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13. ABSTRACT *(Maximum 200 Words)*
Apoptosis, a cell death process required for normal brain development, is also aberrantly activated in certain neurodegenerative diseases and following exposure to neurotoxins. We hypothesize that certain components of the signaling pathways activated by different physiological and pathophysiological stimuli might be shared and could serve as targets for the development of therapeutic approaches. In our application, we proposed to compare the signaling pathways activated by four different apoptotic stimuli using cultures of rat cerebellar granule neurons with the goal of identifying common signaling molecules. During the first three years, our goal was to use one of these apoptotic stimuli - potassium (K+) deprivation - and examine the role of four different apoptosis-regulatory molecules. We have now confirmed that NF-κB is a molecule central to neuronal survival. We have also gathered evidence indicating that p38-α activation promotes neuronal death. Furthermore, we provide evidence suggesting that Akt, a serine-thronine kinase believed to be importannt for the inhibition of apoptosis, is not required for K+ mediated neuronal survival. As a step towards identifying molecules that represent convergent points in the signaling pathways activated by different neurotoxins, we have established conditions for inducing cell-death using two neurotoxins - β-amyloid protein and methylmercury.

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INTRODUCTION

Apoptosis is a cell-suicide process that is required for the normal development of the nervous system. Aberrant and inappropriately regulated apoptosis can, however, lead to undesirable neuronal loss such as that seen in certain neurodegenerative diseases. Apoptosis can also be induced in various neuronal populations by chemical and biological neurotoxins. The intracellular pathways by which these different physiological and pathophysiological stmuli cause neuronal death has not been characterized. We hypothesize that certain components of the signaling pathways activated by different apoptotic stimuli might be shared. Our expectation is that once identified, such molecules could serve as ideal targets for the development of approaches to protect or treat individuals against the actions of neurotoxic agents. In our grant application, we had proposed to compare the signaling pathways activated by four different apoptosis-inducing stimuli using cultures of cerebellar granule neurons from the rat brain with the goal of identifying common molecular components. Specifically, we proposed to induce apoptosis in neuronal cultures by (i) potassium (K+)-deprivation, (ii) treatment with the environmental neurotoxin, methyl mercury, (ii) treatment with b-amyloid protein (βAP) accumulation, which has been implicated in Alzheimer's disease, and (iv) overstimulation with the neurotransmitter, glutamate. We proposed to examine the involvement of four signaling molecules - NF-κB, p38 MAP kinase, caspases, and mGluR4 - in the regulation of cell-death by the different apoptotis-inducing stimuli.

In a request for supplemental funding that was submitted last year, we had proposed to extent our investigation to another well-studied apoptosis-regulatory molecule - the serine-threonine kinase, Akt.

BODY

During the first three years we had proposed to examine whether four molecules - NF-κB, p38 MAP kinase, caspases, and mGluR4 - are causally involved in the regulation of low K+ (LK)-mediated neuronal apoptosis. As reported in our first annual report that was submitted last year, we obtained evidence that NF-κB and p38 MAP kinase were involved in the regulation of LK-mediated apoptosis. In contrast and contrary to the hypothesis presented in our original proposal, caspases are not involved in LK-induced apoptosis. During the past year, we have strengthened our data regarding NF-κB and p38. Furthermore, we have strengthened our data on the role of Akt in the survival of granule neurons. Akt was the focus of our request for supplemental funding submitted in October 2000. Our results are described below:

NF-κB

Although not outlined in our original proposal, in response to the review of our previous annual report we have performed additional experiments to strengthen our results regarding NF-κB. Using transcriptional activity assays, we find that NF-κB activity is dramatically reduced within 6 h after the switch to LK-medium (Fig. 1A), consistent with our earlier conclusion that NF-κB is necessary for the survival of granule neurons. Further evidence supporting this conclusion is our finding that the expression of bcl-2 and bcl-XL, both of which are transcriptionally regulated by NF-κB, is reduced during LK-mediated apoptosis (Fig. 1B).

We had reported in our last report that neither the levels of the five NF-κB proteins, nor their intracellular localization, is altered in neurons primed to undergo apoptosis by LK-treatment. Although not proposed in our original grant application, we have performed experiments to gain

insight into the mechanism by which the DNA-binding and transcriptional activity is regulated in granule neurons induced to undergo apoptosis by LK-treatment. Briefly, we find that that the NF-kB protein, p65 /RelA interacts with the transcriptional coactivator, CREB-binding protein (CBP), in healthy granule neurons. The decrease in NF-κB transcriptional activity induced by LK treatment is accompanied by a reduction in the interaction between p65 and CBP (Fig. 2A). The reduced interaction is not due to decreased CBP expression (Fig. 2B), but due to an increase in the phosphorylation of CBP (Fig. 2C). Preliminary evidence suggests that the hyperphosphorylation of CBP is due to decreased activity of protein phosphatase-2A (not shown). As indicated below, these results are the basis of a grant application, which is presently under review at the NIH.

