Growth Factors Protect PC12 Cells against Ischemia by a Mechanism That Is Independent of PKA, PKC, and Protein Synthesis

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We have established an *in vitro* model of ischemia incorporating the combination of anoxia with glucose deprivation, which is toxic to PC12 cells. In this model, nerve growth factor (NGF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) improve PC12 cell survival. K252a, a specific inhibitor of NGF-induced trk p140 autophosphorylation, did not alter the neuroprotection provided by EGF or bFGF, yet it completely abolished the protection provided by NGF. Activation of protein kinase A (PKA) with dibutyryl-cAMP also protected during ischemia, although it was not additive with the effect provided by growth factors. Furthermore, growth factors protected a PKA-deficient mutant as effectively as the parental cell line; thus, activation of PKA is protective against ischemia but is not necessary for the action of peptide growth factors. Neither the stimulation of protein kinase C (PKC) with acute phorbol ester treatment nor the downregulation of PKC with chronic high-dose phorbol ester treatment resulted in an altered response to growth factors in either the PC12 wild type or PKA-deficient mutant. Thus, protection by peptide growth factors depends on neither PKA nor PKC. Furthermore, downregulation of PKC alone was protective, indicating that PKC may contribute to toxicity. Interestingly, treatment with the kinase inhibitor H-7 was neuroprotective and may have enhanced the neuroprotective effect of NGF. In contrast, staurosporine, a broadly acting kinase inhibitor, inhibited the neuroprotective effect of NGF, but not of EGF or FGF. The protective effects of the peptide growth factors did not depend upon concurrent protein synthesis, as the inhibition of protein synthesis by anisomycin did not significantly reduce the protective effects of EGF, bFGF, or NGF. Although their precise biochemical nature has yet to be identified, the signal transduction pathways that are regulated by peptide growth factors to protect cells against ischemia are potentially important therapeutic targets.

[Key words: NGF, basic fibroblast growth factor, epidermal growth factor, kinase inhibitors, anisomycin]

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Ischemia can be caused by a decrease in tissue perfusion that results in an inadequate supply of oxygen, glucose, and other metabolites. Prolonged ischemia can result in the death of neurons and other cells. The mechanisms leading to cell death are not fully understood, although a number of important aspects of the process have been studied (Rehncrona et al., 1981; Auer and Siesjo, 1988; Kirschenbaum and Pulsinelli, 1990). For example, brain ischemia results in an increase in the release of the excitatory neurotransmitter glutamate, which has been shown to be a mediator of neuronal cell death (Rehncrona et al., 1981; Benveniste et al., 1984; Rothman and Olney, 1986; Goldberg et al., 1987; Westerberg et al., 1989; Choi, 1990). Postsynaptic binding of glutamate to its receptor results in a complex signaling cascade that includes an increase in intracellular calcium (MacDermott et al., 1986; Mayer et al., 1987) and enhanced degradation of phospholipids with the formation of the protein kinase C (PKC) activators diacylglycerol (DAG) and arachidonic acid (for a review, see Bockaert et al., 1990). While it is postulated that PKC and calcium/calmodulin-dependent kinase may play a role in neuronal cell death, the precise cascade of events that lead to cell death during ischemia is not known (reviewed in Saitoh et al., 1991).

Peptide growth factors provide trophic support for both PNS and CNS neurons (Morrison et al., 1988b; Stemple et al., 1988; Walicke, 1988; Ernsberger et al., 1989; Cattaneo and McKay, 1991; Gage et al., 1991). In addition, they have been shown to promote neuronal cell survival in *in vitro* and *in vivo* models of neuronal injury (Otto et al., 1987; Lipton et al., 1988; Otto and Unsicker, 1990; Faktorovich et al., 1992) and neural injury causes changes in the expression of some peptide growth factors (Finklestein et al., 1988, 1990a,b; M. Endoh and J. A. Wagner, unpublished observation; Logan et al., 1992). NGF is a target-derived polypeptide growth factor that provides necessary trophic support for sensory and sympathetic neurons of the PNS (Bottenstein et al., 1980; Thoenen and Barde, 1980). In the CNS, NGF is located in highest concentration in the hippocampus, where it exerts a trophic effect on cholinergic neurons projecting there from the basal forebrain (Korsching et al., 1985; Johnston et al., 1987). Administration of NGF into the rat brain results in an increase in ChAT levels in the hippocampus, septum, and striatum (Gnahn et al., 1983; Hefti et al., 1984; Mobley et al., 1985). In addition, intraventricular injection of NGF in the rat after fimbria-fornix transection has been shown to protect against the loss of cholinergic neurons in the septum and diagonal band (Hefti, 1986; Williams et al., 1986; Kromer, 1987).

