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Multiple Agents Rescue PC12 Cells from Serum-free Cell Death by Translation- and Transcription-independent Mechanisms

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Past studies revealed that NGF and fibroblast growth factor (FGF) prevent the death of PC12 pheochromocytoma cells that otherwise occurs in serum-free medium. Additional agents were tested here for their abilities to promote long-term survival of naive and NGF-pretreated (primed) PC12 cells in serum-free conditions. Forskolin and permeant cAMP analogs effectively prevented serum-free cell death, as did micromolar levels of insulin and 10–100-nM levels of insulin-like growth factors I and II. In contrast to NGF and FGF, none of these agents caused neuronal differentiation of naive cells or neurite regeneration by primed cells. Each of the agents also prevented rapid cell death in a balanced salt solution, thus apparently ruling out a mechanism dependent on regulation of nutrient uptake. Epidermal growth factor and elevated K^+ appeared to slow the rate of cell death, but did not promote long-term survival; phorbol ester, dexamethasone, or vanadate did not prevent cell death. Each of the survival-promoting agents was effective even when macromolecular synthesis was blocked. Because the synthesis inhibitors themselves did not significantly prevent cell death, such findings indicate that survival was promoted by mechanisms that do not require synthesis of RNA or protein. In addition, various lines of experimental evidence (using the kinase inhibitor K-252a or PC12 cell variants deficient either in protein kinase A activity or in responsiveness to NGF) further suggested that the effective agents maintain survival by independent initial pathways. Regulation of protein kinase activity appears to be a common feature of each pathway and may therefore play a key convergent role in mediating prevention of cell death.

Among the major actions of trophic agents is the maintenance of cell survival/prevention of cell death. For instance, in the nervous system, both *in vitro* (Levi-Montalcini and Angeletti, 1963) and *in vivo* (Levi-Montalcini and Booker, 1960; Gorin and Johnson, 1979) experiments have revealed that NGF is required for the survival of sympathetic and developing sensory neurons. While inroads are being made on this issue (Martin et al., 1988; Koike et al., 1989; Oppenheim et al., 1990), the mechanism whereby NGF and other agents promote neuronal survival is presently unclear.

We and others have employed the PC12 line of rat pheochromocytoma cells to study the mechanisms of NGF action and as a model for various aspects of neuronal behavior (Greene and Tischler, 1976, 1982; Levi et al., 1988). PC12 cells have been especially useful for this because they respond to NGF by undergoing morphological and biochemical differentiation similar to that shown by maturing sympathicoblasts. With respect to the subject of NGF-dependent survival, PC12 cells differ from sympathetic neurons in that they survive without NGF if cultured in presence of serum (Greene and Tischler, 1976). However, in a serum-free nutrient medium such as RPMI 1640, PC12 cells rapidly die unless NGF is present (Greene, 1978). Subsequent studies have shown that fibroblast growth factor (FGF), an agent with trophic actions on certain neurons (Morrison et al., 1986; Walicke et al., 1986) and on PC12 cells (Togari et al., 1985; Rydel and Greene, 1987), can also rescue PC12 cells from serum-free cell death (Rydel and Greene, 1987). Findings of this type have suggested that PC12 cells may be utilized in the serum-free paradigm to study the mechanisms by which neuronal survival and cell death are regulated.

The present study addresses several issues related to the subject of cell survival. First, we have asked what agents in addition to NGF and FGF may promote PC12 cell survival in a defined medium and what these agents may share in common with respect to possible mechanism. In addition, we have defined agents that fail to support PC12 cell survival and thereby have ruled out certain mechanistic pathways. Further experiments deal with the issue of whether macromolecular synthesis is required for the survival-promoting actions of NGF and other factors. Finally, our data permit comparison of NGF-deprived sympathetic neurons and serum-deprived PC12 cells.

Materials and Methods

Materials. NGF was prepared from male mouse submaxillary glands as previously described (Mobley et al., 1976). Bovine brain basic FGF (bFGF), insulin-like growth factors I and II (IGF-I and IGF-II), dibutyryl cyclic AMP, and 8-(4-chlorophenylthio) cyclic AMP (CPT-cAMP) were purchased from Boehringer-Mannheim. Insulin, dexamethasone, actinomycin, forskolin, phorbol 12-myristate 13-acetate (PMA), anisomycin, and camptothecin were purchased from Sigma Chemical Company. K-252a was the kind gift of Dr. Gordon Guroff, National Institutes of Health.

Cell culture. Stock PC12 cultures (passages 26–31) were maintained as previously described (Greene and Tischler, 1976, 1982) in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum. For serum-free experiments, the cells in

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stock cultures were washed three or four times with serum-free RPMI 1640 medium (supplemented with 50 U/ml of penicillin and 50 μg/ml streptomycin). The cells were then detached from the dishes by repeated trituration and washed again in serum-free medium by three to five cycles of centrifugation/resuspension. The washed cells were resuspended in RPMI 1640 medium and plated at a density of 2–6 × 10^4 cells per well in 24-well (16-mm diameter) plastic culture dishes. The bottom surfaces of the wells were precoated with rat-tail collagen as described elsewhere (Greene and Tischler, 1982), and the volume of medium per well was 0.5 ml. For experiments involving "primed" PC12 cells, cultures were treated for 1–2 weeks with NGF in RPMI 1640 medium supplemented with 1% heat-inactivated horse serum. The cells were then passaged into serum-free medium as described above, except that six washes by centrifugation/resuspension were used to enhance removal of residual NGF. The medium in all serum-free cultures was replaced every 3–4 d.

The A126-1B2 variant line of PC12 cells (Van Buskirk et al., 1985) was kindly provided by Dr. John Wagner, Harvard Medical School. The UR61 line of N-*ras*–inducible U7 cells (a PC12 cell variant; Guerrero et al., 1988) was kindly provided by Dr. David Burstein, Mt. Sinai School of Medicine. Both lines, as well as PC12nnr5 cells (Green et al., 1986) were cultured and treated as described for PC12 cells.

When various agents were supplied to the serum-free cultures, these were added from 10–1000-fold concentrated stocks, except for KCl, for which additions were made by mixing RPMI 1640 with the appropriate amounts of a 160-mM solution of KCl.

Cell counts. For cells growing in multiwell dishes, the culture medium (along with most cellular debris) was removed by aspiration and replaced with 0.5 ml of a detergent-containing lysing solution that dissolves cell membranes and cytoplasm and that provided a uniform suspension of single, intact nuclei (Soto and Sonnenschein, 1985). The latter were quantified by counting in a hemacytometer. Broken or damaged nuclei were not included in the counts. In all cases, triplicate wells were scored, and counts are presented as means ± SEM.

There was only a minimal change in cell number in the NGF-treated serum-free cultures over time (recoveries ranged between 94% and 125% of cells plated by up to 1 week), probably because of the stringent washing procedures used to remove bound serum components. Likewise, both counts and the absence of debris generated by cell death indicated little loss of cells in the NGF-treated cultures over the course of the experiments. For this reason, and to permit convenient comparison of data from different experiments, results are presented relative to cell numbers in NGF-treated cultures rather than in absolute cell numbers.

Results

Multiple agents support PC12 cell survival in serum-free medium. Past studies showed that PC12 cells degenerate within a few days of culture in serum-free medium (Greene, 1978) and that NGF (Greene, 1978) and FGF (Rydel and Greene, 1987) support their long-term survival under such conditions. A variety of additional agents to which PC12 cells are known to respond were tested for their capacities to maintain the cells in serum-free RPMI medium for 1 week. Without additives, essentially all of the cells were dead by this time, whereas all survived in the presence of NGF (Figs. 1, 2). Figures 1 and 2 show that 8-(4-chlorophenylthio) cAMP promotes long-term survival of the cells with a maximal effect at about 100 μM. Dibutyryl cAMP (not shown) and forskolin, an activator of adenylate cyclase (Fig. 2) also effectively prevented cell death.