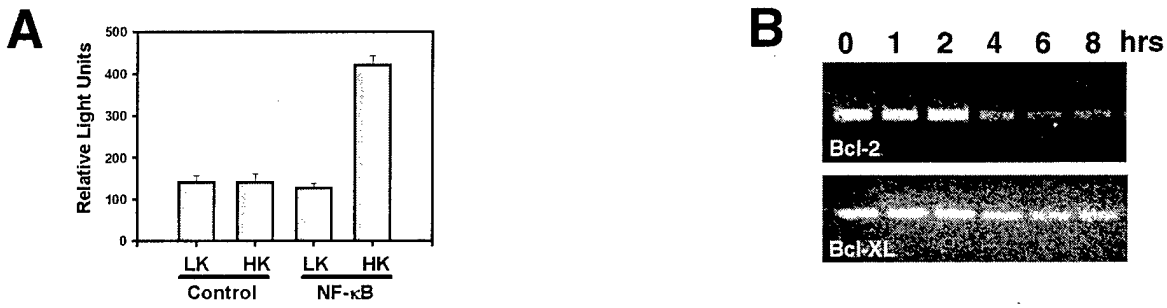


FIG. 1: NF-κB transcriptional activity is reduced in LK.

A. Transcriptional activity of NF-κB-Luciferase reporter plasmid. Neuronal cultures were transfected with NF-κB-Luciferase (NF-κB), or an identical plasmid lacking the NF-κB elements (Control). The following day the cultures were switched to LK or HK medium for 6h and luciferase assays performed using a kit (Promega) and measured with a Lumicount Luminometer. Results shown are from two independent experiments performed in triplicate.

B. Reduced Bcl-2 and Bcl-X mRNA. Expression of Bcl-2 and Bcl-XL mRNA was analyzed by RT-PCR and agarose gel electrophoresis using 1 ug of total mRNA from neuron cultures switched to LK medium for 0, 1, 2, 4, 6, and 8 h.

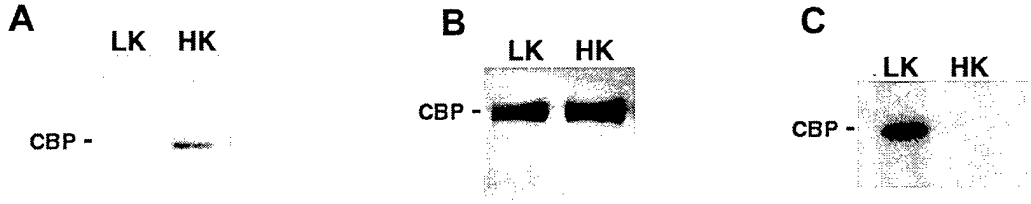


Fig. 2: *p65 interacts with CBP, the phosphorylation of which increases in apoptotic neurons.*

A. Interaction between p65 and CBP is reduced in LK. Cultures were treated with HK and LK medium for 6h. After cell lysis, CBP was immunoprecipitated and the precipitate analyzed by Western blotting using a p65 antibody

B. CBP is expressed in granule neurons but its level is unchanged by K+ deprivation. Western blot analysis of whole-cell lysates switched to LK or HK medium for 4h using a CBP antibody.

C. CBP is hyperphosphorylated following K+ deprivation. Phosphorylated proteins were labeled in vivo by the addition of [^{32}P] orthophosphate to cultures switched to LK and HK medium for 4h. Whole-cell lysates were prepared and CBP immunoprecipitated and analyzed by SDS-PAGE followed by autoradiography.

We have thus completed fully the experiments concerning the role of NF-κB in low K+ (LK)-induced apoptosis and conclude that this transcription factor is necessary for neuronal survival. Although most studies have found that NF-κB activity promotes neuronal survival, some studies have shown an association of this factor with apoptosis. While more work is needed to determine how a single factor can have opposing effects on cell viability, possible explanations could include the specific cell-type and apoptotic stimulus, the composition of the NF-κB dimer, or other proteins that may interact with NF-κB and modulate its activity.