Other peptide growth factors share some of the characteristics of NGF. Like NGF, basic fibroblast growth factor (bFGF) pro-

motes survival of axotomized cholinergic neurons of the basal forebrain after fimbria-fornix transection (Anderson et al., 1988). bFGF promotes the survival of severed optic axons (Sievers et al., 1987) and provides trophic support for primary cultures of CNS neurons. bFGF expression has been shown to be increased after stroke and is thought to play a role in wound healing and functional recovery (Baird and Walicke, 1989; Finklestein et al., 1990b; M. Endoh, personal communication). Epidermal growth factor (EGF) elicits many of the responses common to NGF and bFGF in PC12, including stimulating protein phosphorylation, activating second messenger systems and specific enzymes, and inducing immediate-early genes (Greenberg et al., 1985; Heasley and Johnson, 1989; Chao, 1992). In PC12, however, EGF is not a differentiating factor but a mitogenic factor (Huff et al., 1981). In other cell populations, EGF has a neuroprotective effect. EGF protects primary hippocampal cultures from glutamate toxicity (Pauwels et al., 1989) and anoxia (Maiese et al., in press), and it also protects cortical cultures from anoxia (Kinoshita et al., 1990). How peptide growth factors exert their survival-promoting effect is not yet known. Determining the signal transduction pathways involved in their effect is of obvious clinical interest, as peptide growth factors have been implicated in the process of brain development, neuronal plasticity, and repair.

The rat pheochromocytoma cell line PC12 (Greene and Tischler, 1976), which displays phenotypic traits associated with both adrenal chromaffin cells and sympathetic neurons, is a useful model system for exploring signal transduction pathways. Upon treatment with NGF or FGF, PC12 cells develop features of sympathetic neurons; that is, they stop dividing, develop processes, and demonstrate increased electrical excitability and increased levels of ChAT (Dichter et al., 1977; Greene and Tischler, 1982). In addition to stimulating differentiation, NGF or bFGF promotes survival of PC12 cells in serum-free medium, and the activation of intracellular protein kinases plays a key role in this process (Greene, 1978; Rukenstein et al., 1991). NGF has been shown to activate several protein kinases, including its cell surface receptor (Klein et al., 1991; Meakin and Shooter, 1991), N-kinase (Volonte et al., 1989), the S6 kinases (Blenis and Erikson, 1986; Hashimoto and Hagino, 1990), a proline-directed kinase (Vulliet et al., 1989), MAP2/ERK kinases (Miyasaka et al., 1990; Boulton et al., 1991), and PKC (Hama et al., 1986; Heasley and Johnson, 1989). Activators of protein kinase A (PKA, i.e., the cAMP-dependent protein kinase) have been shown to mimic some of NGF's effects, including protection against serum deprivation. While PKA is not essential for many of NGF's key effects, it has been implicated in the NGF-induced activation of tyrosine hydroxylase (Cremers et al., 1986), the induction of voltage-dependent sodium channels (Kalman et al., 1990; Ginty et al., 1992), and the downregulation of calmodulin-dependent kinase activity (Brady et al., 1990). bFGF stimulates the differentiation of PC12 cells in a way that is reminiscent of NGF by activating a cell surface protein tyrosine kinase as well as a cascade of intracellular protein kinases (reviewed in Schlessinger and Ullrich, 1992). Because of the well-characterized signaling pathways in PC12, we elected to use this line as well as a PKA-deficient PC12 mutant to examine the effects of ischemia, the potential neuroprotective effect of peptide growth factors, and the role of second messenger systems in mediating cell death and cell survival. If the activation of defined second messenger systems modulates cell death during ischemia, then a careful study of these signaling pathways

will increase our understanding of the cascade of events that leads to cell death.

