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Puromycin Induces Apoptosis of Developing Chick Sympathetic Neurons in a Similar Manner to NGF-deprivation

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ABSTRACT—Survival of sympathetic neurons of chick embryo depends on nerve growth factor (NGF), and removal of NGF from culture medium caused apoptosis. We found that puromycin, an analogue of aminoacyl-tRNA, also induced apoptosis in a manner quite similar to that caused by NGF-deprivation. First, transcriptional inhibitors such as actinomycin D and α-amanitin effectively prevented both apoptoses, suggesting a requirement of gene expression. Second, the apoptoses were effectively prevented by translational inhibitors acting on peptidyltransfer reaction, such as anisomycin and blasticidin S. On the other hand, emetine, an inhibitor of translocation of peptidyl-tRNA, was not effective. Finally, NGF-deprivation and puromycin-addition affected the same three phosphorylated proteins to undergo dephosphorylation or to be eliminated. Besides, these changes were suppressed by anisomycin and blasticidin S, but not by emetine. Based on these findings, we discuss the mechanism to induce apoptosis of chick sympathetic neurons.

INTRODUCTION

Programmed cell death (PCD) in nervous system is considered to be a step whereby the size of neuronal population is adjusted to the size of target tissue [10, 11]. During neurogenesis, twice as many neurons are produced than compared to adult organism, and half of them undergo PCD while making functional connections with their target cells. The survival of neurons during PCD period depends on a specific neurotrophic factor which is produced by the target cells and delivered to neurons through the synaptic junctions. Therefore, only the neurons that managed to have established a functional junction with their correct target cells and received the factor would survive [7].

When neurons are transferred to culture, a relevant neurotrophic factor is required for the neurons to survive, and deprivation of the factor causes apoptosis of neurons [4]. In some cases, inhibitors of transcription and translation such as actinomycin D, cycloheximide, and puromycin have been reported to prevent the apoptosis [7, 8]. From these observations, neuronal death caused by neurotrophic factor-deprivation is considered to be an active process requiring expression of certain genes. However, the mechanism by which expression of those genes is induced is not known.

In this study, we have found that puromycin as well as NGF-deprivation induced apoptosis of developing chick sympathetic neurons in a quite similar manner. The induction of apoptosis by these causes required transcription, and was suppressed by a class, but not by another class, of translational inhibitors. In addition, the same phosphorylated proteins were dephosphorylated or eliminated by these causes. Based on these findings, we discuss the mechanism by which the apoptosis of neurons is induced.

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MATERIALS AND METHODS

Cell Culture

Sympathetic ganglia of thoracic regions were dissected from 10–15 chick embryos (White Leghorn) at 11 to 11.5 embryonic-day and collected in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (HBSS (−)). The obtained 100–150 ganglia in 100 ml of HBSS (−) were incubated with 0.05% trypsin (GIBCO) at 37°C for 10 min and then gently triturated by pipetting. After centrifugation for 10 sec at 1000 rpm, the supernatant containing dissociated cells was transferred to a tube containing 1 ml of culture medium supplemented with 30 ng/ml mouse 2.5S NGF (Chemicon), and this procedure was repeated until dissociation was completed. Collected supernatant was centrifuged at 10°C for 10 min at 1000 rpm and cell pellet was suspended in 4 ml of culture medium and plated on non-coated 100 mm plastic dish (Corning). The dish was kept at 37°C for 1.5 hr in a humidified 5% CO_2 incubator. During this incubation, most non-neuronal cells attached to the plastic surface, whereas neurons remained in suspension. The collected suspension was diluted with the same medium to $6.0–7.5\times10^5$ cells/ml and aliquots were plated on a poly-lysine coated culture dish (Corning), followed by addition of NGF at the final concentration of 30 ng/ml. Culture medium was composed of 90% Eagle's minimum essential medium (Nissui Inc.) and 10% dialyzed and heat-inactivated FCS (JRH Biosciences) supplemented with 18 μM fluorodeoxyuridine and 10 μM uridine (Nakarai Tesque Inc.) to kill dividing cells such as Schwann cells and fibroblasts.

Neurons began to extend neuritic processes within 10 hr after plating. Cell bodies aggregated in clusters of 3–10 cells and were connected by neurite to each other in 2 days. The number of cells in culture was reduced to approximately 50% after 2 days, because cells that had been severely damaged before plating degenerated within the first 2 days (they adhered to living cells as debris). Although the culture initially contained non-neuronal cells comprising 15% of the total cells, most of them were killed by fluorodeoxyuridine during the 5-day incubation so that finally more than 98% of the cells in the culture were neurons. Every 2.5–3 days, two-thirds of culture medium was exchanged for NGF-free medium and NGF was immediately added at 25 ng/ml. Neurons required NGF for the first 2 weeks, and thereafter, they began to lose the NGF-dependency.