As shown in Figure 2, insulin is an additional agent that promotes PC12 cell survival. Maximal activity occurred at about 3 μM, a concentration far higher than that required for other direct actions of this hormone. This suggested that insulin might be acting via receptors for IGF-I and/or IGF-II. Also, functional receptors for IGFs have been demonstrated on PC12 cells (Dahmer and Perlman, 1988; Nielsen and Gammeltoft, 1988; Dahmer et al., 1989). In consonance with this, both IGFs supported PC12 cell survival and did so at concentrations of 10–100 nM (Figs. 1, 2). Insulin and the IGFs also promoted at least a degree of cell division under the serum-free conditions because the cell number at 1 week was in each case higher than the number initially plated. Visual inspection of cultures indicated that the cAMP analogs, insulin and the IGFs (in addition to NGF and FGF), maintained survival for at least 2 weeks under the serum-free conditions. However, in contrast to NGF, the other agents did not cause morphological differentiation (Fig. 1).

Agents that do not support long-term survival. A variety of additional substances that have been identified as promoters of cell or neuronal survival in other systems were also tested in the serum-free paradigm. Epidermal growth factor (EGF), which can support survival of certain CNS neurons (Morrison et al., 1987) and for which PC12 cells bear receptors and responses (Huff et al., 1981; Lazarovici et al., 1987), exerted a relatively small effect on long-term survival (Figs. 1, 3). Inspection of the cultures at times shorter than 1 week revealed that, though EGF did not ultimately prevent cell death, it did slow the rate of cell loss (data not shown). The small number of cells that were present after 1 week with EGF did not appear to constitute a responsive subpopulation because essentially no cells survived in the presence of this factor by 2 weeks of treatment.

PMA, a tumor promoter and potent activator of protein kinase C, has been reported to maintain the survival of chick peripheral neurons (Montz et al., 1985). In contrast, when tested over a wide range of concentrations, it did not support long-term PC12 cell viability (Fig. 3), nor did it prevent cell death at very early times of culture (data not shown). Moreover, it did not substantially diminish the ability of NGF to prevent cell death, even at concentrations up to 10 μM.

Prolonged exposure to depolarizing levels of K^+ has been recognized as an effective means to prevent the death of a variety of neuronal types, including sympathetic neurons (Wakade et al., 1983; Koike et al., 1989). However, as shown in Figures 1 and 3, this treatment was relatively ineffective in promoting PC12 cell survival for 1 week; after 2 weeks, no survival was apparent (data not shown). Like EGF, elevated K^+ appeared to somewhat delay, rather than prevent cell death. The data in Figure 3 show that high K^+ concentrations also somewhat diminished the survival effects of NGF.

In addition to the above agents, dexamethasone also was unable to prevent PC12 cell death. Glucocorticoids have been shown to antagonize a variety of NGF actions (Doupe et al., 1985; Leonard et al., 1987) but, except at a very high concentration, did not significantly reduce the survival-promoting actions of NGF (Fig. 3). Instances have been reported in which the application of vanadate (Rawson et al., 1990) prevents cell death, presumably by inhibition of phosphotyrosine phosphatase activities. However, this agent, at concentrations from 0.1 nM to 1 μM, did not prevent PC12 cell death (data not shown).

Survival effects on "primed" PC12 cells. The above experiments were performed on PC12 cells that had no prior exposure to NGF. Because NGF treatment can substantially alter cellular properties, experiments were performed in which cultures were preexposed to NGF for 1–2 weeks in the presence of serum to permit neuronal differentiation (i.e., primed; Burstein and Greene, 1978) and then detached from the substrate and passaged into serum-free medium with NGF or the various treatments described above. After 4 d, numbers of surviving cells were assessed. The data in Figures 4 and 5 show that removal of serum and NGF resulted in a substantial degree of cell loss. In contrast, NGF supported both serum-free cell survival and neurite regeneration; FGF also supported survival and, as pre-

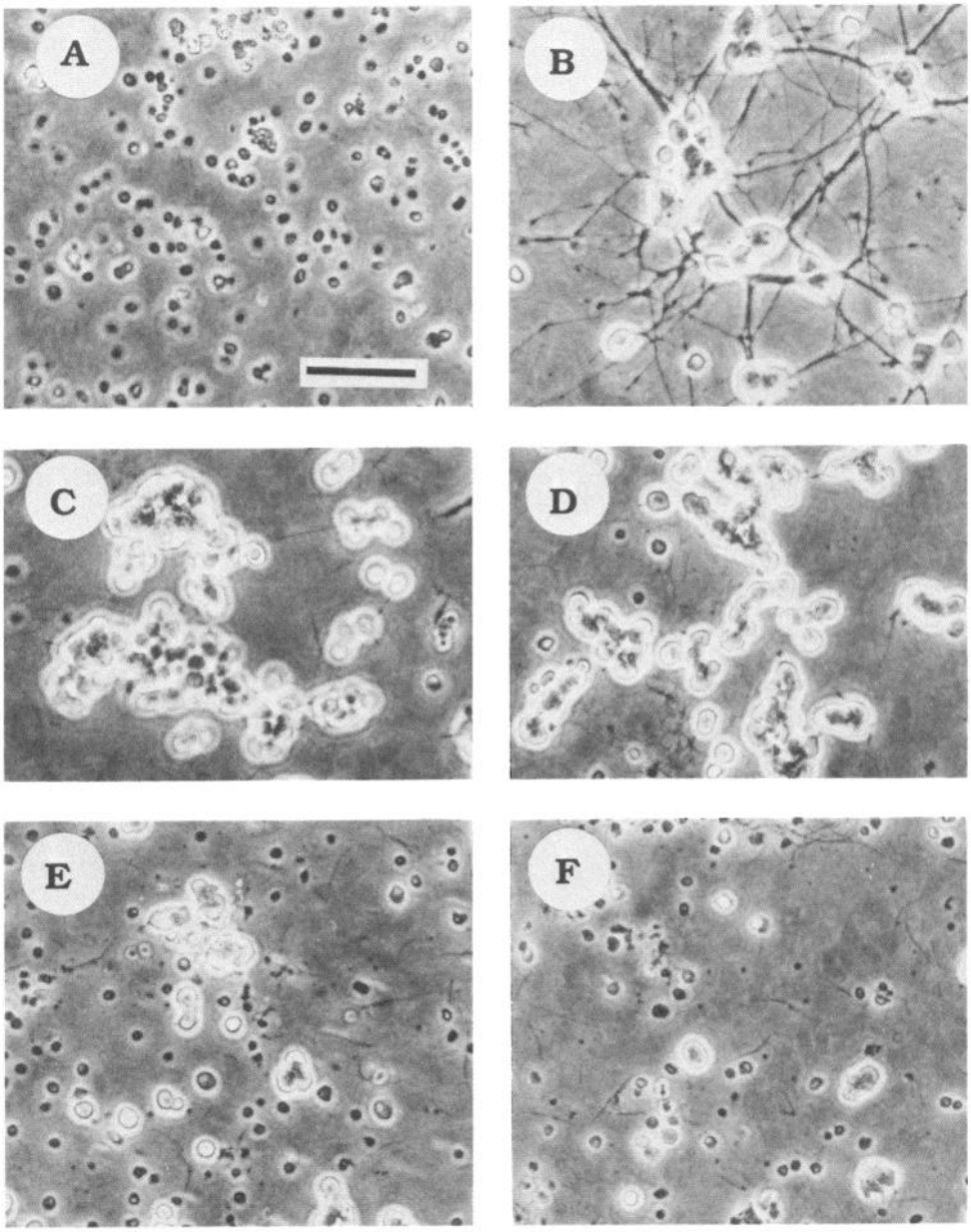


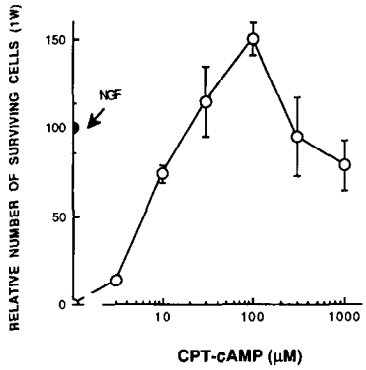
Figure 1. Morphology of PC12 cells maintained for 1 week in serum-free RPMI 1640 medium supplemented with various agents: no additives (*A*), 50 ng/ml NGF (*B*), 100 μM CPT-cAMP (*C*), 10 nM IGF-I (*D*), 1.3 nM EGF (*E*), or 43 mM K^+ (*F*). Cells were washed and plated in RPMI 1640 medium as described in Materials and Methods. Phase-contrast optics. Scale bar, 50 μm.

