J. Biochem. 121, 122-127 (1997)

Induction of Bip mRNA upon Programmed Cell Death of Differentiated PC12 Cells as Well as Rat Sympathetic Neurons[1]

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Received for publication, September 5, 1996

We have found that expression of the Bip (immunoglobulin heavy chain binding protein)/GRP78 (glucose regulated protein 78) gene is markedly enhanced specifically among the heat shock protein (HSP) 70 gene family during the neuronal cell death of PC12 (22a) cells, that is induced by removal of nerve growth factor (NGF) and blocked by a transcription inhibitor, actinomycin D. The Bip mRNA induction is suppressed when the NGF-deprivation-dependent cell death of PC12 (22a) cells is inhibited by cAMP, cycloheximide or high K^+. The Ca^{2+} ionophore, A23187, caused neuronal cell death accompanied by up-regulation of Bip, HSP90, and HSP70 mRNAs. In addition, a chelator of intracellular Ca^{2+} (BAPTA) elevated Bip mRNA and induced cell death in a low Ca^{2+} medium. Alterations of intracellular calcium homeostasis thus appear to induce Bip mRNA expression as well as apoptosis in PC12 (22a) cells. However, release of Ca^{2+} from intracellular stores by thapsigargin induced Bip mRNA expression but not cell death, indicating that Bip mRNA induction is not sufficient for neuronal death. Induction of Bip mRNA in association with apoptosis was also observed for NGF-deprived sympathetic ganglion cells in primary culture. These lines of evidence suggest that selective induction of Bip mRNA may play an important role in the programmed cell death of neurons deprived of neurotrophic factors and could be a landmark of the neuronal programmed cell death.

Key words: apoptosis, Bip, calcium homeostasis, heat shock protein, PC12.

Programmed cell death (PCD) is a highly regulated process that is essential to the development of the nervous system. Approximately half of the neurons generated in many neuronal populations die before or shortly after birth, especially during the period when synapses are being formed between neurons and their targets. Neurons are thought to compete for sufficient amounts of trophic factors provided by target tissues. Neurons that are less successful as to connection with target tissues receive insufficient quantities of trophic factors and thus die (*1, 2*). A typical example of neuronal PCD takes place in the rat superior cervical ganglia in the perinatal period, during which approximately 40% of the neurons die. Neurons isolated from fetal rats and maintained with nerve growth factor (NGF) were induced to die by removal of NGF through a process that exhibits the hallmarks of apoptosis (*3*).

Neuronal PCD *in vitro* and *in vivo* was shown to be suppressed by inhibitors of RNA or protein synthesis, leading to the hypothesis that PCD depends on the activation of a genetic program that induces the synthesis of cell death proteins or genes (*3*). However, no such gene products have been identified. This is partly because only small amounts of mRNAs are available from sympathetic neurons deprived of NGF *in vitro*, which have been considered to be the best model system for neuronal PCD. It is, therefore, important to use an *in vitro* neuronal cell death system that consists of a homogeneous neuronal population available in large quantities.

In the present paper, we report studies to identify transcriptionally activated genes upon PCD using a subline of PC12 cells, PC12 (22a), whose death can be induced by NGF removal and blocked by actinomycin D. We found that expression of the Bip (immunoglobulin heavy chain binding protein)/GRP78 (glucose regulated protein 78) gene is associated with neuronal PCD induced by not only NGF deprivation but also modulation of the intracellular calcium concentration.

MATERIALS AND METHODS

Materials—Mouse NGF (2.5S) was isolated from male mouse submaxillary glands (*7*). Antiserum against mouse 2.5S NGF was kindly donated by E.M. Johnson, Jr. (Washington Univ., St. Louis). The reagents used for the inhibition of apoptosis were actinomycin D-mannitol (Sigma Chemical, St Louis, MA), cycloheximide (Sigma), and 8-(4-chlorophenylthio) cyclic AMP (CPT-cAMP) (Boehringer Mannheim, Tokyo). 1,2-Bis (2-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) tetrakis (acetoxymethyl)ester was purchased from Dojin Laboratories (Kumamoto). The probes for Northern blot analysis were

[1] This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan.

