Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 10989–10993, December 1993
Neurobiology

Induction of apoptosis in cerebellar granule neurons by low potassium: Inhibition of death by insulin-like growth factor I and cAMP

(programmed cell death/trophic factor/cell survival/model system)

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Communicated by Rita Levi-Montalcini, July 8, 1993

ABSTRACT **High levels of extracellular K^+ ensure proper development and prolong survival of cerebellar granule neurons in culture. We find that when switched from a culture medium containing high K^+ (25 mM) to one containing a low but more physiological K^+ concentration (5 mM), differentiated granule neurons degenerate and die. Death induced by low K^+ is due to apoptosis (programmed cell death), a form of cell death observed extensively in the developing nervous system and believed to be necessary for proper neurogenesis. The death process is accompanied by cleavage of genomic DNA into internucleosome-sized fragments, a hallmark of apoptosis. Inhibitors of transcription and translation suppress apoptosis induced by low K^+, suggesting the necessity for newly synthesized gene products for activation of the process. Death can be prevented by insulin-like growth factor I but not by several other growth/neurotrophic factors. cAMP but not the protein kinase C activator phorbol 12-myristate 13-acetate can also support survival in low K^+. In view of the large numbers of granule neurons that can be homogeneously cultured, our results offer the prospect of an excellent model system to study the mechanisms underlying apoptosis in the central nervous system and the suppression of this process by survival factors such as insulin-like growth factor I.**

The mammalian nervous system is critically dependent on trophic support for proper development and survival (for review, see refs. 1 and 2). Among the molecules shown to influence neuronal differentiation and survival are the neurotrophins [nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4 (NT3 and NT4)], the fibroblast growth factors (FGFs), ciliary neurotrophic factor (CNTF), insulin, and the insulin-like growth factors (IGFs) (1–5). In culture, however, neuronal survival can also be supported by a variety of agents in the absence of any neurotrophic factor. One of these agents is K^+. High levels of K^+ promote survival of several types of cultured neurons (refs. 6 and 7; for review, see ref. 8). Besides supporting neuronal survival, high K^+ has also been suggested to influence neuronal development and phenotypic characteristics (9–11). Although the ability of high K^+ to promote neuronal survival is well established, the mechanism by which it acts is unclear.

Among the various cell types known to be dependent on high K^+ for survival *in vitro* are cerebellar granule neurons (6, 12, 13). These cells constitute the most abundant neuronal population in the mammalian brain. When cultured from early postnatal rats, cerebellar granule cells differentiate, acquiring several morphological, biochemical, and electrophysiological characteristics of mature neurons (12–15). Cultured cerebellar granule neurons express excitatory amino acid receptors and upon depolarization selectively release L-glutamate and L-aspartate (15).

In this study, we have attempted to examine the mechanisms by which survival of mature cerebellar granule neurons is promoted and cell death is prevented by high K^+. Initially we investigated the effect of a lower but physiological level of extracellular K^+ on neuronal survival. We found that lowering the K^+ concentration induces apoptosis (a form of programmed cell death) in these neurons. The death process is dependent on new RNA and protein synthesis. IGF-I and cAMP can protect these neurons from apoptosis in low K^+. It is known that IGF-I is synthesized and secreted by cerebellar Purkinje cells (16, 17). Further, the IGF-I receptor is present in granule neurons (17–19). IGF-I may therefore serve as a survival factor for these neurons *in vivo*.

Apoptosis occurs in cultured sympathetic neurons of the rat superior cervical ganglion after NGF deprivation (20, 21). Our results suggest an alternative cell culture model system, using primary neurons from the central nervous system. The relatively large numbers of cerebellar granule neurons that can be homogeneously cultured (10–15 $\times$ 10^6 per rat), as well as the finding that lowering of extracellular K^+ induces apoptosis, render this an excellent system to investigate the biochemical and molecular mechanisms underlying apoptosis in the central nervous system. Moreover, the ability of IGF-I and cAMP to support survival of these neurons permits studies of the mechanisms involved in the suppression of this process by survival factors.