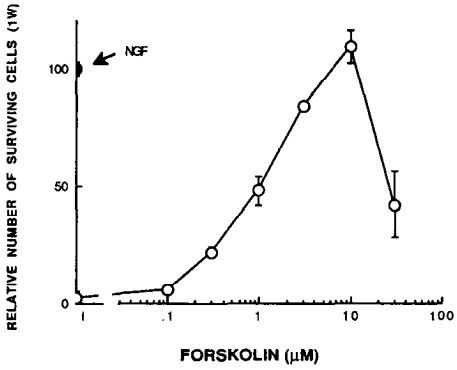
viously reported (Togari et al., 1985; Rydel and Greene, 1987), a degree of process outgrowth. CPT-cAMP, insulin, IGF-I, and IGF-II, as with naive cells, maintained survival of the primed cells, but did not promote significant levels of neurite regrowth (Figs. 4, 5). Furthermore, EGF and elevated K^+, as with naive cells, did not provide robust support of the primed cells (Figs. 4, 5).

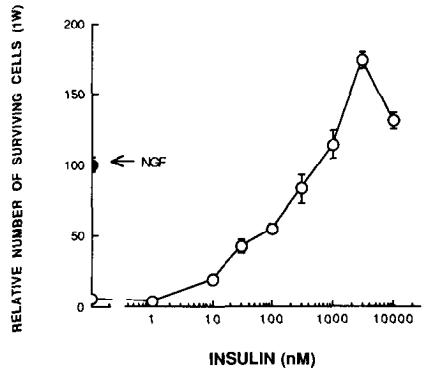
Role of RNA transcription and protein synthesis in prevention of cell death. The abilities of the various agents to promote survival were tested in the presence of high concentrations of inhibitors of protein and RNA synthesis. The inhibitors were applied 0.5–1 hr prior to the survival factors, and the cultures were evaluated for cell number 16–20 hr later. Under these conditions, approximately 60–90% of the cells die in serum-free medium alone. At the concentrations employed, actinomycin and camptothecin inhibit PC12 cell RNA synthesis by approximately 95% and 85% (Burstein and Greene, 1978), respectively, and anisomycin inhibits PC12 cell protein synthesis by over 99% (Greenberg et al., 1986). The data in Figures 6 and 7 show that the inhibitors did not substantially block the capacities of the various agents tested (NGF, CPT-cAMP, insulin, IGF-I, and IGF-II) to rescue the cells from death. Similar results were obtained with "primed" cells (data not shown).

The inhibitors of macromolecular synthesis were also tested in the absence of survival agents. At the concentrations used above, the inhibitors did not show substantial rescue of either naive (Fig. 6) or primed cells (not shown) from death. This was also the case when the inhibitors were tested at serial dilutions (by factors of one-third) down to 100-fold lower concentrations.

Promotion of survival in nutrient-free medium. One potential mechanism for the promotion of cell survival/prevention of cell death is regulation of the uptake of required nutrients from extracellular sources (Horii and Varon, 1977). To test this, cells were plated in serum-free Hank's balanced salt solution with or







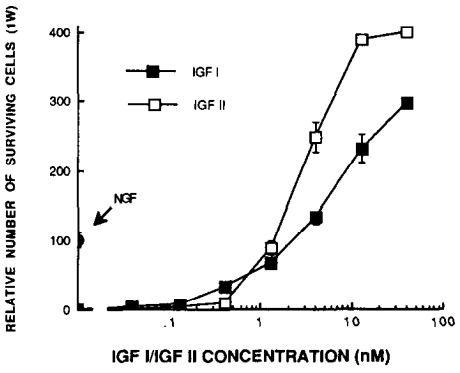
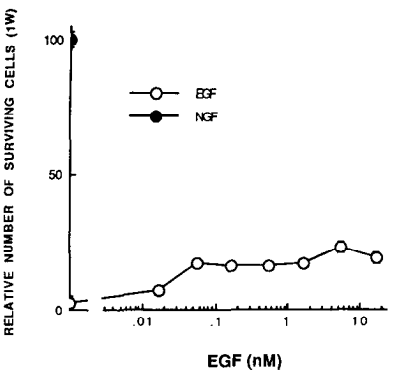
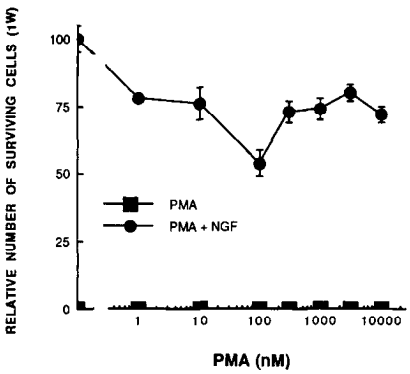
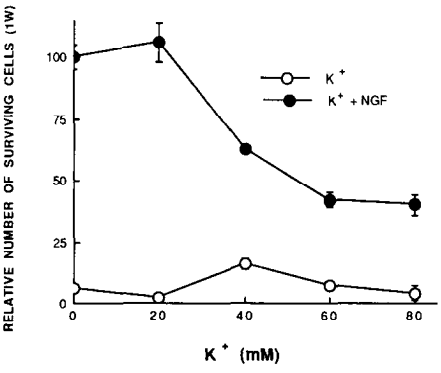


Figure 2. Dose–response relationships for 1-week survival of PC12 cells in serum-free RPMI 1640 medium supplemented with the indicated additives (CPT-cAMP, forskolin, insulin, IGF-I, or IGF-II). Cell survival data are expressed relative to that in NGF-treated sister cultures (indicated by *arrows*). Recoveries in the NGF-treated cultures ranged between 96% and 114% of the numbers of cells originally plated. *Error bars* represent SEM (n = 3).







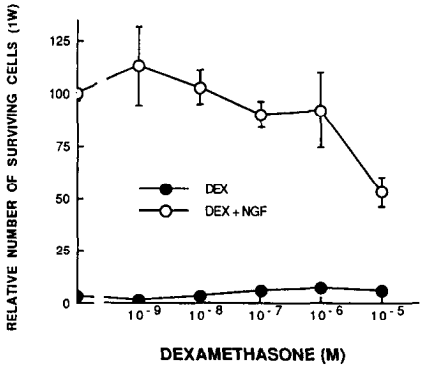


Figure 3. Dose–response relationships for 1-week survival of PC12 cells in serum-free RPMI medium supplemented with the indicated additives (EGF, PMA, KCl, or dexamethasone) and with or without NGF. Cell survival data are expressed relative to that in sister cultures treated with NGF alone. *Error bars* represent SEM (n = 3).