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described previously (*4*): human HSP70 probe (the *Bam*HI-*Hin*dIII fragment of pH 2.3), mouse HSP25 probe (the *Xho*I-*Eco*RI fragment of pUC19Hx/B-E), human HSP90 probe (the *Pst*I-*Bam*HI fragment of PMH47a), and mouse β-actin (the *Eco*RI-*Eco*RI fragment of pMAβ-3′ut), as an internal control. Murine GRP94 probe is the *Bam*HI-*Eco*RI fragment from pGEM99.2 (*5*).

Cell Culture—PC12 (22a) cells were grown at 36.5°C on collagen-coated plates in RPMI 1640 medium containing 5% fetal bovine serum, and in 10% heat-inactivated horse serum (JRH Biosci, Lexena, KS) as described (*6*). Cells that had been previously treated with NGF for 2 weeks were deprived of NGF by adding goat antiserum against mouse NGF (1%) (*6*). Dissociated sympathetic neurons were prepared from superior cervical ganglia of newborn Wistar rats (*7*). These cells were typically plated on collagen-coated dishes (Costar Data Packaging, Cambridge, MA). Cultures were grown for 7 days in Eagle's minimal essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 20 μM fluorodeoxyuridine, and 20 μM uridine to kill non-neuronal cells, and 50 ng/ml 2.5S NGF. To ensure rapid and complete NGF deprivation, polyclonal antiserum against NGF was added to the culture medium at a final concentration of 1% (*7*). High-K^+ media were prepared by increasing the KCl concentration from the normal value of 5.4 mM to 25 and 45 mM with the omission of the corresponding concentration of NaCl to maintain the osmolarity (*7*).

Evaluation of Neuronal Survival after NGF Deprivation—The amount of a cytoplasmic enzyme, lactate dehydrogenase (LDH), released into the medium was spectrophotometrically assayed using NADH as a substrate (*6*). The total enzymic activity was defined as the sum of the activity in the medium and that in cell lysates prepared with 0.1% Triton in 0.1 M HEPES-NaOH buffer, pH 7.0. The enzyme leakage was represented as the percentage of the activity in the medium, taking the total enzymic activity as 100%. Normally, 3-8% of the total activity was released into the medium in cultures of healthy cells; this value increased to 20-50% upon cell degeneration caused by NGF deprivation, depending on when the cells were harvested for the assay (*6*).

RNA Isolation and Analysis—Total cellular RNA was isolated from cultured cells by standard methods (*8*), and poly(A)$^+$ RNA was selected by oligotex (Takara, Kyoto). Approximately 20 μg of total RNA samples were used for standard Northern blot analysis (*8*).

Construction of a cDNA Library—Total cellular RNA was prepared from PC12 (22a) cells previously treated with NGF for 2 weeks and cultured for 16 h without NGF by adding antiserum against NGF. Five micrograms of poly(A)$^+$ RNA was used to synthesize cDNA primed with an oligo (dT)-adapter primer using a cDNA synthesis kit (Pharmacia). The cDNA was ligated into the *Eco*RI site of λgt10. This library contained 10^6 independent clones carrying inserts of more than 1.5 kb on average, with a range of 0.7-5.5 kb.