Materials and Methods

Cell culture. Wild-type PC12 cells and the PKA-deficient PC12 mutant AB11.0 (Ginty et al., 1991) were maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) horse serum (HS) and 10% (v/v) fetal bovine serum (FBS) at 37°C in an atmosphere of 10% CO₂ and 90% room air.

In vitro ischemia model. To study cell survival after ischemia, PC12 or AB11.0 cells were plated in DMEM/5% HS/10% FBS on polylysine (Sigma)-coated 24-well Nunc plates at 2×10^5 cells per well and allowed to adhere overnight. To provide a glucose-free environment, the cells were washed with a glucose-free Earle's Balanced Salt Solution (EBSS) at a pH of 7.4 and supplemented with penicillin (50 U/ml) and streptomycin (50 mg/ml), and then placed in fresh glucose-free EBSS. To provide an anoxic environment, cells were then placed into an incubator without oxygen, containing 95% (v/v) nitrogen and 5% (v/v) CO₂ at 37°C for a period of 8 hr. At the end of the exposure period, cells were returned to DMEM/5% HS/10% FBS supplemented with penicillin and streptomycin and an atmosphere of 10% CO₂/90% room air at 37°C. Cell death was assayed using the trypan blue exclusion method 24 hr later. Briefly, trypan blue was added to a final concentration of 0.04% and cultures were counted after a period of 20–60 min using a phase-contrast microscope. Cells were counted in random fields until a total of between 200 and 500 cells (viable plus nonviable) was reached. We chose to determine the extent of cell death at 24 hr after the cells were restored to normal medium as there is a population of cells that is destined to die yet does so only after a delay.

PKC was downregulated by pretreating with 1 μ M phorbol myristate acetate (PMA) in 0.06% dimethyl sulfoxide (DMSO) (v/v; Sigma) for 24 hr prior to the exposure period with continued treatment throughout the exposure and postexposure period. This procedure results in a population of cells that has less than 1% of the normal level of PKC activity and that is functionally deficient in PKC-dependent increases in transcription (Damon, 1990, #31; additional data not shown). Kinase inhibitors H-7 (60 μ M; Sigma), staurosporine [10 nM in 0.002% DMSO (v/v; Sigma), and K252a [200 nM in 0.009% DMSO (v/v; Kamiya Biomedical Co.)] were added 12 hr prior to the ischemic period and the cells were maintained in their presence throughout the ischemic and postischemic periods. Dibutyl cyclic AMP (1 mM; Sigma) and PMA (100 nM; Sigma) were added to stimulate PKA and PKC, respectively, at the initiation of ischemia and treatment continued throughout the postischemic period. Use of vehicle controls containing an equivalent concentration of DMSO did not alter cell survival. Growth factors were added 11 hr prior to the exposure period and cells were maintained in their presence throughout the experimental period. Human recombinant EGF (10 ng/ml) and human recombinant bFGF (10 ng/ml) were obtained from R & D Systems; NGF (100 ng/ml) was isolated and purified from mouse submaxillary gland according to the method of Mobley et al. (1976).

Protein synthesis. To quantify protein synthesis, PC12 cells were plated in DMEM/5% HS/10% FBS on 24-well plates and were treated with the appropriate dose of the protein synthesis inhibitor anisomycin for 2 hr, after which they were washed and replaced with leucine-free medium (ICN Flow). ³H-leucine (2 μ Ci/ml; Amersham) was added in the presence or absence of anisomycin and cells were incubated for 1 hr. Nonspecific binding was tested by using a 1 min exposure to ³H-leucine prior to washing the cells. The cells were washed with PBS and detached by the addition of 750 μ l of 0.1 M NaOH followed by transfer to a 1.5 ml Eppendorf tube. Protein was precipitated by the addition of 250 μ l of 100% (w/v) trichloroacetic acid (TCA) and 10 μ l of bovine serum albumin (10 mg/ml) at 4°C for 90 min. Precipitants were collected by filtration onto Whatman glass microfiber filters, washed once with 10% TCA, twice with 5% TCA, and twice with 95% EtOH, and then allowed to dry prior to scintillation counting. Experiments testing the effect of anisomycin itself on cell viability utilized a 24 hr exposure period to the protein synthesis inhibitor. For the effect of anisomycin on PC12 cells during ischemia, anisomycin was added 1 hr prior to the exposure to ischemia, and growth factors were added at the time of ischemia. Cells were maintained in anisomycin in the presence and absence of growth factors throughout the exposure and postexposure periods. Determination of cell viability was performed 24 hr later.