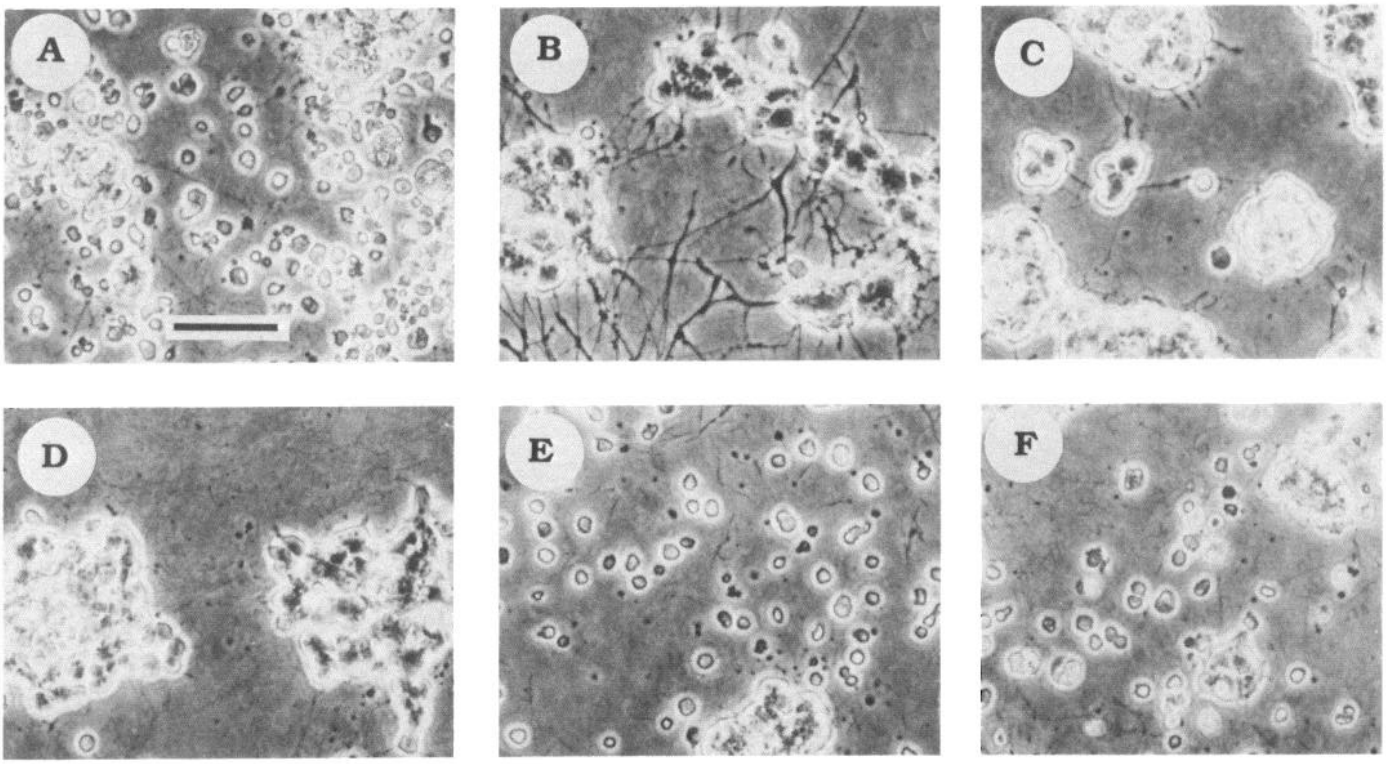


Figure 4. Morphology of NGF-pretreated PC12 cells maintained for 4 d in serum-free RPMI 1640 medium with no additive (*A*), NGF (*B*), 100 μM CPT-cAMP (*C*), 3 μM insulin (*D*), 43 mM K^+ (*E*), or 1.3 nM EGF (*F*). Cells were pretreated with NGF in serum-containing medium for 8 d, then washed and passaged into RPMI medium and maintained for 4 d with the indicated additives as described in Materials and Methods. Phase-contrast optics. Scale bar, 50 μm.

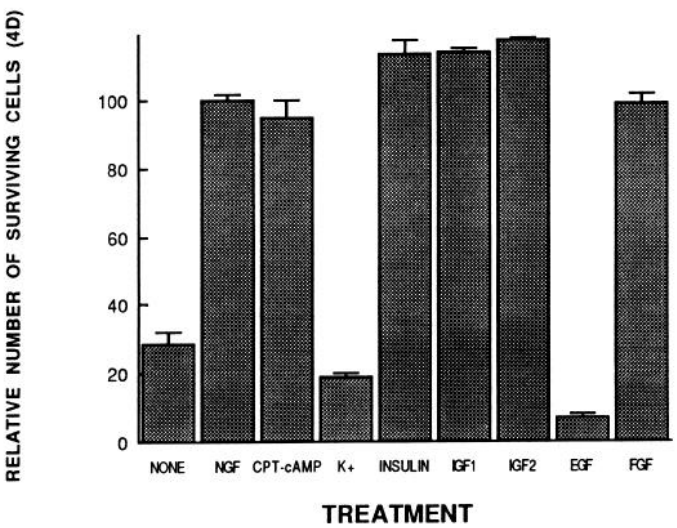


Figure 5. Survival of NGF-pretreated (primed) cells maintained in serum-free medium for 4 d in presence of the indicated additives. Cells were pretreated with NGF in serum-containing medium for 8 d, then washed and passaged into RPMI medium and maintained for 4 d with the indicated additives as described in Materials and Methods. Cell numbers are expressed relative to those in cultures treated with NGF. Recovery of the latter averaged 94% of the number of cells plated. Concentrations were NGF, 2 nM; CPT-cAMP, 100 μM; K^+, 43 mM; insulin, 3 μM; IGF-I and IGF-II, 10 nM; EGF, 1.3 nM; FGF, 10 nM. *Error bars* represent SEM ($n = 3$).

without various agents and counted 1 d later. Without additives, 60–95% of the cells died; good survival was apparent, however, when NGF, FGF, insulin, IGF-I, IGF-II, CPT-cAMP, or forskolin were present (Fig. 8). These agents maintained survival for 2–3 d before cell death occurred, presumably because of the absence of essential nutrients. EGF and elevated K^+ showed partial prevention of cell death at 24 hr (Fig. 8). This appears to be consistent with their delay, rather than prevention, of cell death. These data thus suggest that the agents tested here do not promote cell survival by controlling the uptake of metabolites from the extracellular environment.

Potential role of protein kinases A and C in prevention of cell death. The observation that cAMP analogs prevent PC12 cell death suggests a potential role for protein kinase A in this action. To test this, as well as the possible role of PKA in the survival action of other agents, the A126-1B2 line, a variant of PC12 cells that is defective in PKA activity (Van Buskirk et al., 1985), was employed. The data in Figure 9 show that CPT-cAMP was ineffective in promoting survival of A126-1B2 cells; this strongly suggests that activation of PKA is involved in the maintenance of serum-free cell survival by cAMP analogs. In contrast to CPT-cAMP, NGF, insulin, IGF-I, IGF-II, and FGF all promoted long-term survival of A126-1B2 cells in serum-free medium. Such findings indicate that PKA neither mediates nor is required for the survival actions of these growth factors.

With regard to PKC, the above findings that PMA does not promote cell survival indicate that activation of this kinase is