Differential Screening of the cDNA Library—The library was plated at a density of $\sim 3 \times 10^3$ PFU per 30×30 cm^2 plate. A total ~12,000 clones were screened. Two nitrocellulose filter replicas were made from each plate. ^{32}P-labeled cDNA probes derived from mRNA were prepared from 5 μg of poly(A)$^+$RNA using random hexamers and Superscript II reverse transcriptase, followed by removal of the RNA template by alkali hydrolysis using the standard protocol (*8*). The first set of filters was hybridized with the cDNA probes from healthy PC12 (22a) cells treated with NGF for 2 weeks. The second set was hybridized with the cDNA probes from dying PC12 (22a) cells, that had been previously treated with NGF for 2 weeks, followed by deprivation of NGF by adding antiserum against NGF. Hybridization with the probes was carried out by standard methods (*8*). Plaques exhibiting different hybridization signal intensities between the two probes were picked up. These clones were prescreened and plaque-purified by two subsequent differential screenings. Six independent clones were picked up and the cDNA inserts were subcloned into Bluescript SK(−) plasmid vectors (Stratagene). These plasmid DNA templates were analysed using an ABI 373 DNA sequencer (Applied Biosystems, ABI). The partial sequences were compared with nucleic acid sequences in GenBank.

RESULTS

Characterization of PCD of a Neuronally Differentiated PC12 Subline by NGF Derivation—PC12 (22a) cells differentiated on culturing in the presence of NGF for 2 weeks. Such treatment gave rise to cultures comprising flattened, attached, phase-bright cells with an extensive network of robust neurites (*6*). Subsequent withdrawal of NGF by adding antiserum against NGF to the medium led to cell death within 48-72 h. Morphological observation revealed that these differentiated PC12 (22a) cells underwent massive degeneration characterized by the disappearance of growth cones, neurite dilation, phase-dark cellular atrophy, and abundant floating debris (*6*). Their cell death was characterized by apoptotic changes including DNA fragmentation, shrinkage of the cells with preservation of the organelles, and blebbing in the cytoplasm (data not shown).

To determine whether RNA synthesis is required for the apoptotic cell death of differentiated PC12 (22a) cells, we treated the cells with actinomycin D. Actinomycin D almost completely blocked the apoptotic cell death, that was monitored as the LDH release (Fig. 1A). The disappearance of growth cones, and dilatation and disintegration of neurites occurred rapidly without RNA synthesis (data not shown), and these changes were prevented by treating the cells with cysteine protease inhibitors. Actinomycin D prevented PCD that typically follows withdrawal of neurotrophic factors but not the deterioration of the neurites, suggesting the presence of both transcription-dependent and -independent pathways for the degeneration of PC12 (22a) cells.

To determine when PC12 (22a) cells become committed to die, actinomycin D was added at various times after NGF deprivation. The released LDH in each culture was measured at 45 h after NGF deprivation (Fig. 1B). The data show that about half of the dead cells at the plateau level are committed to die around 12 h after the removal of NGF because actinomycin D could no longer prevent their death.

Selective Induction of mRNA for Bip upon PCD of PC12 (22a) Cells—As actinomycin D blocked the apoptotic cell death of NGF-deprived PC12 (22a) cells, we assumed that mRNAs essential to PCD might be induced during PCD in

this system. The strategy used to isolate cDNA clones for such mRNAs was differential screening, as described under "MATERIALS AND METHODS." Six independent clones were picked up, and three of the six clones were shown to be induced during PCD in this system by Northern blotting (data not shown). The partial nucleotide sequences of these three cDNA clones showed that they were derived from the same gene encoding Bip, a member of the heat shock protein (HSP) 70 family, although the other three clones proved to be artifacts.

Bip mRNA is induced during PCD in PC12 (22a) cells (Fig. 2). Glucose-regulated-protein 94 (GRP94) and Bip/GRP78 are predominantly localized in the endoplasmic reticulum, and both are induced by similar treatments, such as glucose starvation and calcium ionophore treatment (*9, 10*). Therefore, we examined GRP94 mRNA expression in PCD and found that this gene was also induced. Although mRNAs of all HSP family members examined are induced during the ischemic change in rat brain (*4*), none of them, except for Bip and GRP94, changed over the 36 h time course of NGF deprivation (Fig. 2). Expression of HSP45 mRNA was not detected in PC12 cells (data not shown). In control experiments, expression of β-actin mRNA gradually decreased, whereas the amounts of ribosomal 28S and 18S RNA remained almost constant. We also examined the expression patterns of several PCD-related genes; the mRNA levels of the Ich1/nedd-2 and trkA genes did not change during PCD of PC12 (22a) cells, whereas expression of p75NGFR mRNA decreased between 8 and 16 h after NGF deprivation, and then increased after 20 h.