Therefore, we used the 5 to 6-day-old culture for all of the following experiments.

Deprivation and Readdition of NGF

For the sake of rapid and complete removal of the activity, IgG fraction from the sheep immunized by mouse NGF (CIDtech Research Inc.) was added to the culture at the final concentration of 12.5 μg/ml, after exchanging two-thirds of culture medium for NGF-free medium. For readdition of NGF, two-thirds of culture medium was exchanged for NGF-free medium and then NGF was added at 40 ng/ml. Thereafter, every 2.5–3 days medium was exchanged in the same manner and NGF was added at 25 ng/ml.

Analysis of phosphorylated proteins

Each 48-well containing $1.2–1.5\times10^4$ neurons received 7.4 MBq of [^{32}P]orthophosphate for 6–7 hr (ARC Radiochemicals) and cells were lysed with a buffer containing 1% Triton X-100 and 1% sodium deoxycholate. After centrifugation at 100,000 rpm for 1 hr in a Beckman model TLA 120.2 rotor at 4°C, the precipitates were electrophoresed through a 10–20% linear polyacrylamide gradient gel containing 0.1% SDS and 7 M urea. The gel was rinsed with 50% methanol-10% acetic acid, dried and exposed to an image plate of Fujix Bas 2000 to take an autoradiography.

Inhibitors

Inhibitors were purchased from the following corporations; actinomycin D (WAKO Pure Chemical), anisomycin and puromycin (Sigma Chem. Co.), a-amanitin (Boehringer Ingelheim), blasticidin S (Funakoshi Co. Ltd.) and emetine (Nakarai Tesque Inc.). For use of each translational inhibitor, a dose which inhibited approximately 80% of [^{35}S]Met/Cys incorporation in 3 hr was determined and defined as a minimal dose.

RESULTS

NGF-deprivation and puromycin induced apoptosis of developing chick sympathetic neurons

Apoptosis of developing neurons after NGF-deprivation has not been characterized *in vitro*, except for rat sympathetic neurons [4]. In order to investigate the mechanism of apoptosis caused by NGF-deprivation without limit to mammalian system, we established the culture system of sympathetic neurons from chick embryo. Observation of morphological alterations under a microscope revealed that most cell bodies fragmented into pieces between 45–65 hr after NGF-deprivation (Fig. 1). This and other morphological alterations (refer to the legend of Fig. 1) were quite similar to those during apoptosis of rat sympathetic neuron after NGF-deprivation [4]. Like the death of rat sympathetic neuron, this death was also apoptotic, because DNA degradation characteristic to apoptosis occurred. When total DNA extracted from NGF-deprived neurons was analyzed by agarose gel electrophoresis, a DNA ladder, each band of which corresponded to a multimer of nucleosome-sized DNA, was detected with DNA extracted from neurons deprived of NGF for longer than 12 hr (Fig. 2). In addition, DNA longer than 20 Kb was very scarce in the total DNA from neurons at 48 hr, indicating that extensive degradation of DNA had occurred.

During the course to examine effects of translational inhibitors on apoptosis caused by NGF-deprivation (see below), we found that puromycin itself caused fragmentation of cell bodies between 30 to 50 hr eventually after morphological alterations similar to those with apoptosis induced by NGF-deprivation (Fig. 5D). This death was also apoptotic, because DNA degradation was discernible by 12 hr (Fig. 2). Apoptosis was induced by puromycin in the dose-dependent manner: 1, 2 or 3 μM puromycin induced fragmentation of cell bodies of 30%, 80% or more than 95% neurons, respectively.

The decrease of protein synthesis rate preceded committment to Apoptosis

Preceding to DNA degradation, an activity of protein synthesis of neurons was found to decrease rapidly after NGF-deprivation. Figure 3 shows that the incorporation rate of [^{35}S]Met/Cys into the TCA-insoluble fraction began to decrease by 6 hr and reached approximately 30% level at 18 hr after NGF-deprivation. Taking into account the activity of NGF-independent cells that comprised 10–15% in this culture (refer to the legend for Fig. 1), NGF-dependent neurons appear to lose approximately 80% of their original protein synthesis activity within 18 hr.