MATERIALS AND METHODS

Primary Neuron Cultures. Cultures enriched in granule neurons were obtained from dissociated cerebella of 8-day-old Wistar rats (Charles River Breeding Laboratories) (13). Cells were plated in basal medium Eagle (BME; GIBCO) supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine (GIBCO), and gentamicin (100 μg/ml; GIBCO) on dishes (Nunc) coated with poly(L-lysine). Cells were plated at 3 $\times$ 10^5 per cm^2 (2.5 $\times$ 10^6 cells per 35-mm dish or 7 $\times$ 10^6 per 60-mm dish). 1-β-D-Arabinofuranosylcytosine (10 μM) was added to the culture medium 18–22 hr after plating to prevent replication of nonneuronal cells. Immunocytochemical analysis of such primary cultures has shown that they contain >95% granule neurons (12).

Treatment of Cultures. Culture medium was replaced with serum-free medium 6–7 days after plating. Cells were washed

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Abbreviations: BDNF, brain-derived neurotrophic factor; FGF, fibroblast growth factor; aFGF, acidic FGF; bFGF, basic FGF; IGF, insulin-like growth factor; LDH, lactate dehydrogenase; NGF, nerve growth factor; NT-3, neurotrophin 3; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate.

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twice and maintained in serum-free BME (normally containing 5 mM KCl) supplemented with glutamine, gentamicin, and 1-β-D-arabinofuranosylcytosine at the concentrations indicated above. Control cells were washed identically and maintained in serum-free medium supplemented with KCl at 25 mM.

Recombinant human IGF-I, IGF-II, acidic FGF (aFGF), basic FGF (bFGF), and platelet-derived growth factor (PDGF-A) were from Boehringer Mannheim. NT-3 was a gift from G. D. Yancopoulos (Regeneron Pharmaceuticals, Tarrytown, NY). All other agents were from Sigma.

Neuron Survival. Neuron survival was quantified by staining with fluorescein diacetate (10 μg/ml; Sigma) (22). The stained (viable) cells were examined with a Leitz Dialux 22 fluorescence microscope. Two randomly chosen fields from each dish were photographed and the number of surviving cells (indicated by bright green color) was computed. Typically, in healthy cultures (maintained in high K^+) there were 80–100 viable cells per field.

When indicated, lactate dehydrogenase (LDH) released into the culture medium was used as a measure of cell death. LDH activity was determined spectrophotometrically (23). LDH release is expressed as a percent of total LDH (LDH released plus intracellular LDH).

DNA Fragmentation Analysis. Fragmentation of DNA was analyzed as described (24). After treatment with RNase A (50 ng/ml) at 37°C for 30 min, the soluble DNA was subjected to electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

RESULTS

K^+ Deprivation Induces Neuronal Apoptosis. Cerebellar granule neurons of >95% purity can be cultured from postnatal rats in fetal bovine serum-containing medium supplemented with 25 mM K^+ (13). The viability of these neurons is prolonged, from ≈5 days in 5 mM K^+, to about 15 days in 25 mM K^+. Although high K^+ is known to support differentiation and survival of immature cerebellar granule cells (6, 11, 12), the effect of a more physiological level of K^+ on fully differentiated neurons has not been well studied.

To investigate the effect of low K^+, neurons were allowed to develop for 6–7 days in culture medium with 10% serum and 25 mM KCl (6, 13). The mature neurons were then shifted to serum-free medium containing 5 mM KCl (BME). Control cells were maintained in serum-free medium with 25 mM KCl. Serum-free medium was chosen for several reasons. (*i*) After maturation (4–5 days *in vitro*), cerebellar granule neurons are not dependent on serum for survival (ref. 6; C.G. and T.C., unpublished results). (*ii*) Addition of medium containing fresh serum has a toxic effect on cerebellar granule neurons, resulting in rapid and extensive cell death (25). (*iii*) The role of high K^+ in supporting survival and the mechanism by which it acts can be better assessed in the absence of the growth factors and other polypeptides present in serum.

In the presence of 25 mM K^+ the neurons were healthy, with round cell bodies and a complex network of neurites (Fig. 1*a*). Within 8 hr of switching to 5 mM K^+ however, vacuoles had formed. Some of the cell bodies were smaller, with fragmented neurites. After 24 hr in low K^+, most of the neuronal bodies were no longer well-rounded and had lost their phase-bright appearance. The cell bodies were shrunken with condensed nuclei. The neurites were fragmented and their density had decreased. The presence of vacuoles and cellular fragmentation was clearly evident (Fig. 1*c*). By 48 hr, cellular fragmentation was extensive, and cells had begun detaching from the substrate. By 72 hr few cells survived, and neurites were not visible (Fig. 1*d*).