Figure 1. Growth factors promote survival of PC12 cells during glucose deprivation. PC12 wild-type cells were exposed to either no growth factor, EGF (10 ng/ml), bFGF (10 ng/ml), or NGF (100 ng/ml), and the normal medium was replaced with a balanced salt solution containing the same growth factor for the period indicated in the *abscissa*, so that the cells were deprived of glucose, vitamins, amino acids, and serum. Afterward, the cells were then returned to normal medium and maintained in the presence of the same added growth factors for an additional 24 hr before viability was determined utilizing the trypan blue exclusion method. Data represent the means $\pm$ SEM of three trials from one of two experiments that gave equivalent results.

Results

Peptide growth factors promote PC12 cell survival during glucose deprivation. In the initial series of experiments, PC12 cells were incubated in a glucose-free EBSS for increasing periods of time. Thus, this model consisted not only of glucose deprivation but also vitamin, amino acid, and serum deprivation. Viability was assayed 24 hr following the ischemic period. In this model cell viability was greater than 90% during the first 24 hr, but cell viability decreased dramatically in the next 24 hr period to 50–60% (Fig. 1). NGF, bFGF, and EGF all promoted cell survival at each interval in this period, although EGF was somewhat less efficacious than NGF and bFGF (Fig. 1). All the growth factors were very effective until 40 hr, after which even the growth factor-treated cultures began to lose viability (Fig. 1). As shown below (Figs. 2, 3), NGF, bFGF, and EGF were also neuroprotective when PC12 cells were exposed to 8 hr of anoxia in a glucose-free balanced salt solution, and we decided to use an ischemic model including both hypoglycemia and anoxia to study the protective effects of peptide growth factors and second

Figure 2. Growth factors improve cell survival during ischemia by more than 50%, and the protective effect of NGF is abolished by K252a. PC12 cells were exposed to either EGF (10 ng/ml), bFGF (10 ng/ml), or NGF (100 ng/ml) or maintained in the absence of any exogenous growth factor in the presence or absence of K252a (200 nM), as described in Materials and Methods, and the medium was replaced with a balanced salt solution in an oxygen-free environment. After an 8 hr period of ischemia, cells were maintained in the presence of the same growth factors $\pm$ K252a for an additional 24 hr in serum-containing medium supplemented with the same growth factors and then scored for viability. Data represents the means $\pm$ SEM of a total of four trials taken from two separate experiments.

messenger systems in greater detail. Although this is a reasonable model of ischemia, it lacks many of the aspects of ischemia that occur *in vivo*, including acidosis, hyperkalemia, and other ionic imbalances.

Peptide growth factors are protective against the combination of oxygen and glucose deprivation, and NGF's neuroprotective effect occurs through a K252a-sensitive cell surface receptor. When PC12 cells were deprived of oxygen and glucose for a period of 8 hr, about 30% of the cells died (Fig. 2), but EGF, NGF, and bFGF all substantially increased cell survival (Fig. 2). The kinase inhibitor K252a prevents many of NGF's effects on PC12, including neuriteogenesis, and this effect is probably caused by blocking the NGF-dependent phosphorylation of its cell surface receptor (Hashimoto, 1988; Koizumi et al., 1988; Tischler et al., 1990; Berg et al., 1992; Ohmichi et al., 1992). To assess whether K252a also affects NGF's neuroprotective effect, PC12 cells were exposed to ischemic conditions (i.e., combined oxygen and glucose deprivation) in both the presence and absence of K252a (Fig. 2). K252a completely abolished NGF's neuroprotective effect (Fig. 2), and it blocked neuriteogenesis in NGF-treated cells (data not shown). K252a did not alter survival in either the control population (by itself it was not toxic) or the EGF-treated or bFGF-treated populations, showing that it affected a pathway that is unique to NGF. As has been reported (Koizumi et al., 1988; Hashimoto and Hagino, 1989), K252a potentiated the neuritogenic effect of bFGF (data not shown).