Next, we examined the time course of commitment to apoptosis. The commitment is defined as entering an irreversible step to apoptosis, which is not suppressed by NGF [4]. At various timepoints after NGF-deprivation, NGF was readded to measure the percentage of neurons that could be rescued. The results shown in Figure 3 indicate that the survival began to decrease at 8 hr and reached to approximately 25% at 24 hr after NGF-deprivation. Taking into account NGF-independent neurons in this culture, NGF-dependent neurons become committed to apoptosis during 8–24 hr.

To examine the time course of commitment to apoptosis triggered by puromycin, neurons were exposed to puromycin for various periods of time and then puromycin was removed by exchanging culture medium. Figure 4 presents the survival at day 3 (closed circles) and the incorporation rate of [^{35}S]Met/Cys (open circles) after puromycin-addition, and indicates that the commitment to apoptosis started soon after the decrease in protein synthesis.

Transcription was required for apoptosis

Inhibitors of transcription and translation are reported to block or delay apoptosis induced by deprivation of neurotrophic factors, proposing the requirement of gene expression for such dying processes [7]. To assess the requirement of transcription for apoptosis of chick sympathetic neurons, transcriptional inhibitors were added simultaneously with either NGF-deprivation or puromycin-addition and then the survival at day 3 was measured. The results indicate that by addition of 20 nM actinomycin D, approximately 85% or 70% of neurons remained intact (Fig. 3 open triangle at time 0 and Fig. 5 A) or puromycin-addition (Fig. 4, open triangle