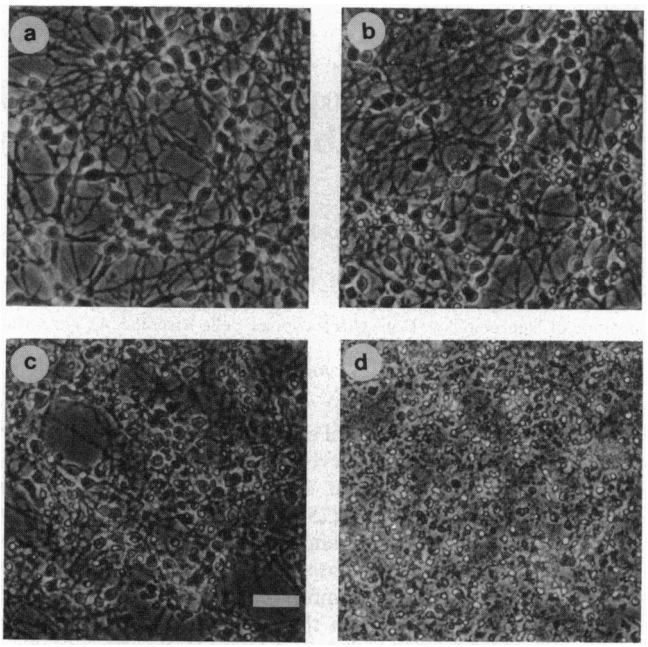

FIG. 1. Cerebellar granule neurons die when maintained in low K^+. Cerebellar granule neurons were allowed to mature for 6 days after plating in BME containing 10% fetal bovine serum and 25 mM KCl. The neurons were then switched to serum-free medium (normally containing 5 mM KCl). Phase-contrast micrographs show neurons maintained in 5 mM KCl for 8 hr (*b*), 24 hr (*c*), and 72 hr (*d*). Control cells (*a*) were maintained for 24 hr in serum-free medium containing 25 mM KCl. (Bar = 25 μM.)

The morphological changes accompanying death of granule neurons in low K^+ are characteristic of cells undergoing apoptosis or programmed cell death (for review, see refs. 26 and 27). To verify that death induced by K^+ was due to apoptosis, we examined the DNA from these cells. Withdrawal of K^+ led to cleavage of DNA into oligonucleosomal-sized fragments (Fig. 2), a hallmark of apoptosis (27). Based on the intensity of ethidium bromide staining, the extent of fragmentation was greater at 24 hr of deprivation than at 48 or 72 hr. At 24 hr, 40–50% of the neurons were still viable as judged by microscopic examination (and as described below), suggesting that DNA fragmentation is an early event in the death process.

Neuronal death was also observed when K^+ is lowered in the presence of serum. The morphological changes occurring during low K^+-induced death in the presence of serum were identical to those seen in serum-free cultures (data not shown). More importantly, DNA fragmentation was also evident in K^+-deprived neurons maintained in serum-containing medium (data not shown). These results show that cell death is not due to serum deprivation and that lowering of K^+ alone is sufficient to induce apoptosis.

Time Course of Neuronal Death Following K^+ Withdrawal. Initially, we estimated cell survival by the fluorescein diacetate staining method (26, 27). Cell death was not detectable at 8 hr of K^+ deprivation, but <50% of the cells were viable at 24 hr (Fig. 3*a*). The number of viable cells continued to decrease and was <5% by 96 hr. In contrast, parallel cultures containing high K^+ showed no change in number of viable neurons over the 96-hr treatment (data not shown).

In addition to quantifying the viable cells, we estimated cell death by measuring release of LDH into the medium. LDH release into the culture medium was low (≈2% of total LDH activity) until 16 hr of K^+ deprivation but increased to >12% by 24 hr (Fig. 3*b*). LDH release continued to increase gradually to about 25% by 96 hr. Taken together, the results of these two assays indicate that neuronal death begins after