Association of Bip mRNA Induction with PCD of PC12 (22a) Cells—To determine whether Bip mRNA induction is coupled with the signal transduction pathway of PCD, we

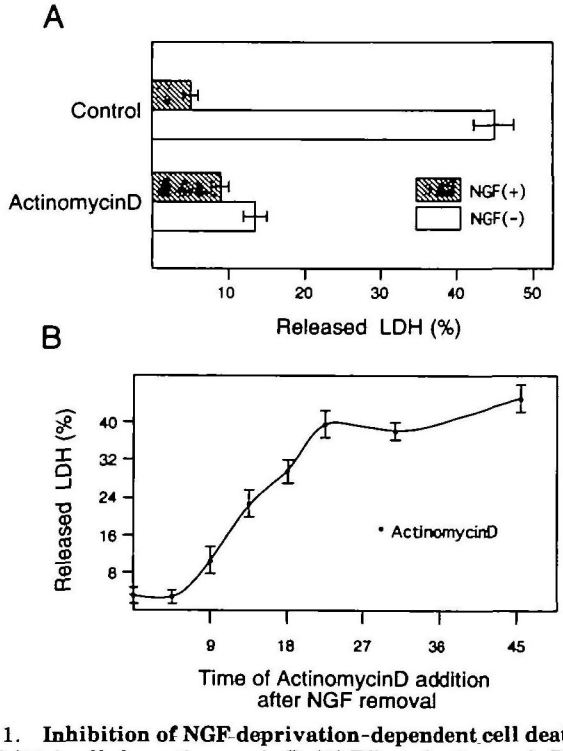

Fig. 1. **Inhibition of NGF-deprivation-dependent cell death of PC12 (22a) cells by actinomycin D.** (A) Effect of actinomycin D (0.3 μM) on neuronal death caused by NGF removal. Data were taken at 48 h after the addition of antiserum against NGF (1.0%), and represent means (±SE) for three different experiments performed in triplicates. Actinomycin D was added at 0 h after the removal of NGF (50 ng/ml). (B) Death commitment time of PC12 (22a) cells on NGF removal. Differentiated PC12 (22a) cells were deprived of NGF for 45 h in total, and then subjected to assaying for LDH. Actinomycin D (0.3 μM) was added at 0, 6, 9, 15, 18, 24, 32, and 45 h after the removal of NGF. Released LDH was determined at 45 h after the removal of NGF (see "MATERIALS AND METHODS"). Data are means±SE (n=3).

