring the number of surviving cells and the length of the longest neurite. Table 1 shows the results of two independent experiments in which various doses of APP were used. Neuronal survival was enhanced with increasing doses of APP, with optimal survival observed at 100 nM. There appeared to be no difference in survival-promoting effect between APP592 and APP667. The difference in cell survival between control cultures in the two experiments may be due to cellular damage caused during dissociation. Both types of APP also increased the length of the longest neurite in a dose-dependent manner, and the increase in the neurite length was significant at concentrations higher than 40 nM. Interestingly, slight decline in neuronal survival was observed at 200 nM of APP, whereas neurite outgrowth was still stimulated. This observation suggest a possibility that neuronal survival and neurite formation are regulated independently. It was noted that the extent of the outgrowth of shorter neurites was not altered much by the addition of APP. The long neurite may correspond to an axon, and short neurites dendrites, but further examination will be necessary for precise characterization. In addition, there were no GFAP-positive glia in the culture even in the presence of APP, suggesting that the effects of APP in our study were not modified by glial cells.

We have clearly shown that secreted APP has a trophic effect on cultured cerebral cortical neurons in terms of neuronal survival and neurite extension. Since the APP species used in this study lack C-terminal 103 a.a. of the parent APPs, and both APP592 and APP667 caused essentially the same effects, we can conclude that (i) the extracellular domain of APP excluding the β-protein region has the trophic effect and (ii) the KPI-domain is irrelevant to this effect. In addition, we have observed that a 72 a.a. fragment of APP770 including the KPI-

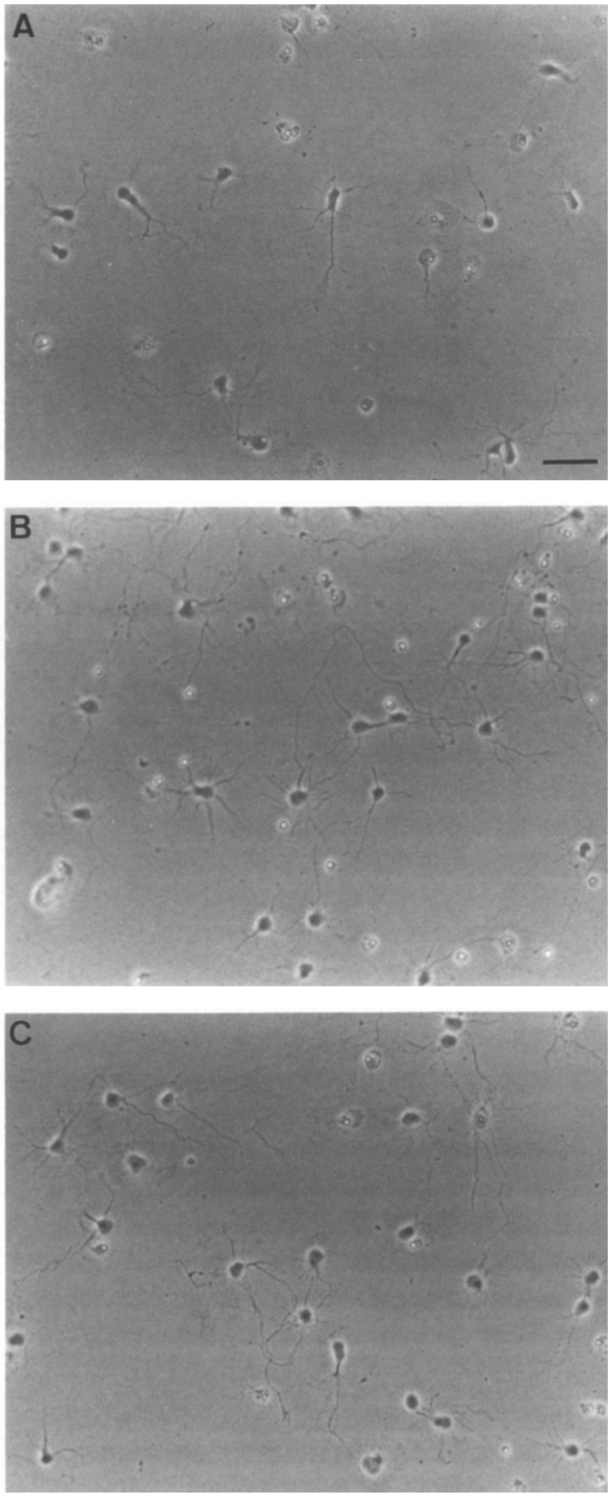

Fig. 2. The effects of APP592 and APP667 on cultured cortical neurons. Phase-contrast micrographs of Day 3 control culture (A) and cultures treated with 100 nM APP592 (B) or 100 nM APP667 (C). Bar = 50 μm.

domain (APPI-72) (22) had no clear effect on these cultured neurons (data not shown). These results suggest that APP exerts its effect by a different mechanism from that of glia-derived nexin (GDN), in which the neurite-promoting effect may involve protease inhibition (23).

The mechanism of the trophic effect of APP remains to be clarified, but at least two possible assumptions can be made. First, the APP effect might result from enhancement of cell adhesion. Schubert et al. (13) have reported that both APP695 and APP751 are able to stimulate adhesion of PC12 cells to substrata. Second, APP might interact with a specific cell-surface receptor, activating a second messenger system. It is consistent with this idea that a similar trophic effect on cortical neurons is produced by basic fibroblast growth factor (bFGF) most likely by a receptor-mediated mechanism (24).

The results of the present *in vitro* study suggest that APP may have a neurotrophic effect *in vivo*. The APP is expressed not only in neuronal cells but also in glial cells in the brain (25). In addition, cerebrospinal fluid and brain tissue appear to contain secreted forms of APP as in the case of certain cultured cells (7,8,21,26). Thus, secretory forms of APP may exist in the brain, and have trophic activity for central nervous system neurons possibly by an autocrine mechanism. Whether there is any relationship between the neurotrophic activity of APP and the pathogenesis of AD remains to be investigated.

In conclusion, our results have demonstrated that secreted APP produces a trophic effect on primary cerebral cortical neurons, and strongly indicate that APP has important physiological activities in the central nervous system.

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