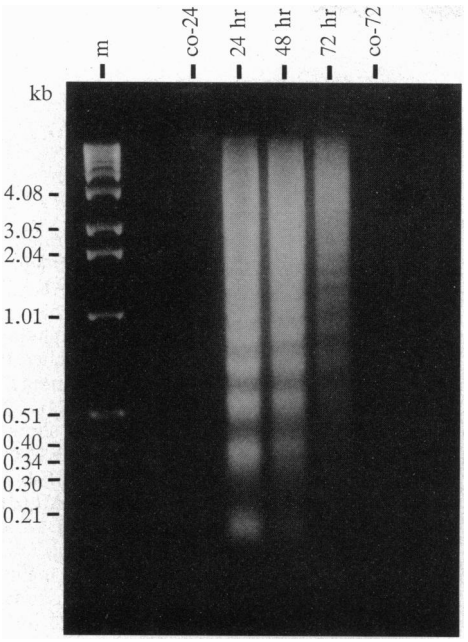

FIG. 2. DNA fragmentation occurs in dying neurons. Soluble DNA was extracted from neurons switched to serum-free culture medium containing low K^+ (5 mM) for 24, 48, or 72 hr, as indicated above the lanes. Lanes CO-24 and CO-72 contain DNA from control cells maintained in high K^+ (25 mM) for 24 or 72 hr, respectively. DNA from equal numbers of plated cells (6×10^6) was loaded in each lane. Lane m shows DNA molecular size markers. The sizes of the various DNA fragments are indicated at left.

≈16 hr of K^+ withdrawal and proceeds rapidly within the next 8 hr.

Apoptosis Induced by Low K^+ Requires mRNA and Protein Synthesis. In apoptosis, the death program is thought to be activated by the expression and action of "killer genes" (26, 27). Evidence for this idea includes the observation that apoptosis can generally be inhibited by the suppression of gene expression. To examine whether this was true for granule neurons, K^+ was withdrawn in the presence of transcriptional and translational inhibitors. The presence of the transcriptional inhibitor actinomycin D (1 μg/ml) significantly inhibited neuronal death induced by low K^+ (Figs. 4 and 5*e*), ≈75% of the cells viable at 48 hr. At 24 hr, actinomycin D was capable of totally preventing cell death (data not shown). It is likely that the cell death observed at 48 hr with actinomycin D was not due to apoptosis but the result of a toxic effect commonly observed following long-term exposure to this drug. Protection at 48 hr in low K^+ was also observed with the translational inhibitor cycloheximide (10 μg/ml) (Fig. 5*f*). Quantification of survival confirmed that, like actinomycin D, cycloheximide completely prevented cell death at 24 hr and greatly inhibited it at 48 hr (data not shown). These results suggest that macromolecule synthesis is required for induction of apoptosis in cerebellar granule neurons after K^+ withdrawal.

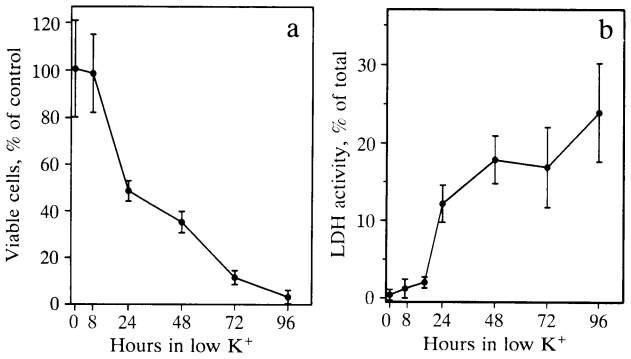

FIG. 3. Time course of neuronal death caused by low K^+. (*a*) Fluorescein diacetate staining of viable neurons. Cerebellar granule neurons were cultured in medium with 10% fetal bovine serum and 25 mM KCl for 6 days. The medium was then replaced with a serum-free medium containing 5 mM KCl. At the indicated times after the change of medium, the number of viable neurons was determined by fluorescein diacetate staining. Control cells were switched to a serum-free medium containing 25 mM KCl for 24 hr. Results represent the mean ± SD of five fields taken from three culture dishes. (*b*) LDH activity in the extracellular medium. Six days after plating, neurons were switched to serum-free medium containing low K^+ (5 mM). LDH activity in the culture medium was measured at the indicated times. Results represent mean ± SD of triplicate cultures and are expressed as percent total LDH activity (LDH released plus intracellular LDH). In control cultures, neurons were maintained in serum-free medium with high K^+ (25 mM KCl). LDH activity in the extracellular medium was undetectable even after 96 hr.