The role of second messengers in cell survival. Activation of

Figure 3. Neuroprotection by growth factors during ischemia is independent of PKA and PKC. *a*, Stimulation of PKA promotes survival of PC12 cells during ischemia without altering the response to growth factors. Cells were treated with dibutyryl-cAMP (*dbcAMP*) (1 mM) in the presence or absence of EGF (10 ng/ml), bFGF (10 ng/ml), or NGF (100 ng/ml) at the initiation of 8 hr of ischemia. Cells were maintained in the presence of the same agents for 24 hr postischemia, after which cell viability was assayed via trypan blue exclusion. *b*, Stimulation of PKC does not alter survival of PC12 cells during ischemia or the response to peptide growth factors. PC12 cells were treated with 100 nM PMA in the presence or absence of growth factors at the initiation of 8 hr of ischemia and throughout the 24 hr postischemic period, after which cell viability was assayed. *c* and *d*, Downregulating PKC with PMA is neuroprotective, but does not alter the survival-promoting effect of peptide growth factors in PC12 parental cells (*c*) or AB11.0 mutants (*d*). PC12 cells (*c*) and AB11.0 mutant cells (*d*) were pretreated with 1 μ M PMA for 24 hr prior to 8 hr of ischemia, and treatment was continued throughout the ischemic and postischemic periods. Exposure to peptide growth factors in the presence or absence of PMA began 12 hr prior to the ischemic period and continued throughout the ischemic and postischemic periods. Data represent means $\pm$ SEM from a total of six trials taken from two separate experiments for *a* and *b*, a total of four trials taken from two separate experiments for *c*, and a total of five trials taken from two separate experiments for *d*. **, $p < 0.02$; ^^, $p > 0.10$. ANOVA followed by Tukey's test for pairwise comparisons confirmed statistical significance as indicated by ** with $p = 0.01$.

PKA can induce neuritogenesis and promote the survival of rat neonatal sympathetic and embryonic sensory neurons in culture (Rydel and Greene, 1988). To study the role of PKA in promoting cell survival during ischemia, PC12 cells were treated with the membrane-permeable analog dibutyryl-cAMP and exposed to ischemic conditions. Activation of PKA alone provided significant protection, although it was not as effective as the peptide growth factors (Fig. 3*a*). Activation of PKA in the presence of peptide growth factors did not provide any additional

protective effect beyond that provided by EGF, bFGF, or NGF alone (Fig. 3*a*).

Stimulating PKC with phorbol esters decreased the survival of cortical neurons during serum deprivation, while downregulating PKC resulted in improved survival (Morrison et al., 1988a). To determine what role PKC may have in PC12 cell survival during ischemia, PKC was stimulated by acute treatment with the phorbol ester PMA (Fig. 3*b*). Stimulating PKC did not affect cell survival in the absence of exogenous growth factors, and

Figure 4. Kinase inhibitors H-7 and staurosporine promote survival of PC12 cells during ischemia in the absence of growth factors, but the combination of staurosporine and NGF is not neuroprotective. PC12 cells were exposed to H-7 (60 μ M) or staurosporine (10 nM) 12 hr prior to an 8 hr ischemic period and treatment continued through the 24 hr postischemic period. Cells were treated with EGF (10 ng/ml), bFGF (10 ng/ml), or NGF (100 ng/ml) in the presence or absence of kinase inhibitors 11 hr prior to ischemia (i.e., 1 hr after addition of the kinase inhibitors) and treatment continued throughout the ischemic and post-ischemic periods. Cell death was assayed 24 hr after ischemia via the trypan blue exclusion method. Data represent means $\pm$ SEM of a total of five trials taken from two separate experiments. ANOVA followed by Tukey's test for pairwise comparisons confirmed statistical significance; **, $p < 0.05$ in comparing combined NGF-treated and H-7-treated experiments with both NGF-treated alone and H-7-treated alone experiments.

the small effect of PKC on the protective effects of EGF, bFGF, and NGF was not statistically significant (Fig. 3b).