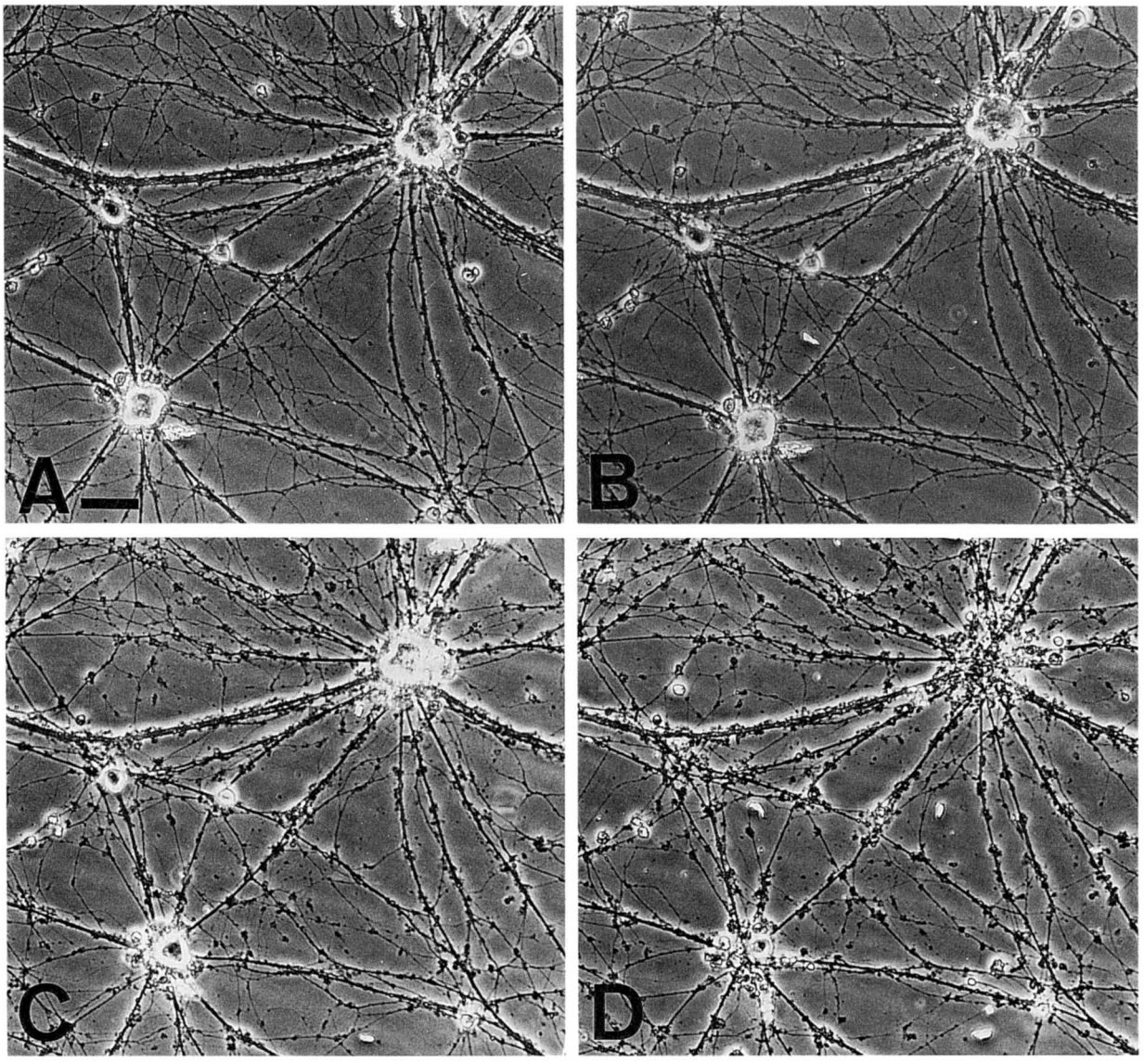

FIG. 1. Phase-contrast micrographs of cultured sympathetic neurons after NGF-deprivation. NGF was deprived at time 0 (A). The first alterations were detected at 25 hr; the neurites became slightly thinner and locally disrupted in places, leaving behind bits of neuritic debris (B). Cell bodies became phase-dark, and the distortion of nuclear perimeter became noticeable in 45 hr (C). At 65 hr, most intact cell bodies disappeared and were replaced by scattered debris (D). The neurons comprising 10–15% of initial culture remained intact at 65 hr and for at least 6 more days in the absence of NGF (data not shown), indicating that the culture initially contained 10–15% neurons whose survival did not depend on NGF. Scale bar in photograph (A), 50 μm.

at time 0). 20 nM actinomycin D suppressed approximately 80% of [^{3}H]uridine incorporation within 3 hr, and inhibitory effect continued throughout the experimental period. We also found that a-amanitin (13.6 nM) rescued 70–80% of NGF-deprived or puromycin-exposed neurons from apoptosis.

The above results suggest that transcription is required for apoptosis of either NGF-deprived or puromycin-exposed neurons. In order to determine when transcription is essential for both apoptoses, actinomycin D was added at various timepoints after triggering of apoptosis. Open triangles in Figure 3 indicate that addition of actinomycin D within 6 hr effectively prevented apoptosis triggered by NGF-deprivation, but thereafter, the effect rapidly diminished by addition at 18 hr only 20% neurons remained intact. Considering 10–15% NGF-independent neurons in this culture, this result indicates that addition of actinomycin D at 18 hr is almost unable to prevent neuronal apoptosis. These results strongly suggest that the transcription during 6–18 hr after NGF-deprivation is indispensable for apoptosis. Open triangles in Figure 4 represents the results obtained from the same experiment except that apoptosis was triggered by puromycin. The results suggest that transcription between 6–18 hr after puromycin-addition is crucial for neurons to be committed to apoptosis.

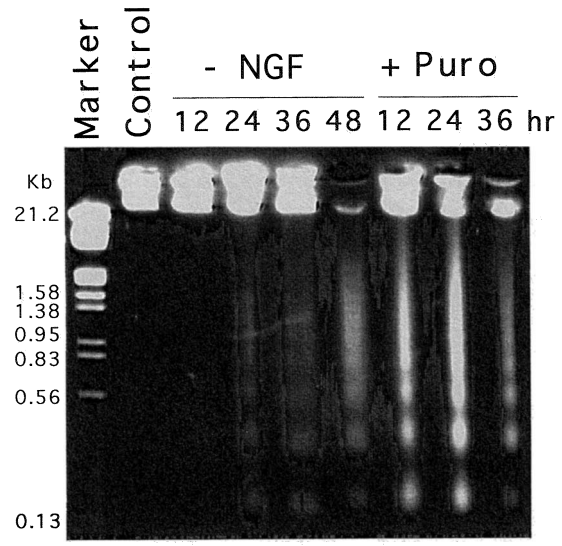

FIG. 2. Apoptotic DNA degradation after NGF-deprivation or puromycin-addition. Each 35 mm plastic dish containing 6.0–7.5×10^5 neurons was deprived of NGF or received 3 μM puromycin at time 0. At various timepoints indicated above the figure, neurons were collected and lysed with 20 μl of buffer containing 0.2% SDS. After incubation at 37°C for 3.5 hr with 0.5 mg/ml proteinase K and further incubation for 1 hr with 0.2 mg/ml pancreatic RNaseA, all the lysate was electrophoresed through a 2% agarose gel in TAE buffer and DNAs were stained with ethidium bromide. DNA size markers were Lambda phage DNAs prepared by digestion with two restriction enzymes, *Eco*RI and *Hin*dIII.