Apoptosis by Low K^+ Can Be Prevented by IGF-I. Although high K^+ can promote survival of cerebellar granule neurons in culture (7, 12), the concentration necessary for this effect is unphysiological. It was possible therefore that like sympathetic neurons, which are NGF-dependent (1, 21) but are capable of surviving *in vitro* in high K^+ (7), survival of granule neurons *in vivo* depended on one or more neurotrophic factors.

We examined the ability of IGF-I, IGF-II, aFGF, bFGF, NT-3, and PDGF-A to support survival of these neurons in low K^+. These factors were chosen because they are synthesized in the cerebellum and can promote survival of other neuronal populations (28–31). Moreover, the receptors for all these factors are present in the cerebellar granule layer (17, 19, 32, 33).

When added to cultures of fully mature granule neurons, bFGF and PDGF-A failed to significantly prevent death caused by low K^+ (Fig. 4). A similar inability to prevent death was also seen with aFGF and IGF-II (data not shown). NT-3 had a small but statistically significant effect on neuronal survival (Fig. 4). In contrast to these factors, IGF-I strongly inhibited neuronal death at 25 ng/ml (Fig. 5*c*). As judged by fluorescein diacetate staining, >80% of the neurons were

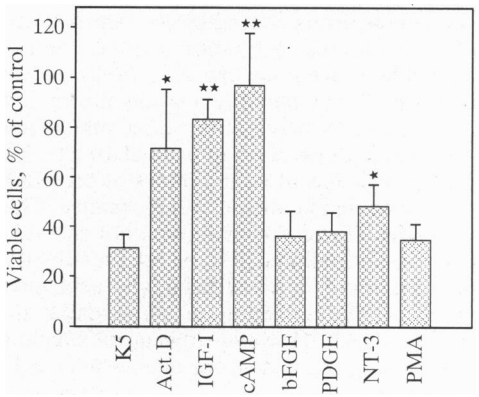

FIG. 4. Survival of neurons after treatment with various agents in low K^+. Neurons were switched from culture medium containing 10% fetal bovine serum and 25 mM KCl to serum-free medium containing 5 mM K^+ and no additives (K5) or with actinomycin D (Act. D, 1 μg/ml) IGF-I (25 ng/ml), forskolin (10 μM; to increase cAMP), bFGF (100 ng/ml), PDGF-A (20 ng/ml), NT-3 (50 ng/ml), or phorbol 12-myristate 13-acetate (PMA, 100 nM). Control represents survival in serum-free medium containing 25 mM KCl. Survival was quantified by fluorescein diacetate staining 48 hr after treatment. Each bar represents mean ± SD of five randomly chosen microscopic fields taken from three culture dishes. Statistically significant differences from K5 were estimated by the Student *t* test: ⋆, $P < 0.01$; ⋆⋆, $P < 0.001$. The experiment was repeated three times with similar results.