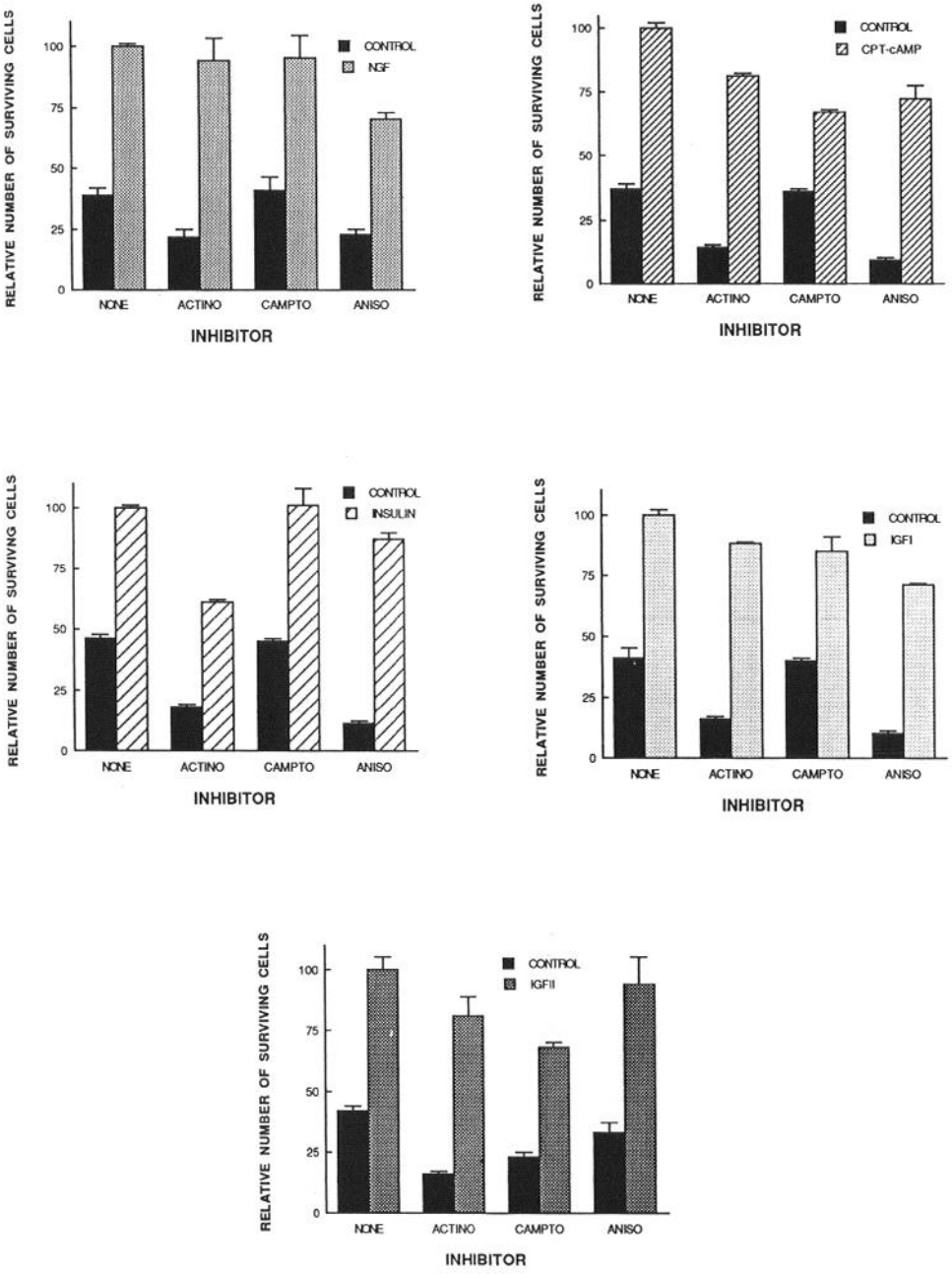


Figure 6. Effects of macromolecular synthesis inhibitors on the capacities of NGF (2 nM), CPT-cAMP (100 μM), insulin (3 μM), and IGF-I and IGF-II (each 10 nM) to promote PC12 cell survival in serum-free RPMI 1640 medium. Cells were washed and plated in RPMI 1640 medium and pretreated where indicated for 2 hr with 10 μM actinomycin-D (*ACTINO*), 20 μM camptothecin (*CAMPTO*), or 100 μM anisomycin (*ANISO*), and then with or without the indicated growth factors for an additional 16–20 hr. Cell numbers are expressed relative to those in cultures treated with each factor alone. *Error bars* represent SEM ($n = 3$).

not sufficient to prevent serum-free cell death. To explore further the role of PKC in survival, serum-containing PC12 cultures were pretreated with 5 μM PMA for 3 d and then exposed to NGF, insulin, or CPT-cAMP in serum-free medium with or without the continued presence of the phorbol ester. Prior experiments have established that exposure of PC12 cells to such concentrations of PMA leads to a substantial downregulation of their PKC levels (Matthies et al., 1987). The data in Figure 9 show that, despite this treatment, each of the tested agents retained the capacity to prevent cell death. This suggests that PKC also neither mediates nor is required for the survival-promoting actions of NGF, CPT-cAMP, or insulin.

Differential actions of K-252a on survival. The drug K-252a has been shown at low concentrations (200 nM) to block a variety of actions of NGF on PC12 cells, but not to effect at least certain responses to FGF and other agents (Koizumi et al., 1988). The data in Figure 10 show that 200 nM K-252a itself does not affect the course of serum-free cell death and that it does completely suppress the ability of NGF to maintain survival. In contrast, the drug did not block the survival-promoting actions of CPT-cAMP, insulin, IGF-I, IGF-II, or FGF.

*Promotion of survival by expression of N-*ras. The expression of v-*ras* or N-*ras* in serum-supported PC12 cells leads to process outgrowth and other manifestations of neuronal differentiation (Noda et al., 1985; Guerrero et al., 1986). It has been suggested that cellular ras protein may be involved in the primary mechanism of action of NGF (Hagag et al., 1986). Furthermore, injection of oncogenic ras protein into a variety of chick embryo peripheral neuron types promotes their survival *in vitro* (Borasio et al., 1989). To test the potential effect of ras expression in the

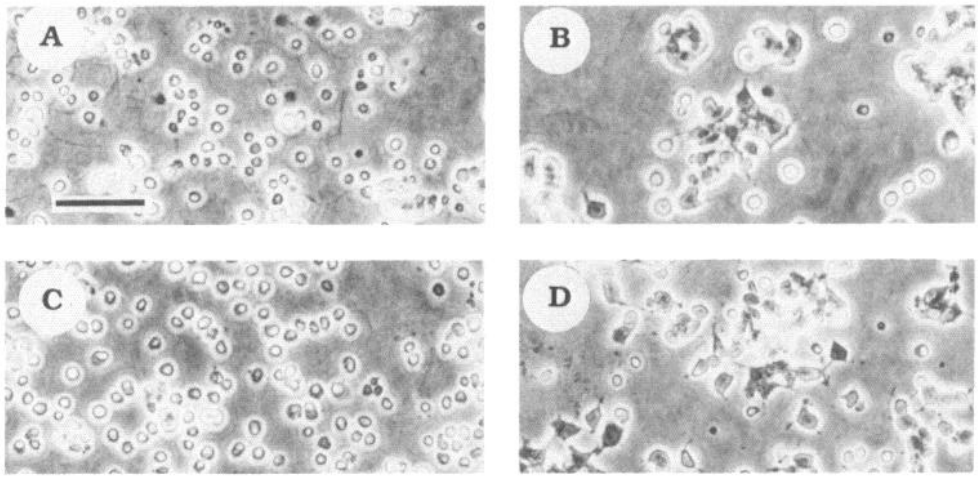


Figure 7. Morphology of PC12 cells maintained in serum-free RPMI 1640 medium for 1 d with either no additives (*A*), NGF (*B*), 10 μM actinomycin-D (*C*), or NGF plus 10 μM actinomycin-D (*D*). Cells were washed and plated in medium and pretreated for 2 hr where appropriate with actinomycin before NGF addition. Phase contrast optics. Scale bar, 50 μm.

present serum-free paradigm, experiments were performed using the UR61 cell line. The latter is derived from a variant line of PC12 cells and possesses a dexamethasone-inducible N-*ras* (Guerrero et al., 1988). UR61 cells were preexposed in serum-containing medium to 1 μM dexamethasone for 5 d to induce N-*ras* and then passaged into serum-free medium for 4 d with or without NGF. For dexamethasone-pretreated cells, the corticosteroid was also present during culture in serum-free medium. The data in Figure 11*A* show that dexamethasone treatment promoted survival to a degree similar to that achieved with NGF. Because dexamethasone itself does not prevent serum-free cell death (Fig. 3), these findings show that induction of N-*ras* promotes cell survival. Figure 11*B* shows the effect of K-252a on serum-free survival by *ras*-induced UR61 cells. In contrast to its effects on NGF-promoted survival, K-252a did not significantly diminish the ability of *ras* to prevent cell death. This suggests that, if ras proteins were to play a role in the NGF mechanism of survival promotion, then they would do so at steps downstream from the step affected by K-252a.