Anisomycin and Blasticidin S, but not Emetine, Prevented Apoptosis

Following to transcriptional inhibitors, the rescue effect of translational inhibitors was tested. Since translational inhibitors have complicated effects on cellular physiology, inhibitors whose action was well characterized were selected. Puromycin at the minimal dose (3 μM, see Materials and Methods) and 20 μM, puromycin did not prevent apoptosis caused by NGF-deprivation (Fig. 5C). Anisomycin and blasticidin S were effective on prevention. At the minimal dose (10 μM for anisomycin and 300 μM for blasticidin S) and 10-fold higher dose, simultaneous addition of anisomycin or blasticidin S with NGF-deprivation rescued 70–80% of neurons from apoptosis (as for anisomycin, see Fig. 3 closed triangle at time 0 and Fig. 5B). However, at the one-fifth of each minimal dose, they could not rescue the neurons at all (data not shown). Figure 3 indicates the rescue effect of anisomycin which was added at various timepoints after NGF-deprivation: the rescue effect declined when added after 6 hr, and addition at 24 hr was no longer effective at all. Similarly to NGF-deprived neurons, anisomycin and blasticidin S had a rescue-effect on puromycin-exposed neurons: 3 days after simultaneous addition of anisomycin and puromycin, approximately 65% of neurons remained intact (Fig. 5E for anisomycin). Addition of blasticidin S rescued approximately 80% of neurons from apoptosis. These results indicate that anisomycin and blasticidin S apparently antagonize

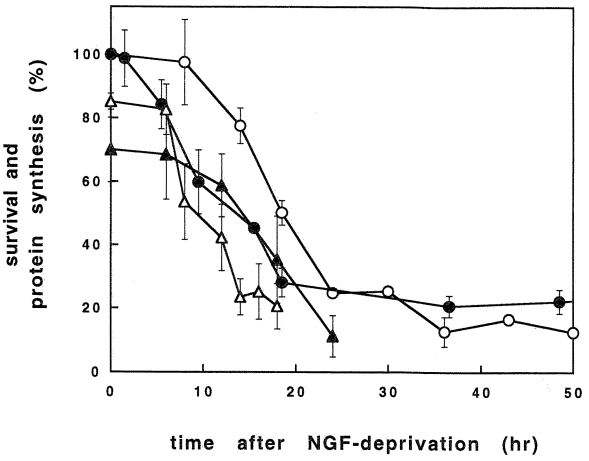

FIG. 3. Characterization of apoptosis after NGF-deprivation. Each 96 half-area well containing 2.5-3.0×10^3 neurons was deprived of NGF at time 0. To examine the activity of protein synthesis (●), each well received 185 KBq of [^{35}S]Met/Cys (41.7 MBq/mM, 70% of L-methionine and 30% of L-cystein, ARC Radiochemicals) for 20 min. After removing the medium, cells were washed twice with ice-cold HBSS (−) and lysed with a solution of 10 mM EDTA, 0.2% SDS. The radioactivity incorporated into the TCA-insoluble fraction was measured as described previously [16] and was corrected by subtracting the radioactivity obtained from cell-free wells. To examine the time course of commitment to apoptosis (○) NGF was readded at the timepoints indicated in abscissa, and to assess rescue effects of actinomycin D and anisomycin either 20 nM actinomycin D (△) or 10 μM anisomycin (▲) was added at the timepoints indicated in abscissa. The number of intact neurons at day 3 was counted and expressed as the percentage of that for control cultures which received 25 ng/ml NGF instead of anti-NGF antibody. Results represent the mean±SD of 2–3 separate experiments each done in duplicate.