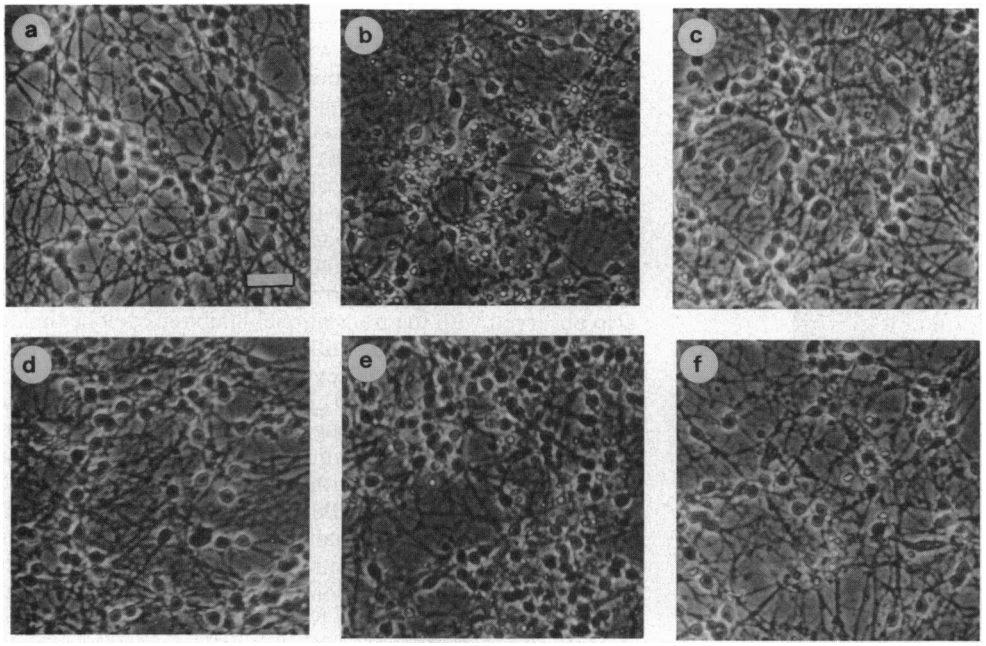

FIG. 5. Appearance of neurons following treatment with various agents in low K^+. Neurons were cultured for 6 days after plating in medium containing 25 mM KCl and then switched to serum-free medium containing 5 mM KCl and various agents. Phase-contrast micrographs show neurons after 48 hr in 5 mM KCl containing no additives (*b*), IGF-I (25 ng/ml) (*c*), forskolin (10 μM) (*d*), actinomycin D (1 μg/ml), (*e*) or cycloheximide (10 μg/ml) (*f*). Control neurons (*a*) were maintained in serum-free medium containing 25 mM KCl. (Bar = 25 μM.)

viable after 48 hr in low K^+ (Fig. 4). A lower but significant level of protection was also observed with IGF-I at concentrations as low as 5 ng/ml (data not shown).

cAMP-Elevating Agents/Analogues, But Not Phorbol Ester, Prevent Neuron Death Induced by Low K^+. The IGF-I receptor is a tyrosine-specific protein kinase. It was possible therefore that the protective effect of IGF-I and that of K^+ was mediated by phosphorylation of specific cellular proteins. To examine whether similar protection of granule neurons could be observed by activation of the protein kinases A and C, we examined the effects of forskolin (an activator of adenylate cyclase causing an increase in intracellular cAMP) and PMA, an activator of protein kinase C. Forskolin (10 μM) protected >95% of neurons from death (Figs. 4 and 5*d*) for at least 48 hr. Similar protection was observed when the cAMP analogue dibutyryl-cAMP was used (unpublished observation).

In contrast, PMA (100 nM) failed to significantly inhibit death of granule neurons caused by K^+ deprivation (Fig. 4). This result suggests that activation of PKC cannot prevent death of granule neurons in low K^+. Prolonged treatment with PMA results in the down-regulation of protein kinase C activity. However, we have observed a similar inability of PMA to inhibit death even when used at 10 nM. Moreover, K252a and sphingosine—two inhibitors of protein kinase C activity—failed to significantly affect the ability of high K^+ or IGF-I to support neuron survival (data not shown).

Apoptosis can be inhibited in some cell types by the general endonuclease inhibitor aurintricarboxylic acid, presumably by preventing DNA fragmentation, an essential step in the death process (34, 35). However, this agent was found to be toxic to cerebellar granule neurons even when used at only 5 μM (data not shown).

DISCUSSION

During the development of the vertebrate nervous system a large proportion of neurons that are generated die by a process referred to as programmed cell death. Although widespread in the nervous system and believed to be critical for normal neural development, the molecular mechanism underlying this process is unknown, and few examples of neuronal apoptosis *in vitro* have been described. Apoptosis, a form of programmed cell death, has clearly been shown to occur in sympathetic neurons following NGF deprivation (20, 21). In this report we show that fully differentiated cerebellar granule neurons undergo apoptosis when the extracellular level of K^+ is lowered. The death process is accompanied by morphological changes characteristic of death by apoptosis. Indeed, fragmentation of DNA, a hallmark of apoptosis, is observed in dying neurons. Interestingly, DNA fragmentation is detectable before degeneration is maximal, at about 24 hr, when a significant proportion of neurons are still viable. In fact, DNA fragmentation is detectable as early as after 8 hr in low K^+ (unpublished data), suggesting that this is an early event in the death process. However, a more detailed analysis of the temporal relationship between DNA fragmentation and neuronal death is necessary to demonstrate this point more convincingly. In contrast to death by K^+ withdrawal, DNA fragmentation is not observed in cerebellar granule cells killed by glutamate neurotoxicity, a process occurring via necrosis (unpublished observation and ref. 36).