Promotion of survival of PC12 cell variants lacking high-affinity NGF receptors. PC12nnr5 cells are a variant line with low- but not high-affinity NGF receptors (Green et al., 1986). These cells cannot endocytose NGF and lack a variety of responses to NGF, including the capacity to be maintained in serum-free medium (Green et al., 1986). The data in Figure 12 confirm the lack of survival response to NGF and show that, despite this, FGF, insulin, and CPT-cAMP can each prevent serum-free cell death of the variant cells. These findings indicate that the defective element in the variants that otherwise confers NGF receptor functionality does not play a required role in the mechanisms by which FGF, insulin, and cAMP derivatives prevent cell death.

Discussion

This study has explored several aspects of the mechanisms by which trophic agents prevent cell death of neuronal-like PC12 cells in a controlled milieu. We have used these cells both before and after NGF-promoted neuronal differentiation and, in each case tested, found comparable results. In part because PC12 cells share a number of properties in common with sympathetic neurons and their precursors (Greene and Tischler, 1976, 1982), one aim here has been to examine the suitability of this model for extending neuronal cell death studies. Our findings suggest both similarities to and differences from normal neuronal behavior.

Multiple agents and multiple primary mechanisms support survival. The paradigm used here has been to test various agents for their abilities to rescue PC12 cells from death brought about by serum deprivation. Exposure of PC12 cells to serum-free medium can be viewed as withdrawal of trophic agents. Unlike sympathetic neurons, PC12 cells can survive in presence of serum. This most likely is due to their capacity to respond to serum-borne trophic agents, and this in turn probably reflects the immature/neoplastic nature of pheochromocytoma cells. For instance, though mature sympathetic neurons do not respond to FGF, both sympathico-adrenal precursors (Stemple et al., 1988) and PC12 cells do. Thus, our assay system may reveal defined agents that either normally act as neurotrophic factors or that ultimately mimic the mechanistic actions of such molecules.

Past work established that NGF and FGF provide long-term rescue of PC12 cells after serum deprivation. These factors also induce PC12 cell neuronal differentiation. Among the present findings is that several agents that do not promote the differentiation of PC12 cells, namely, insulin and IGFs as well as mimics or inducers of intracellular cAMP, will also rescue them from death. There is precedence for these results. PC12 cells have other known responses to each of the death-prevention agents (Greene and Tischler, 1982; Togari et al., 1985; Dahmer and Perlman, 1988; Nielsen and Gammeltoft, 1988; Rowland-Gagné and Greene, 1990). Furthermore, the survival-promoting actions of NGF and FGF on various types of neurons have been well documented (cf. Morrison et al., 1986; Walicke et al., 1986; Knusel et al., 1990), and there is evidence for similar effects of insulin, IGFs, and cAMP derivatives (cf. Aizenman and de Vellis, 1987; Gammeltoft et al., 1988; Knusel et al., 1990). With respect to sympathetic neurons, NGF (Levi-Montalcini and Angeletti, 1963), insulin (Recio-Pinto et al., 1986), cAMP derivatives (Rydel and Greene, 1988), and vasoactive intestinal peptide (probably via cAMP; Pincus et al., 1990) may each sustain their survival *in vitro*.

It is notable that the various survival-promoting activities described here employ distinct primary mechanisms of action. For instance, our data indicated that the survival-promoting activity of CPT-cAMP was mediated by protein kinase A, but that such actions of NGF, FGF, insulin, and the IGFs were not.

Similarly, both NGF and CPT-cAMP can maintain rat sympathetic neuron survival in culture, but only in the latter case is activation of PKA required (Rydel and Greene, 1988). In the cases of FGF, insulin, and the IGFs, it is likely that primary mediation involves specific receptor-associated tyrosine kinase activities (Czech, 1989; Lee et al., 1989; O'Hare and Pilch, 1990). NGF also appears to trigger rapid changes in PC12 cell protein phosphotyrosine levels (Maher, 1988). However, despite this as well as the observation that many FGF and NGF actions on PC12 cells are comparable, as in other instances (Koizumi et al., 1988), the protein kinase inhibitor K-252a differentially blocked the supportive actions of NGF, but not those of FGF (or of other agents). Moreover, an NGF-insensitive PC12 cell variant line retained survival responses to FGF, insulin, and CPT-cAMP. These findings all indicate that various independent initial pathways may be employed to prevent cell death in PC12 cells.

While the full mechanisms are unknown by which survival is promoted by each of the agents described here, it is worth noting that each regulates specific cellular phosphorylations in target cells. This raises the speculation that phosphorylation may be the critical regulatory event in control of cell survival/death. A further speculation is that, while various agents may use different primary mechanistic pathways, each may convergently prevent cell death by regulating phosphorylation of the same key substrates.

Agents that do not rescue PC12 cells from cell death. Although several different agents maintained PC12 cell survival, the lack of effect of certain other treatments is informative. PMA neither prevented cell death nor interfered with NGF-promoted survival. Moreover, NGF was effective even after pretreatment with levels of PMA sufficient to cause significant downregulation of protein kinase C. PMA is also ineffective in supporting the survival of cultured rodent sympathetic neurons (Rydel and Greene, 1988). Some actions of NGF have been suggested to be mediated by protein kinase C (Cremins et al., 1986; Hama et al., 1986), while other actions do not appear to require this kinase (Reinhold and Neet, 1989; Rowland-Gagné and Greene, 1990). The present findings appear to rule out a role for protein kinase C in the survival mechanism of NGF, as well as of FGF, cAMP, and insulin/IGFs.

EGF had a slight effect in our assay, but this appeared to be a delay of death rather than promotion of survival. This factor has no known effects on sympathetic neurons. However, though it does not cause their differentiation, it does have a variety of actions on PC12 cells (Huff et al., 1981; Lazarovici et al., 1987), many of which are shared with NGF and with CPT-cAMP and insulin. Thus, the survival mechanism shows a degree of discrimination with respect to trophic agents.

Elevated K^+ was another treatment that was only slightly effective and that appeared to delay rather than prevent PC12 cell death. This apparently differs significantly from findings with cultured chick and rat sympathetic neurons for which NGF may be replaced with depolarizing levels of K^+ (Wakade et al., 1983; Koike et al., 1989). The reasons for this discrepancy are not clear, but among several alternatives could be differences in experimental paradigm. The present studies utilized newly passaged cells. Even for NGF-pretreated cells, neurites were mechanically detached before plating. In contrast, studies with rat sympathetic neurons utilized cultures first established with NGF and then switched into NGF-free medium with elevated K^+ without being subcultured (Koike et al., 1989). In support

