Neurotrophic Effects of Epidermal Growth Factor on Cultured Brain Neurons Are Blocked by Protein Kinase Inhibitors

Kazuho Abe, Megumi Takayanagi and Hiroshi Saito

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113, Japan

Received March 16, 1992 Accepted April 20, 1992

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

Keywords: Epidermal growth factor, Neurotrophic effect, Protein kinase inhibitors

Epidermal growth factor (EGF) is a single-chain polypeptide composed of 53 amino acids and well-known as a potent mitogen for a variety of cell types (1). However, it has recently been found that EGF promotes the survival of primary cultured brain neurons from neonatal and fetal rats (2–4). The survival-promoting effects of EGF on brain neurons were independent of mitogenic action on non-neuronal cells such as glial cells (2). Since EGF has been demonstrated to be present in the brain (5), it is possible that EGF functions as a neurotrophic factor in the brain. However, the cellular mechanism underlying the neurotrophic action of EGF is not known at all. We have very recently found that the survival-promoting effects of basic fibroblast growth factor (bFGF) on cultured brain neurons are blocked by protein kinase inhibitors, K-252a and staurosporine, indicating that protein phosphorylation is an essential event for bFGF to exhibit the neurotrophic effect (6). Furthermore, our previous observation that the survival-promoting effect of a combination of bFGF and EGF was the same as that of bFGF (3, 4). Therefore, the neurotrophic effects of EGF on cultured brain neurons may also involve the activation of protein kinases. To verify the possibility, we investigated in the present study the influences of potent protein kinase inhibitors, K-252a and staurosporine (7–9), on the survival-promoting effects of EGF on cultured brain neurons.

Procedures for cell cultures and determination of neuronal survival were the same as described in our previous paper (10). Briefly, hippocampal and cerebellar neurons were dissociated by incubation with trypsin from embryonic day 19–20 rat brains and were plated in polylysine-coated plastic 48-well plates (1 cm²/well) at a cell density of 10⁵/cm² with modified Eagle's medium supplemented with 10% fetal bovine serum (10). Twenty-four hours after plating, the medium was changed to serum-free medium, and then the drugs (EGF, K-252a or staurosporine) were added. The medium for serum-free culture is a 1:1 (v/v) mixture of Dulbecco’s modified Eagle’s medium and Ham’s nutrient mixture F-12 supplemented with transferrin, insulin and progesterone (10). 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 specific immunostaining (an avidin-biotin-peroxidase complex method) with monoclonal antibodies to neurofilament (10). 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 characteristic morphology.

The EGF used in the present study is recombinant human EGF (a generous gift from Wakunaga Pharmaceutical Co., Ltd., Osaka, Japan). Lyophilized EGF 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 concentrations just before application to the cell cultures. Bovine serum albumin (1 ng/ml - 1 μg/ml) and dimethylsulfoxide (0.0001-0.01%) alone did not significantly affect the neuronal survival in the present cultures.

Addition of 1 ng/ml EGF significantly promoted the survival of cultured hippocampal and cerebellar neurons. Figure 1 shows the influences of K-252a (10, 50, 200 nM) on the neuronal survival in the absence or presence of 1 ng/ml EGF. In both hippocampal and cerebellar cultures, K-252a did not affect the neuronal survival in the absence of EGF, but significantly blocked the survival-promoting effects of EGF. 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 EGF. Staurosporine also significantly blocked the survival-promoting effects of EGF in both hippocampal and cerebellar neurons, without affecting the neuronal survival in the absence of EGF. The blocking effects of staurosporine were concentration-dependent.

The concentrations of K-252a and staurosporine effective in blocking the effects of EGF were well-consistent with the concentrations effective in inhibiting protein kinases (9). Since both K-252a and staurosporine did not significantly influence the neuronal survival in the absence of EGF, these agents at the concentrations used in the present study are unlikely to 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 EGF promotes the survival of cultured brain neurons through activation of protein...
kinase.