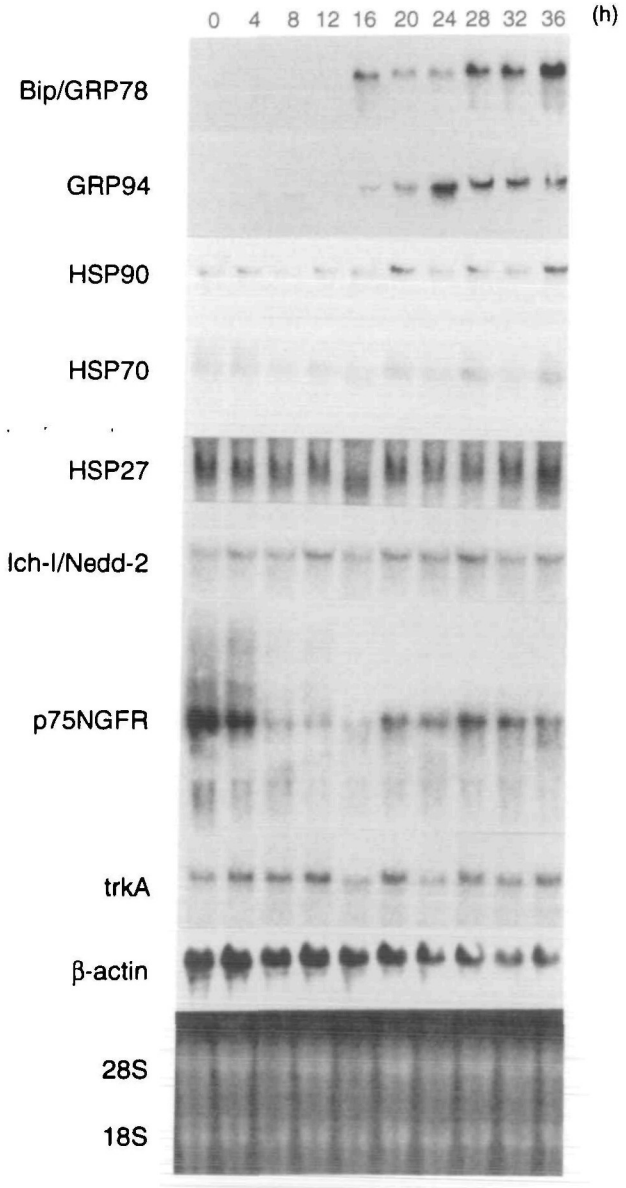

Fig. 2. **Induced expression of mRNAs of HSP family genes in differentiated PC12 (22a) cells deprived of NGF.** To evaluate changes in mRNA levels, PC12 (22a) cells were maintained with NGF (50 ng/ml) for 14 days and then deprived of NGF for the indicated times. Twenty micrograms of total RNA was loaded onto each lane of the gel. After hybridization with the Bip cDNA probe, the filter was rehybridized with the HSP27, 70, 90, GRP94, and β-actin cDNA probes, successively. Another filter obtained by the same method was hybridized with the Ich-1/nedd-2, p75NGFR, and trkA cDNA probes, successively. 28S and 18S rRNA were stained with ethidium bromide.

examined the expression levels of Bip mRNA when the NGF-deprivation dependent PCD of differentiated PC12 cells was blocked by various treatments (Fig. 3). The cAMP treatment inhibited both the expression of Bip mRNA and PCD almost completely at 24 and 48 h after the NGF deprivation. The cycloheximide treatment prevented PCD almost completely and strongly suppressed the level of induction of Bip mRNA. Under depolarizing conditions with elevated K^+ (25 or 45 mM), PCD of PC12 (22a) cells following NGF deprivation was significantly inhibited (Fig. 4A). Under the same conditions the induction of Bip mRNA was almost completely suppressed, while the expression of HSP90 and HSP70 was not affected. These data suggest that Bip mRNA expression may be associated with the PCD signaling pathway in differentiated PC12 (22a) cells.

To determine the effects of the calcium concentration on Bip mRNA expression as well as cell death signaling, we examined the expression level of Bip mRNA and the cell death of PC12 (22a) cells treated with a calcium ionophore (A23187), thapsigargin or BAPTA. Bip mRNA together with HSP70 and HSP90 mRNAs were all elevated on treatment with 10 μM A23187 for 4 h in association with PCD (Fig. 4B), suggesting that the increased intracellular Ca^{2+} level induces non-selective Hsp protein synthesis as well as PCD. Bip mRNA was specifically elevated in differentiated PC12 (22a) cells treated with 100 μM BAPTA but not with 50 μM BAPTA, that decreases the intracellular Ca^{2+} level (Fig. 4A). BAPTA acetoxymethyl ester is membrane-permeant and enters the cell, where it is hydrolyzed to the parent acid which acts as a chelator of intracellular Ca^{2+} (7). Bip mRNA is up-regulated in differentiated PC12 (22a) cells treated with 1.0 μM thapsigargin for 24 h in the presence of NGF without up-regulation of HSP90 and HSP70 mRNAs, although depletion of Ca^{2+} from thapsigargin-sensitive stores does not cause cell death. These results indicate that Bip mRNA up-regulation is associated with but not sufficient for PCD of PC12 (22a) cells.