Neuroprotection by peptide growth factors during ischemia is independent of PKA and PKC. PKC is activated by EGF, bFGF, and NGF, but it is not known if this activation is necessary for the protective effects of these growth factors. To address the possible role of PKC in the survival-promoting effect of peptide growth factors, PKC was chronically activated and downregulated so that the effects of the growth factors could be determined in the absence of PKC. Pretreatment with 1 μ M PMA to downregulate PKC in the parental cell line resulted in a nearly 50% improvement in survival in the absence of any added growth factors (Fig. 3c), and the lack of PKC did not attenuate the ability of the growth factors to promote survival.

To determine whether PKA plays a role in the neuroprotective effects of NGF, bFGF, and EGF, we utilized a PC12 mutant cell line, AB11.0, which is deficient in both PKA I and II (Ginty et al., 1991). There was no difference in the percentage of cell death resulting from 8 hr of ischemia, indicating that the lack of PKA activity did not alter survival during ischemia (Fig.

Figure 5. Effect of anisomycin on cell viability and protein synthesis. PC12 cells were treated for 24 hr with increasing concentrations of anisomycin. The cells were then washed and incubated for an additional 24 hr period, and viability was assayed. Data represent means $\pm$ SEM of four experiments. To quantify protein synthesis, PC12 cells were treated with anisomycin for 2 hr prior to labeling with 3 H-leucine for 1 hr. Protein precipitation and quantification were performed as described in Materials and Methods. Data represent means $\pm$ SEM of a total of six trials taken from three separate experiments.

3c,d). Likewise, the neuroprotective effects of the growth factors were equivalent between these two cell lines, proving that NGF, bFGF, and EGF promote survival independent of PKA (Fig. 3c,d).

Downregulation of PKC in the PKA-deficient mutant resulted in an improved survival in the absence of added growth factors, while the response to growth factors was unaffected (Fig. 3d), as might be expected from experiments with wild-type cells. Taken together, these data indicate that neither kinase is necessary for EGF-, bFGF-, or NGF-induced survival in PC12 cells during ischemia, but the presence of activatable PKC during ischemia is detrimental.

Kinase inhibitors alter cell survival during ischemia. The kinase inhibitor H-7, which is a competitive inhibitor of the ATP binding site of both PKA (K_i of 3 μ M) and PKC (K_i of 6 μ M) (Hidaka et al., 1984), augments NGF's neuritogenic affect on PC12 cells (Posypanova et al., 1990) and the survival-promoting effect of EGF and bFGF on serum-depleted primary rat cortical cultures (Morrison et al., 1988a). During ischemia, H-7 was as neuroprotective as the peptide growth factors in PC12 (Fig. 4) and in AB11.0 cells (data not shown). There is some evidence

for an additional enhancement of H-7's neuroprotective effect in the presence of NGF, but this potential effect was not apparent with either EGF or bFGF.

Staurosporine is a more potent PKA and PKC inhibitor than H-7 (Nixon et al., 1991) and can inhibit some protein tyrosine kinases (Nakano et al., 1987). It inhibits phosphorylation of NGF's cell surface receptor, alters NGF-induced protein phosphorylations, and inhibits NGF-induced neuritogenesis (Hashimoto and Hagino, 1989; Tischler et al., 1990; Ohmichi et al., 1992). Although staurosporine did not alter the neuroprotective effects of either EGF or bFGF, it abolished the protection provided by NGF (Fig. 4). It is surprising that the combination of NGF and staurosporine is not protective. The reason for this is unclear, but it suggests that the protective effect of each agent is antagonized by the other. The neuroprotective effect of NGF apparently depends on a kinase that is sensitive to staurosporine. From our experiments, it is unlikely that this kinase is PKC, but it may be *trk* (Berg et al., 1992; Ohmichi et al., 1992).

