Involvement of Protein Kinase Activation in Neurotrophic Effects of Basic Fibroblast Growth Factor in Cultured Brain Neurons

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ABSTRACT—The influences of K-252a and staurosporine, protein kinase inhibitors, on neurotrophic effects of basic fibroblast growth factor (bFGF) were investigated in dissociated cell cultures of the striatum, hippocampus and cerebellum of fetal rats. Addition of 1 ng/ml bFGF enhanced the survival of cultured neurons of all brain regions tested. Both K-252a (10–200 nM) and staurosporine (1–100 nM) blocked the survival promoting effects of bFGF in a concentration-dependent manner. These results suggest that bFGF exerts its neurotrophic effects through activation of protein kinase(s).

Basic fibroblast growth factor (bFGF) is a single-chain polypeptide composed of 146 amino acid and well-known as a potent mitogen for a variety of cell types (1). Furthermore, it has recently been found that bFGF promotes the survival of primary cultured brain neurons (2, 3) and prevents death of lesioned cholinergic neurons in vivo (4). Since bFGF is present in relatively high concentrations in the brain (5, 6), it is possible that bFGF functions as a neurotrophic factor in the brain. However, little is known about the cellular mechanisms underlying the neurotrophic action of bFGF. It has been shown that nerve growth factor (NGF) promotes the survival and neurite outgrowth of cultured dorsal root ganglion cells through activation of a specific protein kinase (7, 8). It has been reported that bFGF activates protein kinase C in Swiss 3T3 cells (9). Therefore, it is possible that activation of protein kinase is involved in the survival promoting effects of bFGF on cultured brain neurons. The present study was undertaken to investigate the possibility by examining the influences of the most potent but non-specific inhibitors of protein kinases currently available, K-252a and staurosporine (10), on the survival promoting effects of bFGF.

Basic FGF used in the present study is an acid-resistant mutein of human bFGF, CS23 (a generous gift from Takeda Chemical Industries, Ltd.). We have already confirmed that the neurotrophic activity of CS23 in cultured brain neurons is virtually the same as that of wild type human bFGF (11). CS23 was first diluted to a concentration of 10 μg/ml in phosphate-buffered saline supplemented with 1 mg/ml bovine serum albumin and stored at -20°C. K-252a and staurosporine were generous gifts from Kyowa Hakko Kogyo Co., Ltd. Stock solutions of K-252a (2 mM) and staurosporine (2 mM) were prepared in dimethylsulfoxide and stored at -20°C. The stored drugs were further diluted to the desired concentration by adding DF/TIP medium (11) just before application to the cell cultures.
Bovine serum albumin (1 ng/ml - 1 μg/ml) and dimethylsulfoxide (0.0001 – 0.01%) showed no significant effects on the neuronal survival in the present cultures.

Procedures for cell cultures and determination of neuronal survival were the same as described in our previous paper (11). Briefly, the desired brain region was isolated from embryonic (E17–18) rats and dissociated by incubation with 0.25% trypsin and 0.01% DNase I at 37°C for 30 min, followed by pipetting. The brain regions tested in the present study were the striatum, hippocampus and cerebellum. The cell suspensions were plated in poly-L-lysine-coated plastic 48-well plates (1 cm²/well) at a density of 100,000 cells/cm² with modified Eagle’s medium supplemented with 10% fetal bovine serum. Twenty-four hours after plating, the medium was changed to serum-free DF/TIP medium and then the drugs (CS23, K-252a or staurosporine) were added. 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 distinguished from non-neuronal cells by immunostaining (an avidin-biotin-peroxidase complex method) with monoclonal antibodies to neurofilament (11). In the present cultures, more than 90% of the cells were labeled by the antibodies to neurofilament and also identified as neurons judging from their morphology.

The number of surviving neurons in the cultures treated with 1 ng/ml CS23 alone was significantly larger than that without CS23. The survival promoting effects of CS23 alone were observed in all of the cultures from three brain regions tested, i.e., striatum, hippocampus and cerebellum. Figure 1 shows the influences of K-252a (1, 5, 200 nM) on the neuronal survival in the absence or presence of 1 ng/ml CS23. In all of the cultures from three brain regions, K-252a did not affect the neuronal survival in the cultures without CS23, but blocked significantly the survival promoting effects of CS23. The effects of K-252a were concentration-dependent. Figure 2 shows the influences of staurosporine (1, 10, 100 nM) on the neuronal survival in the absence or presence of CS23. Staurosporine also blocked significantly the survival promoting
effects of CS23 in all of the cultures from three brain regions, without affecting the basal neuronal survival. The blocking effects of staurosporine were concentration-dependent.

The concentrations of K-252a and staurosporine effective in blocking the effects of bFGF were well consistent with the concentrations effective in inhibiting the protein kinases (10). The fact that both K-252a and staurosporine did not significantly influence the neuronal survival in the absence of CS23 rules out the possibility that K-252a and staurosporine may have non-specific, cytotoxic effects on the cultured brain neurons. Furthermore, the effects of K-252a and staurosporine were very similar. Therefore, the present results suggest that bFGF promotes the survival of cultured brain neurons through activation of protein kinase.

Many types of protein kinases involved in the signal transduction of various cellular responses are known. K-252a and staurosporine inhibit not only protein kinase C but also a variety of other protein kinases, i.e., cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase, tyrosine-specific protein kinase, myosin light chain kinase, etc. (10). Therefore, from the present data alone, we cannot conclude which type of protein kinase is activated by bFGF. It is possible that bFGF activates two or more protein kinases. We are planning to investigate the effects of other protein kinase inhibitors, e.g., H-89, a selective inhibitor of cyclic AMP-dependent protein kinase (12).

Koizumi et al. (13) have reported that K-252a, at a concentration of 200 nM, completely inhibits NGF-induced neurite outgrowth of PC12 cells, but does not affect FGF-induced neurite outgrowth. Doherty et al. (14) have reported that K-252a specifically inhibits the NGF receptor expression in PC12 cells induced by NGF but not FGF. Since we have already confirmed that NGF has no effect on the survival of primary cultured brain neurons from fetal rats (15), it is unlikely that the survival promoting effects of bFGF observed in the present study are mediated by NGF. It is possible that the actions of bFGF in brain neurons are considerably different from those in PC12 cells as regards to the signal transduction mechanisms.

We have already investigated the effects of bFGF on the survival of cultured neurons
from various brain regions, i.e., cerebral cortex, septum, striatum, hippocampus, thalamus, substantia nigra, colliculus and cerebellum, and found that CS23 promotes the neuronal survival without distinction of brain regions (11). Considering the present results that K-252a and staurosporine blocked the survival promoting effects of bFGF in all brain regions tested, it is probable that the cellular mechanisms underlying the neurotrophic effects of bFGF are also the same among brain regions.

In conclusion, we have shown, for the first time, that bFGF exhibits its neurotrophic effects through activation of protein kinase(s). In order to clarify in detail the cellular mechanisms underlying the neurotrophic effects of bFGF, it is necessary to identify the protein kinase(s) activated by bFGF and the target substrate(s) of the kinase(s).

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