Selective Induction of Bip mRNA upon PCD of Sympa-

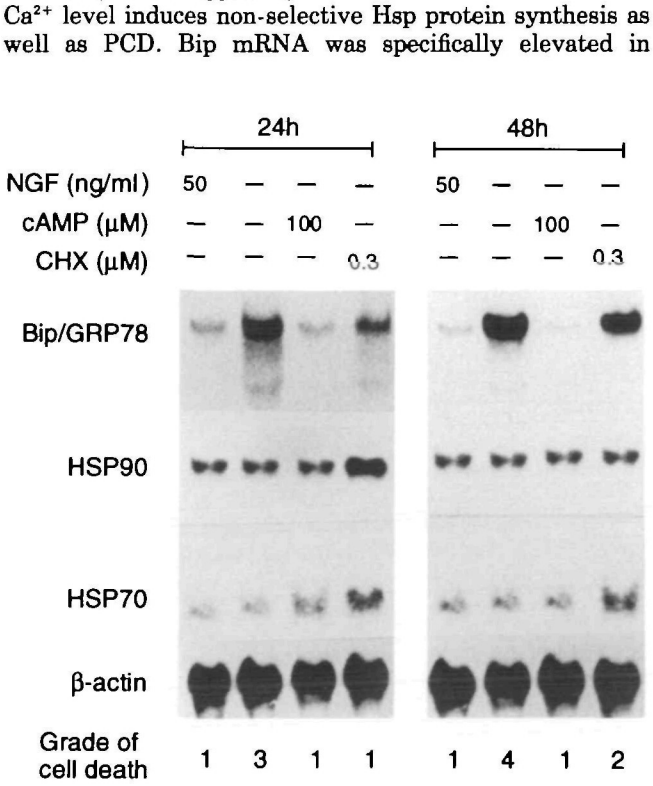

Fig. 3. **Association of Bip mRNA induction with PCD of differentiated PC12 (22a) cells.** Differentiated PC12 (22a) cells in the presence of 50 ng/ml NGF for 2 weeks were deprived of NGF and then supplemented with either 100 μM CPT-cAMP or 0.3 μM cycloheximide. Twenty micrograms of total RNA isolated was loaded on the gel. After hybridization with the Bip cDNA probe, the filter was rehybridized with the HSP70, 90, and β-actin cDNA probes, successively, for 24 or 48 h. The grade of cell death was determined morphologically by light microscopy: 1, no morphological change; 2, ~20% of differentiated PC12 (22a) cells have thinned neurites; 3, 50-70% of the cells have thinned and beaded neurites, and 10-20% of the cell bodies are beginning to change in shape; 4, ~90% of the cells have lost their neurites and some cells are beginning to float into the medium.