Peptide growth factors do not require protein synthesis to promote survival. To determine whether the neuroprotective response to growth factors during ischemia requires protein synthesis, PC12 cells were treated with the protein synthesis inhibitor anisomycin. Preliminary data showed that prolonged treatment (24 hr) with 100 μ M anisomycin is toxic to PC12 cells (see also Fig. 5). We therefore studied the effect of varying concentrations of anisomycin to determine a concentration where there was a substantial effect on protein synthesis without having a pronounced effect on cell viability (Fig. 5). At a concentration of 1 μ M, anisomycin significantly inhibited protein synthesis (86%) yet resulted in a minimal decrease in cell survival (87% viable at 24 hr); higher doses resulted in a marked decrease in cell survival (Fig. 5). EGF, bFGF, and NGF all protected against ischemia in the presence of 0.1 and 1.0 μ M anisomycin (where protein synthesis was inhibited 1% and 86%, respectively) while both bFGF and NGF protected at 10 μ M anisomycin (where protein synthesis was inhibited by more than 95%; Fig. 6). Thus, the protective effects of these three growth factors are largely independent of concurrent protein synthesis.

Discussion

We have established a model of ischemia in PC12 cells and have shown that it can be used to define the signaling pathways involved in modulating cell death during ischemia. We have also shown that modulation of kinase activities can have an effect on cell viability in our model of ischemia. NGF, bFGF, and several other pharmacological agents rescue cells during serum deprivation (Rukenstein et al., 1991), while EGF supported short-term but not long-term survival. In contrast, we have found that the three peptide growth factors protect almost equivalently in our model of ischemia. Activation of PKA, but not PKC, protects against serum deprivation (Rukenstein et al., 1991) as well as in our model of ischemia. Likewise, the protective effects of growth factors are largely independent of protein synthesis in the two models, suggesting a similarity in the two models of cell death. Death of fibroblasts due to serum deprivation is also reduced by the activation of PKA in a translation-independent manner, but, in contrast to PC12 cells, the activation of PKC has a protective effect in this model (Tamm and Kikuchi, 1991).

We found that stimulating PKA during ischemia is indeed neuroprotective, and Rydel and Greene (1988) demonstrated that analogs of cAMP can replace NGF in promoting long-term

Figure 6. Peptide growth factors promote survival during ischemia in the absence of concurrent protein synthesis. PC12 cells were treated with anisomycin (0.1 μ M, 1.0 μ M, or 5.0 μ M) for 1 hr prior to ischemia, and treatment continued throughout the postischemic period. Cell viability was assayed 24 hr after ischemia. Data represent means $\pm$ SEM of three trials from one of three comparable experiments.

survival of rat neonatal sympathetic and embryonic sensory neuronal cultures, yet cells that are deficient in PKA are as sensitive to ischemia as cells that have normal levels of PKA. Furthermore, peptide growth factors are protective independent of PKA, as demonstrated by our experiments with the PKA-deficient mutant AB11.0. Thus, although PKA activation is neuroprotective, this is not the mechanism through which growth factors enhance survival. Similarly, Rukenstein et al. (1991) found that NGF and bFGF continued to exert a survival-promoting effect during serum deprivation even in the absence of PKA activity.

In PC12, NGF stimulates the hydrolysis of phosphoinositides to produce DAG and arachidonic acid, known activators of PKC (Contreras and Guroff, 1987; Chan et al., 1989). NGF also induces the translocation of PKC from the cytosolic to the membrane fraction of PC12 cells (Kondratyev et al., 1990). Thus, PKC is one of the second messengers used by NGF. Nevertheless, neither the stimulation nor the downregulation of PKC altered the growth factor-induced protective effect in either PC12 or the PKA-deficient mutant. Although phorbol esters do not alter survival during serum starvation (Rukenstein et al., 1991), PMA has been shown to potentiate the neuritogenic effect of NGF on PC12 (Burstin et al., 1982; Hall et al., 1988). PKC is

induced during ischemia and may have a role in ischemic cell death. For example, PKC is translocated from the cytosolic to particulate fraction and activated following ischemia in the fetal rat brain (Louis et al., 1988). This was accompanied by an increase in a calcium phosphatidylserine-independent kinase, which is thought to be a cleavage product of PKC. Cardell et al. (1990) observed changes in the location of specific isoforms of PKC including the neuronal isoform PKC γ after ischemia in the adult rat. The precise role PKC may have in neuronal cell death has yet to be determined.