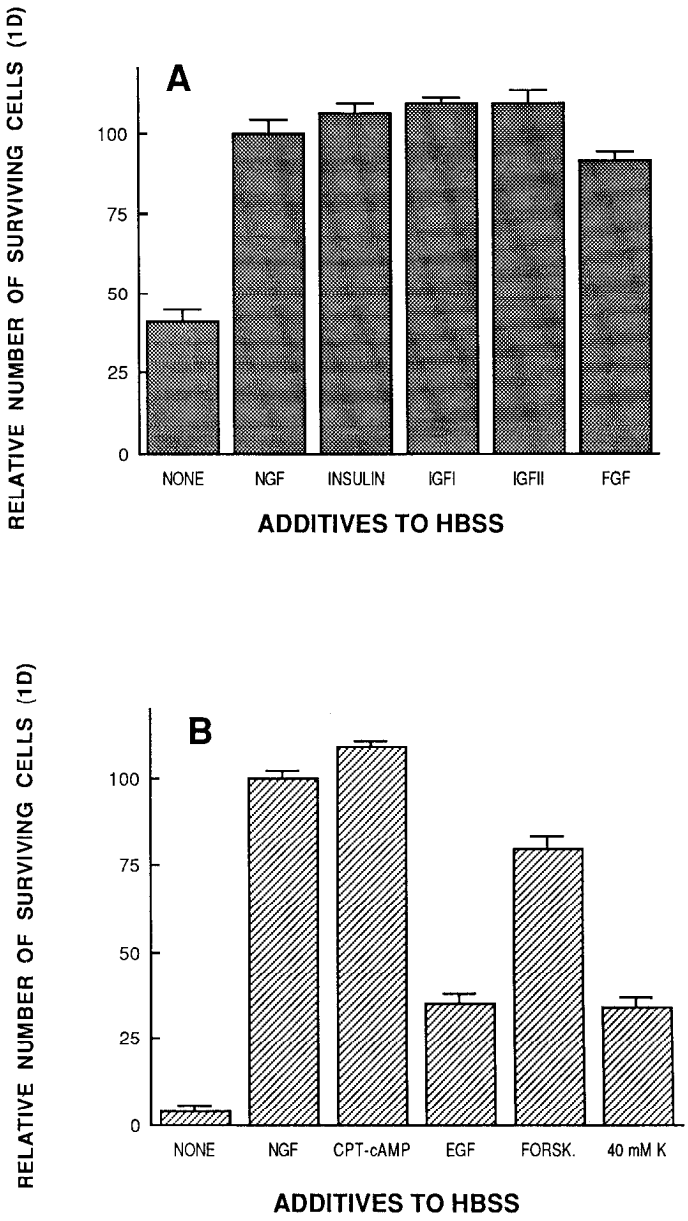


Figure 8. Promotion of PC12 cell survival by various agents in HBSS. Cells were washed and plated in HBSS containing the indicated agents, all as described in Materials and Methods. Concentrations here and in subsequent figures are as given in legend to Figure 5; forskolin (*FORSK.*) concentration was 10 μM. Cell counts were determined after 1 d. The data in *A* and *B* represent independent experiments, and in each case cell numbers are expressed relative to those in cultures treated with NGF (average recovery in *A*, 96%; in *B*, 104%). *Error bars* represent SEM ($n = 3$).

of this possible influence of experimental protocol, we have observed in preliminary experiments that well-established NGF-treated PC12 cells maintain survival and neurites when their medium is exchanged (without passaging) for serum-free medium with 44 mM K^+. Under similar circumstances, but without elevated K^+, the neurites degenerate, and the cells do not survive.

Role of macromolecular synthesis. Studies with PC12 cells and neurons show that some actions of NGF require RNA transcription and that others of its actions do not (Burstein and Greene, 1978). With regard to the present study, two distinct

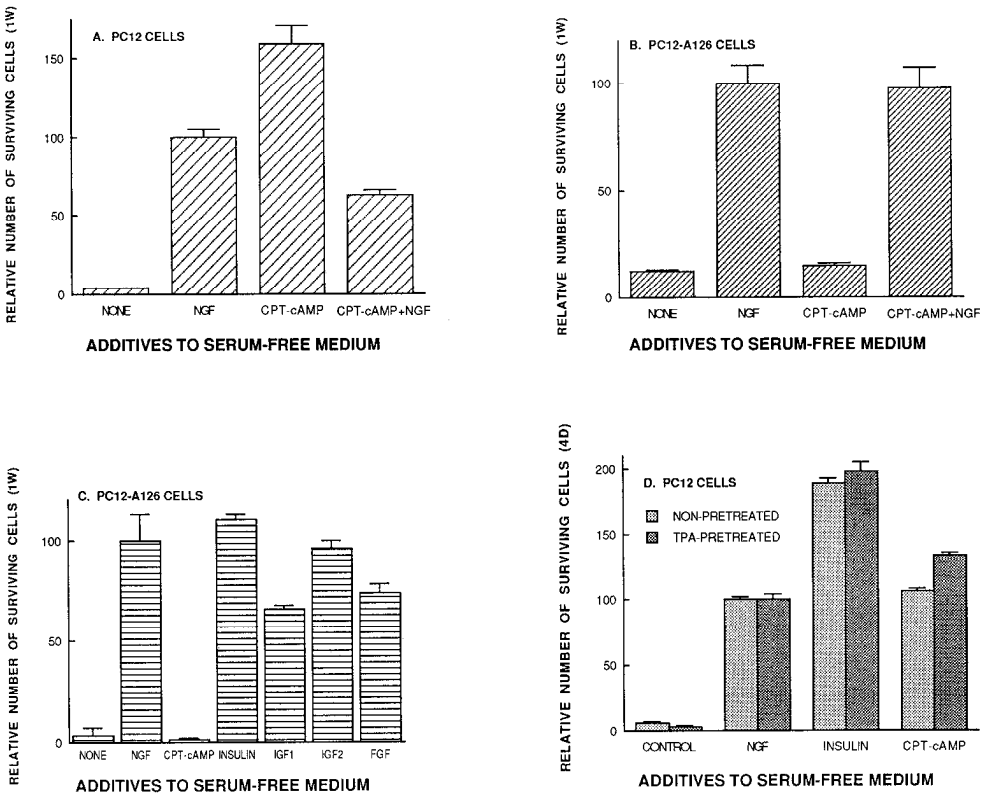


Figure 9. Roles of protein kinase A (*A–C*) and protein kinase C (*D*) in promotion of survival by various agents in serum-free RPMI 1640 medium. *A–C*, PC12 cells (*A*) or PKA-deficient A126-1B2 cells (*B* and *C*) were maintained with the indicated additives for 1 week. *D*, PC12 cells were pretreated for 3 d with or without 5 μM PMA in serum-containing medium, then washed and passaged into serum-free RPMI 1640 medium in the continued presence or absence of 5 μM PMA and the indicated additives. Cell numbers were determined after 4 d. In all cases, cell numbers are expressed relative to those in cultures treated with NGF alone (average recoveries ranged from 96% to 122% of cell numbers plated). *Error bars* represent SEM (n = 3).

issues arise. First, does promotion of survival by NGF and other agents require macromolecular synthesis? Second, does the mechanism by which cells die in the absence of trophic support require synthesis of macromolecules?

The first issue has remained unresolved up to this time. Previous work showed that the ability of NGF to promote the survival of cultured sympathetic neurons is not blocked by inhibition of RNA or protein synthesis (Partlow and Larrabee, 1971). Although these findings appeared to favor a nontranscriptional mechanism for survival maintenance by NGF, recent findings have shown that inhibitors of RNA or protein synthesis themselves prevent or delay the death of cultured sympathetic or sensory neurons even when NGF is withdrawn (Martin et al., 1988; Scott and Davies, 1990). Thus, it has not been possible to discern whether active promotion of neuronal survival by NGF requires macromolecular synthesis. The system employed here appears to differ from that of NGF-deprived neurons. We observed that death of PC12 cells evoked by serum withdrawal is not prevented or significantly delayed by inhibitors of RNA or protein synthesis. The reason for this difference in susceptibility of PC12 cells and neurons to blockade of synthesis is unknown. However, because of this, it was possible here to determine directly that NGF and the other tested agents maintained PC12 cell survival even under conditions in which RNA and protein synthesis were stringently blocked. The conclusion drawn from these results is that the mechanism(s) underlying promotion of survival in the present test system does not require macromolecular synthesis.

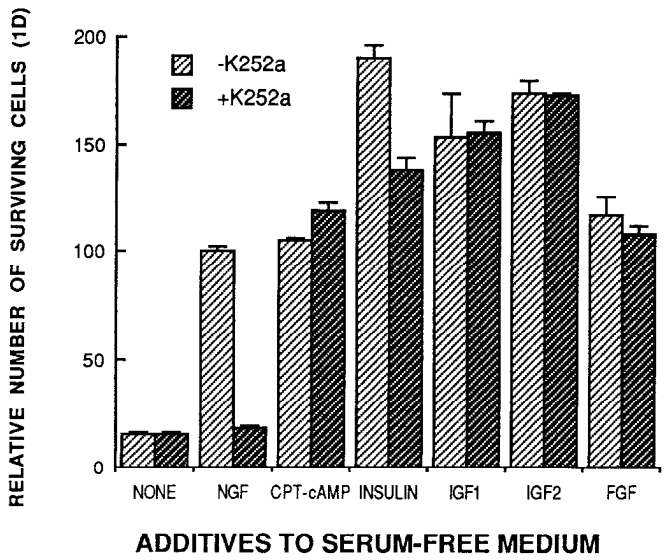


Figure 10. Effect of K-252a (200 nM) on promotion of serum-free PC12 cell survival by various agents. Cells were washed and plated in RPMI 1640 medium and, where appropriate, were pretreated for 2 hr with K-252a before introduction of the indicated agents. Cell numbers were determined at 1 d and are expressed relative to those in cultures treated with NGF alone. *Error bars* represent SEM (n = 3).