The influences of K-252a and staurosporine on the survival-promoting effects of EGF in the present study were very similar to those on the effects of bFGF in our previous study (6). This result further supports the hypothesis that EGF exerts its neurotrophic effects through the mechanisms common to bFGF.

EGF can promote the survival of a wide range of brain neurons, but its effective concentrations were considerably different among the brain regions (3). For example, cerebellar neurons responded to lower concentrations of EGF than hippocampal neurons. The present result that the influences of protein kinase inhibitors on the effects of EGF in hippocampal neurons were very similar to those in cerebellar neurons suggest that the actions of EGF on hippocampal and cerebellar neurons are the same at least in terms of signal transduction mechanisms. The differences in effective concentrations of EGF among brain regions may be attributed to the density of EGF receptors expressed in the neurons or their affinity to EGF.

Many types of protein kinases are known to be involved in the signal transduction mechanisms of various cellular responses. 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. (9). Therefore, from the present data alone, we cannot conclude which type of protein kinase is activated by EGF. It is also possible that EGF activates two or more protein kinases or some unknown type of protein kinase, which can not be distinguished by any protein kinase inhibitor currently available. We are planning to directly identify the phosphorylated protein(s) by EGF stimulation and characterize the protein kinase(s) concerned.

In conclusion, we have shown, for the first time, that the neurotrophic effects of EGF on brain neurons are blocked by protein kinase inhibitors. This evidence will trigger further investigations about the role of protein kinase responses in the neurotrophic action of EGF, and it should be useful for elucidating the essential factors which support the survival of brain neurons.

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REFERENCES

1 Carpenter, G. and Cohen, S.: Epidermal growth factor. Ann. Rev. Biochem. 48, 193–216 (1979)
2 Morrison, R.S., Kornblum, H.I., Leslie, F.M. and Bradshaw, R.A.: Trophic stimulation of cultured neurons from neonatal rat brain by epidermal growth factor. Science 238, 72–75 (1987)
3 Abe, K., Takayanagi, M. and Saito, H.: A comparison of neurotrophic effects of epidermal growth factor and basic fibroblast growth factor in primary cultured neurons from various regions of fetal rat brain. Japan. J. Pharmacol. 54, 45–51 (1990)
4 Abe, K., Takayanagi, M. and Saito, H.: Basic fibroblast growth factor and epidermal growth factor promote the survival of primary cultured cerebellar neurons from neonatal rats. Japan. J. Pharmacol. 56, 113–116 (1991)
5 Fallon, J.H., Seroogy, K.B., Loughlin, S.E., Morrison, R.S., Bradshaw, R.A., Knauer, D.J. and Cunningham, D.D.: Epidermal growth factor immunoreactive material in the central nervous system: location and development. Science 224, 1107–1109 (1984)
6 Abe, K., Iri, Y., Takayanagi, M. and Saito, H.: Involvement of protein kinase activation in neurotrophic effects of basic fibroblast growth factor in cultured brain neurons. Japan. J. Pharmacol. 56, 563–566 (1991)
7 Kase, H., Iwahashi, K. and Matsuda, Y.: K-252a, a potent inhibitor of protein kinase C from microbial origin. J. Antibiot. 39, 1059–1065 (1986)
8 Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F.: Staurosporine, a potent inhibitor of phospholipid/Ca2+ dependent protein kinase. Biochem. Biophys. Res. Commun. 135, 397–402 (1986)
9 Ruegg, U.T. and Burgess, G.M.: Staurosporine, K-252a and UCN-01: potent but nonspecific inhibitors of protein kinases. Trends Pharmacol. Sci. 10, 218–220 (1989)
10 Abe, K., Takayanagi, M. and Saito, H.: Effects of recombinant human basic fibroblast growth factor and its modified protein, CS23, on survival of primary cultured neurons from various regions of fetal rat brain. Japan. J. Pharmacol. 53, 221–227 (1990)