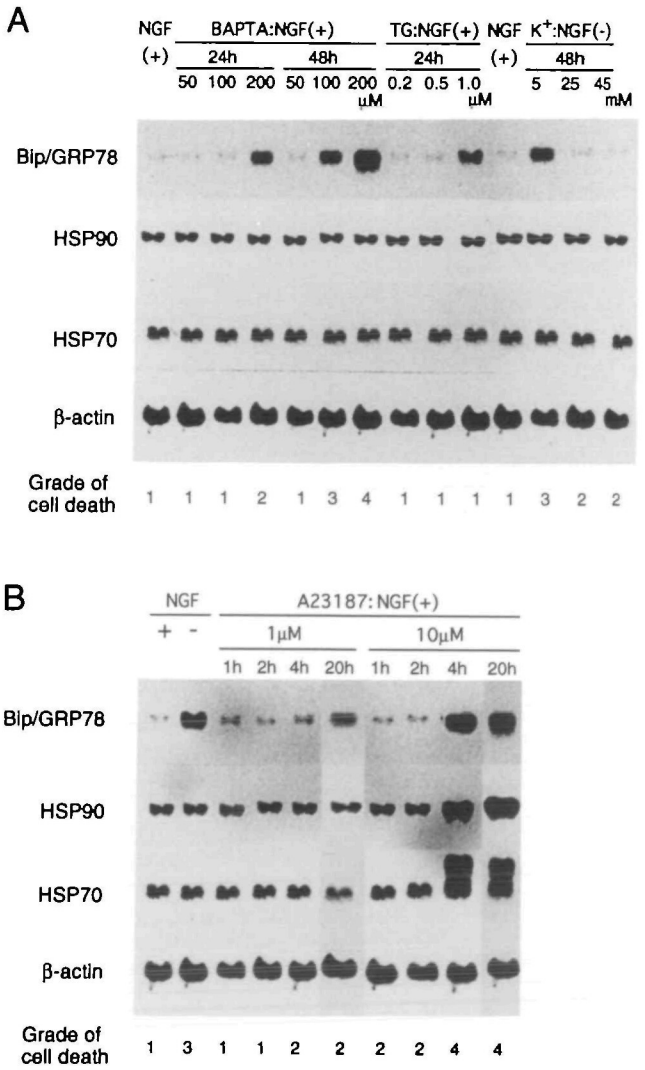

Fig. 4. **Expression levels of Bip mRNA in differentiated PC12 (22a) cells in association of PCD induced by various stimulate.** Differentiated PC12 (22a) cells were previously exposed to 50 ng/ml NGF for 2 weeks, and then BAPTA, Ca^{2+} ionophore (A23187), thapsigargin (TG), or K^+ was added to the culture medium in the presence or absence of NGF, as indicated. Twenty micrograms of total RNA isolated from differentiated PC12 (22a) cells after various treatments was loaded onto each lane of the gel. After hybridization with the Bip cDNA probes, the filter was rehybridized with the HSP 70, 90, and β-actin cDNA probes, successively. The grade of cell death was determined (see legend to Fig. 3).

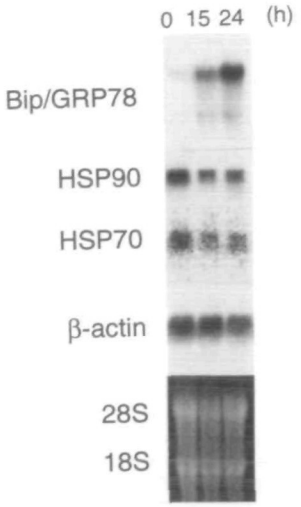

Fig. 5. **Selective induction of Bip mRNA during PCD of sympathetic neurons deprived of NGF.** Total RNA was isolated from sympathetic neurons grown for one week *in vitro* with NGF (50 ng/ml) and then deprived of NGF for 15 or 24 h. Ten micrograms of total RNA was loaded onto each lane of the gel. After hybridization with the Bip cDNA probe, the filter was rehybridized with the HSP70, 90, and β-actin cDNA probes, successively. 28S and 18S rRNA were stained with ethidium bromide.

thetic Neurons—To determine whether selective induction of Bip mRNA also occurs in other neural PCD systems, we examined the expression pattern of HSP mRNAs in sympathetic neurons in primary culture at various times after NGF deprivation. Bip mRNA was markedly induced during PCD of sympathetic neurons deprived of NGF *in vitro*, whereas the mRNA levels of HSP90 and HSP70 did not change (Fig. 5).

DISCUSSION

NGF-deprived sympathetic neurons cultured *in vitro* provide the best characterized system for neural PCD that requires *de novo* RNA synthesis (*3*). However, this system is limited for the identification of molecules involved in neuronal PCD because only a small amount of mRNAs can be obtained. We have confirmed that PCD of a subline of PC12 cells, PC12 (22a), is blocked by actinomycin D (Fig. 1A). The time at which 50% of NGF-deprived PC12 (22a) cells are committed to die was within 12-16 h after NGF deprivation (Fig. 1B), in agreement with that in the case of rat sympathetic neurons cultured *in vitro* (15-18 h) (*3, 11-14*).