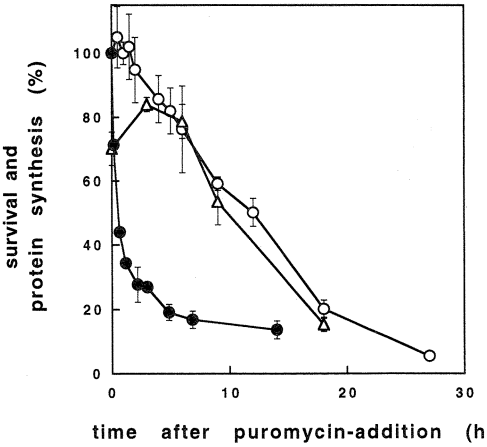

FIG. 4. Characterization of puromycin-induced apoptosis. Culture received 3 mM puromycin in the presence of NGF at time 0. The time course of commitment to apoptosis (○) was investigated as in Fig. 3 except that puromycin was removed by exchanging medium for puromycin-free medium at each timepoints. The incorporation rate of [^{35}S]Met/Cys (●) and the rescue-effect of actinomycin D (△) were measured as in Fig. 3. All the data were expressed as the percentage to that obtained for a control culture which did not receive puromycin. Results represent the mean±SD of 2–3 separate experiments each done in duplicate.