Apoptosis in cerebellar granule neurons induced by low K^+ can be inhibited by suppression of transcription and translation, indicating the necessity for the expression of specific gene products to activate the death program. Several genes have been identified in nonneuronal systems whose expression is increased specifically during examination (27). An examination of some of these genes in degenerating sympathetic neurons as well as cerebellar granule cells revealed no induction of mRNA (37). However, there is evidence that apoptosis may be triggered by posttranslational mechanisms, and not by the action of "death genes" (35).

Death of granule cells by low K^+ can be prevented by IGF-I but not by several other growth and neurotrophic factors. Several pieces of evidence support our contention that this factor is a physiological neurotrophic factor for granule neurons *in vivo*. (*i*) IGF-I mRNA is widely expressed in the vertebrate brain (17, 30) and has been shown to support differentiation, survival, and regeneration of a variety of neurons in culture. (*ii*) IGF-I induces the expression of functionally active glutamate receptors in cerebellar granule neurons (38), suggesting a role for this protein in the development of these cells. Supporting this concept is the finding that even in the presence of high K^+, neutralization of serum IGF-I by a monoclonal antibody reduces by 30–40% the number of granule neurons that mature (38). (*iii*) Although IGF-I is not produced in cerebellar granule neurons at any stage of development, Purkinje cells in the cerebellum synthesize and secrete this polypeptide, especially during early

postnatal life (16, 17, 30). During this period (days 4–10), immature granule cells migrate through the Purkinje cell layer to reach the internal granule layer, whereupon they differentiate. It is likely that the IGF-I secreted by the Purkinje cells is taken up by granule cells and influences the terminal differentiation of these neurons. Indeed, granule cells migrating through the molecular layer transiently display immunoreactivity to IGF-I (16). Most importantly, granule neurons express the IGF-I receptor (17, 19).

Segal *et al.* (39) showed that BDNF and NT-3 enhanced survival of immature cerebellar granule cells from rat embryonic cerebella. Whereas BDNF was more active on the early granule cells in the external germinal layer, NT-3 affected the more mature granule cells (39). We have also observed a small but statistically significant effect of NT-3 on our cultures of fully developed postnatal granule neurons (Fig. 5*a*). BDNF, in contrast, had no survival effect on our neuronal cultures maintained in low K^+ (unpublished observation). It is possible that cerebellar granule neurons are sequentially dependent on BDNF, NT-3, and finally IGF-I for their differentiation and survival *in vivo*.

Apoptosis in cerebellar granule neurons by low K^+ can be prevented by cAMP as well as by IGF-I. This situation is similar to that of sympathetic neurons, which are dependent on NGF for survival *in vivo* but can be supported by either high K^+ or cAMP *in vitro* (7, 40). Like the high-affinity NGF receptor, the IGF-I receptor possesses tyrosine kinase activity. It is conceivable that although effective on different neuronal targets, IGF-I and NGF activate common pathways in the promotion of survival. Phosphorylation of a common cellular substrate by these factors as well as by cAMP and high K^+ is possible. Both IGF-I and NGF, in addition to cAMP and high K^+, were found to prevent apoptosis induced by serum deprivation of the PC12 pheochromocytoma cell line (41). Moreover, IGF-I can inhibit apoptosis in differentiated PC12 cells after NGF withdrawal (unpublished observation).

It is tempting to speculate that the mechanisms involved in low K^+-mediated apoptosis of granule neurons are similar to those operating during neuronal death *in vivo* during development or following a blockade of neuronal activity and *in vitro* by neurotrophic factor deprivation. These mechanisms may also be activated in certain neurodegenerative diseases and during normal aging. In view of their relative abundance, cerebellar granule cells provide an excellent primary cell culture system to investigate the mechanisms underlying neuronal apoptosis in the central nervous system. Moreover, these cells have frequently been used to study neurotoxicity, which is thought to occur via necrosis. The same cells are therefore capable of dying in distinct patterns which almost certainly involve distinct mechanisms. Cerebellar granule neurons therefore are a convenient system to investigate the similarities and differences in the molecular mechanisms underlying these degenerative processes.

Research funds for this project were obtained in part from a Human Frontier Science Program Organization fellowship to S.R.D. Research funds were also obtained from grants by Progetto Finalizzato Chimica Fine and Biotecnologie e Biostrumentazione to P.C.

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