Using differential screening, we found that expression of the Bip gene is induced selectively among Hsp genes in differentiated PC12 (22a) cells when NGF was depleted. Bip shows about 60% amino acid sequence identity with HSP70 (*15*). Members of the HSP70 family have been implicated involved in the translocation of secretory and mitochondrial precursor polypeptides (*16*). In mammalian cells, however, the Bip promoter is devoid of the heat shock element, and Bip is induced by a calcium ionophore (A23187), tunicamycin, glucose starvation, and the accumulation of abnormal proteins in the endoplasmic reticulum (*17-19*). Interestingly, expression of all the other HSP family members examined (HSP90, HSP70, and HSP27) did not change over the 36 h time course of NGF deprivation (Fig. 2), in contrast to the previous report that mRNAs of all HSP family members examined are induced during an ischemic change in rat brain (*4*). Moreover, Bip mRNA is also induced selectively during PCD of cultured sympathetic neurons deprived of NGF, whereas the mRNA levels of HSP90 and 70 did not change (Fig. 5). Similarly, Bip mRNA is induced on postnatal PCD of rat retinal ganglion cells, the growth of which is dependent on brain-derived neurotrophic factors (data not shown). Treatment with cAMP, cyclohexamide, or high K^+ inhibited both the Bip induction and the neuronal PCD (Figs. 3 and 4, and Table I). These observations indicate a close association between Bip mRNA induction and neuronal PCD.

TABLE I. **Effects of various reagents on PCD, and expression levels of Bip and HSP70 mRNAs in differentiated PC12 (22a) cells.**

	Bip mRNA	HSP70 mRNA	PCD	Cytoplasmic Ca^{2+}
NGF deprivation	I	U	I	↓
BAPTA	I	U	I	↓
cAMP	B	U	B	?
Cycloheximide	B	U	B	
High K^+	B	U	B	↑
A23187	I	I	I	↑
Thapsigargin	I	U	N	↑

I, induced; B, induction blocked; U, unaltered; N, not-induced.

Bip mRNA induction may be due to the change in intracellular calcium homeostasis that is associated with PCD. Our preliminary data obtained on fura-2 fluorography suggest that the basal intracellular Ca^{2+} concentration was decreased after the PCD commitment time (12-16 h after NGF deprivation). The induction of Bip mRNA by a Ca^{2+} chelator, BAPTA, and its suppression by high K^+ may be related to Ca^{2+} concentration changes. The prevention of PCD of sympathetic neurons and PC12 cells *in vitro* by high K^+ has been reported to be due to a sustained increase in the cytoplasmic free Ca^{2+} concentration caused by the influx of Ca^{2+} through voltage-gated channels that are activated on K^+ induced chronic depolarization (*11, 20, 21*). Low levels of Bip/GRP78 and GRP94 mRNA induction were reported to be triggered in cells cultured in low Ca^{2+} medium buffered with a Ca^{2+} chelator (*22*). We postulate that reduction of the intracellular Ca^{2+} concentration is associated with the PCD commitment, followed by an increased intracellular Ca^{2+} concentration due to a change in membrane permeability or destruction of the cell membrane. We speculate that BAPTA treatment can mimic the commitment stage and Ca^{2+} ionophore A23187 can induce PCD through a mechanism similar to the later stage of which, in which protein dematuration triggers the expression of all Hsp proteins.

Thapsigargin, which stimulates Bip mRNA induction, could not induce neuronal PCD (Fig. 4 and Table I), indicating that Bip induction is not sufficient for neuronal PCD. Nonetheless, considering all these results together, Bip mRNA may play some important roles in PCD of neurons deprived of neurotrophic factors and could be a landmark of neuronal PCD.

We wish to thank Drs. K. Nagata and M. Green for providing the plasmids, Ms. S. Okazaki, Ms. H. Ohori, Ms. N. Tomikawa, and Ms.

M. Yamamoto for their technical assistance, and Ms. K. Fukui for her help in preparing the manuscript.

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