K252a and staurosporine are both selective inhibitors of the autophosphorylation of *trk* (Berg et al., 1992; Ohmichi et al., 1992), both inhibit the biological effects of NGF (Koizumi et al., 1988; Hashimoto and Hagino, 1989; Tischler et al., 1990; Ohmichi et al., 1992), and both inhibit the protective effects of NGF, but not EGF or bFGF. While K252a enhances FGF's neuritogenic effect (Koizumi et al., 1988; Hashimoto and Hagino, 1989; I. R. Boniece and J. A. Wagner, unpublished observation) and the bFGF- and EGF-induced activity of ornithine decarboxylase (Koizumi et al., 1988), there was no alteration of the survival-promoting effect of either bFGF or EGF. We suggest that the site of action of K252a and staurosporine is probably the receptor for NGF.

H-7 was as protective as the peptide growth factors but the site of action for the protective effects of H-7 is not clear. H-7 is a competitive inhibitor of the ATP binding site of both PKA and PKC (Hidaka et al., 1984), and has been shown to enhance the survival-promoting effect of EGF and bFGF on serum-depleted primary rat cortical cultures (Morrison et al., 1988a), and the neuritogenic response to bFGF and NGF in PC12 (Posypanova et al., 1990). H-7 does protect against ischemia, but it seems unlikely that it is acting through PKA or PKC because of the experiments with the PKA- and PKC-deficient cells that were discussed above. Furthermore, staurosporine, which is a more potent PKA and PKC inhibitor than H-7, did not alter the protective effects of either EGF or bFGF. Together, our data suggest that the enhancement of the survival-promoting effect of peptide growth factors by H-7 occurs through an unknown mechanism.

We have demonstrated that growth factors continue to exert a protective effect during ischemia independent of protein synthesis. Similar results were obtained by Ruckenstein et al. (1991), who demonstrated that the inhibition of RNA or protein synthesis did not alter growth factor-induced survival of PC12 cells during serum starvation. Thus, growth factor-induced protein synthesis is apparently not a necessary element in the events that protect PC12 cells from ischemia or serum deprivation. Interestingly, the protective effect of FGF on glutamate toxicity in hippocampal neurons requires transcriptional activity (Mattson et al., 1989), supporting the idea that different aspects of the signaling mechanism used by growth factors may be required to confer protection under different conditions. It is necessary to consider, however, that PC12 cells may be limited in terms of the importance of conclusions drawn, as this is an immortalized cell line and may respond atypically.

Our experimental model, which deprives cells of energy derived from both oxidative phosphorylation and glycolysis, differs from the serum deprivation model used by Ruckenstein et al. (1991), which presumably leads to cell death because of a deficiency of growth factors. It is complementary to demonstrate, however, that cell survival within these two different models appears to be affected similarly by growth factors and

intracellular signal transduction pathways. Indeed, the commonality of our findings with those of Ruckenstein et al. suggests that growth factors may provide a generalized neuroprotective effect to a variety of metabolic insults through similar mechanisms.

Inhibitors of RNA and protein synthesis have been shown to block apoptosis in certain cases (Cohen and Duke, 1984; Shi et al., 1989), leading to the concept of "death genes" whose expression may be regulated by growth factors. Since continued protein synthesis plays a less significant role during ischemia-induced cell death and in the protective effects of the growth factors, it is likely that apoptosis and ischemic cell death in this model system are fundamentally different processes. We conclude that several kinases play a role in mediating the toxic effects of ischemia and that modification of these kinase activities by growth factors is likely to be an important component of their protective effects. Likewise, pharmacological agents that modify kinase activity may be of use therapeutically; therefore, the identification of the specific kinases that are important is a key step in the development of clinically useful agents.

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