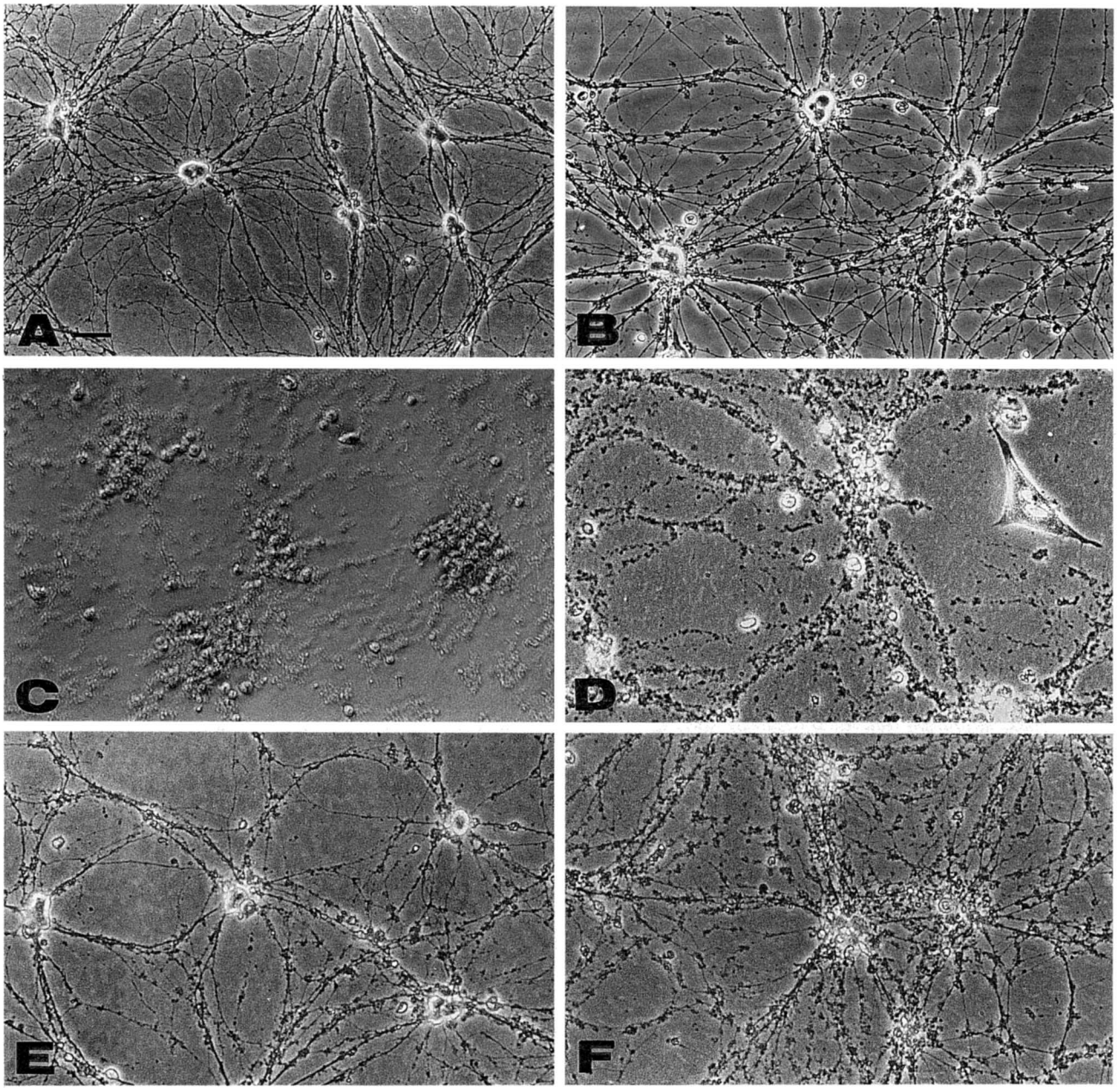

FIG. 5. Phase-contrast micrographs of sympathetic neurons treated with actinomycin D, anisomycin or puromycin in the presence or absence of NGF for 3 days. (A) 20 nM actinomycin D, (B) 10 μM anisomycin or (C) 3 μM puromycin was added simultaneously with NGF-deprivation. In the presence of 25 ng/ml NGF, (D) puromycin or (E) both puromycin and anisomycin was added. The culture which was solely deprived of NGF is also presented in photograph (F). It should be noted that a fibroblast remains intact in photograph (D). Scale bar in photograph (A), 50 μm.

the effect of NGF-deprivation or puromycin on induction of apoptosis.

By contrast, emetine did not prevent apoptosis. When emetine was added at the minimal dose (0.1 μM) or 10-fold higher dose simultaneously with either NGF-deprivation or puromycin-addition, only less than 10% neurons remained intact (data not shown). These results are summarized in Table 1.

Change of phosphorylated proteins after NGF-deprivation and Puromycin-addition

NGF binds to two cell surface receptors and its action is mediated by tyrosine kinase activity associated with one of the receptors [15]. Since this signal transduction would be mediated by serial phosphorylation/dephosphorylation of proteins [15], we addressed phosphorylated proteins before and after NGF-deprivation (Fig. 6, lanes 1–4). Among many phosphorylated proteins detected in a soluble fraction, three phosphorylated proteins (marked with a closed arrow-

TABLE 1. Rescue effects of translational inhibitors on apoptosis

	impaired process in translation	rescue of apoptosis triggered by	
		−NGF	+puromycin
anisomycin	peptidyltransfer	+	+
blasticidin S	peptidyltransfer	+	+
emetine	translocation	−	−
puromycin	retention of growting polypeptide in A site	−	／

Each inhibitor was added to the culture of chick sympathetic neurons with either NGF-deprivation or puromycin-addition. The fraction of neurons rescued from apoptosis was measured after 3 days. +, >60% rescued; −, <10% rescued. See detail in text.

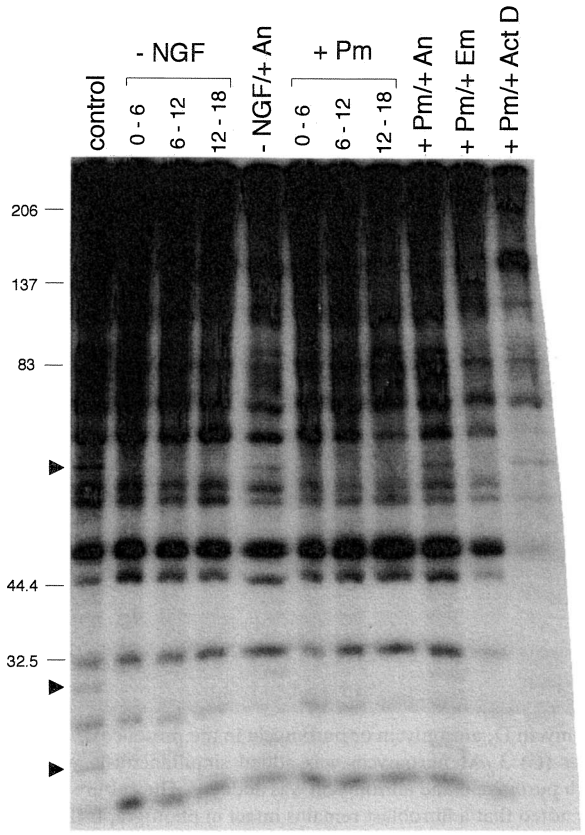

FIG. 6. Change of phorphorylated proteins. Proteins were labeled with [^{32}P]orthophosphate; lane 1, for 6 hr in the presence of NGF; lanes 2–4, during 0–6 hr, 6–12 hr and 12–18 hr after NGF-deprivation, respectively; lane 5, during 12–19 hr after NGF-deprivation in the presence of 20 μM anisomycin; lane 6–8, during 0–6 hr, 6–12 hr and 12–18 hr after addition of 3 μM puromycin, respectively; lane 9, during 12–19 hr after addition of 3 μM puromycin in the presence of 50 μM anisomycin; lane 10, during12–19 hr after addition of 3 μM puromycin in the presence of 0.1 μM emetine; lane 11, during 12–19 hr after addition of 3 μM puromycin in the presence of 30 μM actinomycin D. Phosphorylated proteins were analysed as described in Materials and Methods. Numbers marked in the left margin are molecular weights (Kd) deduced from Kaleidoscope prestained proteins (Bio-Rad).

head) disappeared after NGF-deprivation, indicating that they underwent dephosphorylation or were eliminated.