If the mechanism whereby NGF promotes survival does not require transcription, might cell death have a transcriptional basis? The observation that inhibition of macromolecular synthesis prevents neuronal degeneration both *in vitro* (Martin et al., 1988; Scott and Davies, 1990) and *in vivo* (Oppenheim et al., 1990) has led to the interpretation that cell death is an active process that requires RNA synthesis and that trophic factors prevent cell death by suppressing a transcription-dependent "death program." However, an alternative explanation of such observations is that cell death is caused by rapidly turning-over constitutive component(s) whose potentially lethal activities are

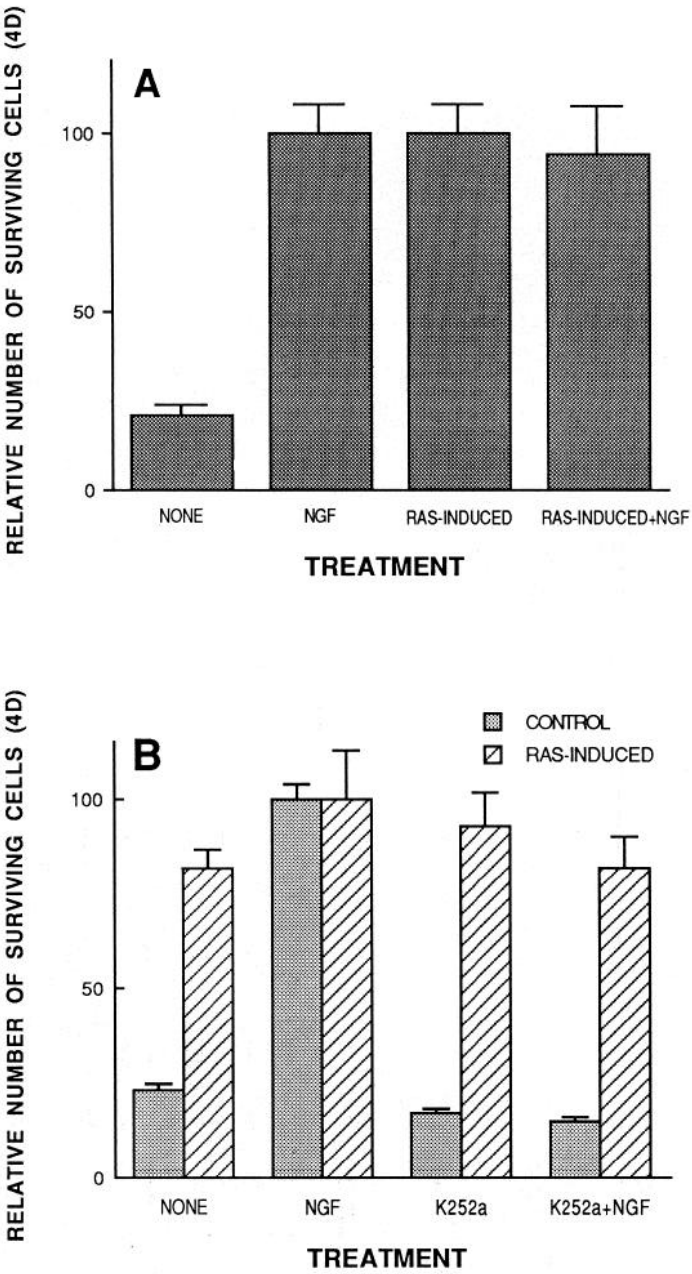


Figure 11. Effect of N-*ras* and NGF on serum-free survival of UR61 cells and differential effects of K-252a thereon. *A*, UR61 cells were pretreated for 5 d in the presence of 1 μM dexamethasone to induce N-*ras*, then washed and plated in RPMI 1640 medium in the continued presence of the dexamethasone and without or with NGF (*RAS-INDUCED* and *RAS-INDUCED+NGF*, respectively). Equal numbers of cells without pretreatment were washed and plated in RPMI 1640 medium either without or with NGF (*NONE* and *NGF*, respectively). *B*, UR61 cells with or without 6 d of pretreatment with dexamethasone were washed and plated in serum-free RPMI 1640. Cells with dexamethasone pretreatment continued to receive the drug. Where indicated, cultures were treated for 2 hr with K-252a, and following this, as indicated, NGF was added. For both *A* and *B*, cell counts were determined after 4 d and are expressed relative to numbers in NGF-treated cultures. *Error bars* represent SEM ($n = 3$).

inhibited in the presence of trophic factors. In this case, withdrawal of a trophic factor would lead to activation of such proteins and cell degeneration, while inhibition of macromolecular synthesis would lead to their depletion and prevention or retardation of cell death. In the present work, inhibitors of

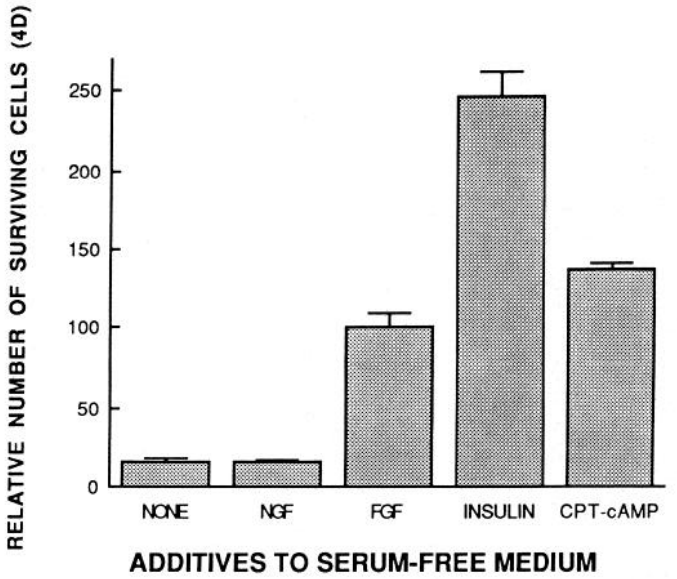


Figure 12. FGF, insulin, and CPT-cAMP prevent serum-free death of NGF-unresponsive PC12nnr5 cells. Cells were washed and cultured for 4 d in serum-free RPMI 1640 in presence of the indicated agents. Cell counts are expressed relative to those in cultures supplemented with FGF (average recovery, 107% of those plated). *Error bars* represent SEM ($n = 3$).

macromolecular synthesis failed to prevent serum-free death of PC12 cells. This raises the possibilities either that PC12 cells, in contrast to neurons, cannot sustain long-term survival without synthesis of macromolecules, that cell death brought about by serum-deprivation and by NGF withdrawal is mediated by distinct mechanisms, or that if cell death is due to nontranscriptional regulation of constitutive proteins, then such molecules have half-lives that are longer in PC12 cells than in neurons.

In summary, our findings indicate that multiple agents can rescue PC12 cells from serum-free cell death and that these agents appear to work by means of independent primary mechanisms. These mechanisms do not require macromolecular synthesis and may be mediated, at least in part, by critical phosphorylations. The serum-free PC12 cell system shows many similarities to responses shown by sympathetic neurons and/or their precursors and appears to be valuable for screening potential survival agents and for uncovering the mechanisms by which trophic factors promote survival/prevent death of neurons. On the other hand, there are also several differences between the survival characteristics of PC12 cells and sympathetic neurons. The significance of these differences remains to be determined.

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