The effect of puromycin on phosphorylation was examined whether puromycin mimicked NGF-deprivation. Lanes 6–8 show that puromycin affected the same three proteins in the same manner as NGF-deprivation did. Thus, the similarity of NGF-deprivation and puromycin in their effects on neurons further extended. Besides, we found that actinomycin D and anisomycin suppressed the disapearance of these phosphorylated proteins (lanes 5, 10, 11), but emetine did not (lane 10). Thus, the effects of these inhibitors on the three phosphorylated proteins paralleled their effects on suppression of apoptosis, implying that these proteins are crucial for apoptosis.

DISCUSSION

In this study, we found that puromycin as well as NGF-deprivation induced apoptosis in developing chick sympathetic neurons. The results with transcriptional inhibitors (Figs. 3 and 4) strongly suggest that induction of apoptosis depends on the expression of certain genes. Several investigators have repeatedly reported that translational inhibitors elicit expression of specific genes in a variety of cells from bacteria to mammals, and that the effect is specific to an individual inhibitor [1, 3, 5, 9, 14]. Among translational inhibitors we tested, only puromycin appears to induce expression of genes specific to apoptosis in neurons. Since low but significant level of translational activity remained after puromycin-addition under the conditions in this study, the activity would be crucial for the expression of such genes. If this is the case, then puromycin may have opposite effects on apoptosis. One is induction of apoptosis as discussed, and the other is suppression; the latter would be discernible when a dose of puromycin is high enough to block cellular translation completely. In this context, the previous report describing suppression of apoptosis of rat sympathetic neurons by puromycin unfortunately did not mention the dose employed in the experiment [8].

In the range of concentrations (0–4 μM) of puromycin, induction of apoptosis correlated to suppression of protein synthesis (data not shown). Although the possibility that apoptosis is induced by unknown effect of puromycin is not excluded, this correlation strongly suggests that induction of apoptosis is a consequence of puromycin-effect on translation. Molecular actions of translational inhibitors used in this study are well characterized [6, 13]. Polypeptide chain elongation is carried out by following two elemental reactions: one is peptidyltransfer by which polypeptide is uncoupled from the tRNA in P-site and joined by a peptide bond to the aminoacyl tRNA in A-site. The other is translocation by which the new peptidyl-tRNA in A-site is translocated to P-site. Puromycin, an analogue of aminoacyl-tRNA, acts as a recipient of peptidyltransfer to form peptidyl-puromycin. The peptidyl-puromycin is immediately released from a ribosome and the ribosome dissociates into subunits (puromycin-

effect). At the doses used in this study, anisomycin and blasticidin S impair the peptidyltransfer and can suppress the puromycin-effect. On the other hand, emetine has an inhibitory effect on the translocation of peptidyl-tRNA and can not suppress the puromycin-effect. Thus, there is a strict correlation between the suppression of puromycin-effect and the suppression of apoptosis caused by puromycin. This correlation prompts us to the idea that the puromycin-effect is essential for the induction of genes required for the apoptosis of chick sympathetic neurons.

The peptidyl-puromycin is potent to compete with the natural substrates in protein degradation mediated by ubiquitin [12], because the compound is rapidly degraded through the ubquitin-dependent pathway [2]. As a consequence, the distortion of cell activities controlled by this pathway might lead to induction of apoptosis-specific genes. Alternatively, the accumulation of dissociated ribosomes would produce a signal to induce the expression of such genes.

The mechanism by which apoptosis is induced after NGF-deprivation is open to be elucidated, because signal transduction pathway triggered by NGF are poorly understood. In the present study, we have found the extensive similarities between the effects of NGF-deprivation and puromycin on neurons. Both apoptoses accompany decrease of translational activity preceding commitment to death (Figs. 3 and 4). The apoptoses induced by these causes require transcription and are suppressible by inhibitors of peptidyltransfer reaction (Table 1). In addition, NGF-deprivation and puromycin induce the same change of phosphorylated proteins (Fig. 6). These facts strongly suggest that puromycin mimics the effects of NGF-deprivation on neuron, activating at least partially the same cascade which leads to neuronal apoptosis.

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