We are happy to inform the USAMRMC that our manuscript describing the involvement of NF-κB in HK-mediated neuronal survival was accepted in the Journal of Neurochemistry and appeared in print recently [Koulich et al. (2001) J Neurochem.76:1188-98].

p38 MAP kinase

As we reported in our last report, pharmacological inhibition of p38 MAP kinase protects granule neurons from LK-mediated apoptosis. We also reported that while the expression of p38-α and p38-β is the same under HK and LK conditions, the phosphorylation of p38-β is reduced following LK-treatment. p38-α phosphorylation, on the other hand, remains unchanged. Finally, results from a preliminary transfection experiment showed that overexpression of p38-α induces apoptotic death in cultures kept in HK-medium whereas similar overexpression of p38-β reduced LK-induced apoptosis. Given that transfection is a very inefficient mode of gene delivery, we have been looking for better alternative ways of expressing the various forms of p38 in granule neurons. As part of a collaboration, Dr. Kim Heindenrich (Univ. Colorado Hlth Ctr) has provided us with adenoviral vectors capable of expressing wild-type (wt) and dominant-negative (dn) forms of p38-α and p38-β. We have just started using these viral vectors. Consistent with p38-α being involved in promoting apoptosis, our preliminary experiments reveal that overexpression of dn-p38-α protects against LK-induced apoptosis. Together with our results from plasmid transfections, we have thus confirmed that overexpression of p38-α is proapoptotic.

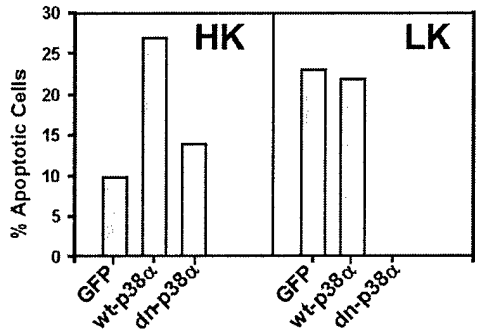
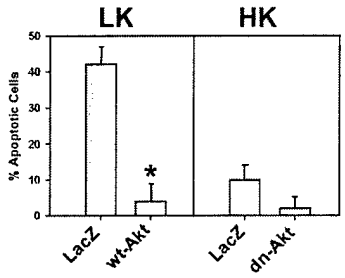


Fig. 3: p38-α may be proapoptotic. Granule neuron cultures were infected with adenoviral vectors expressing GFP, wt-p38α or dn-p38α. The next morning, the cultures were switched to LK or HK medium. Viability was quantified after 24h, by determining the proportion of infected neurons (GFP / FLAG-positive) that displayed condensed or fragmented nuclei (DAPI-staining).

Akt-related results

In our request for a supplement to our grant, we had proposed to investigate the role of the Akt in the regulation of neuronal survival. As shown in our supplement request, Akt is activated by all four survival factors in our system - HK, IGF-1, cyclic AMP, and lithium, albeit by different patterns of phosphorylation. We recently submitted a manuscript reporting these results to Developmental Brain Research and have just been informed that it has been accepted for publication. Akt is known to be necessary for IGF-1 mediated survival of granule neurons. As proposed in our request, we would like to determine if it is also required for the survival effects of HK, cAMP, and lithium. We have been using plasmids expressing constitutively active (ca) and dominant negative (dn) forms of Akt. Overexpression of ca-Akt maintains survival of granule neurons in LK-medium (Fig. 4). Surprisingly, however, the expression of dn-Akt does not reduce HK-mediated survival, suggesting that HK may signal by an Akt-independent pathway (Fig. 4). A manuscript that was published recently by Li et al. (Mol Cell Biol.20: 9356-63), showed that c AMP -mediated survival of granule neurons is also independent of Akt. Taken together, these results raise the possibility that Akt may be required solely for growth factor-mediated survival in neurons.



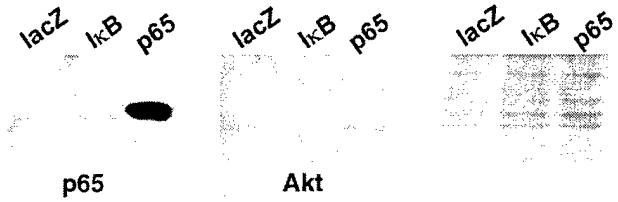


Fig. 4: HK may act in an Akt-independent manner. Neuronal cultures were transfected with plasmids expressing lacZ, wt-Akt, or dn-Akt. The next morning, the cultures were switched to HK or LK medium and the viability assayed 24 h later. For viability, the proportion of transfected cells (FLAG/lacZ positive) that displayed condensed/fragmented nuclei (DAPI-staining) was determined.

Fig. 5: NF-κB activates Akt. HEK293 cells were transfected with vectors expression lacZ, p65, or IκB-α. The next day the cells were switched to serum-free BME medium for 4h to downregulate Akt and then treated for 15 min with 10% serum (FCS). Lysates were prepared and subjected to western analysis using Akt or p65 antibodies.
Left: Western blot using p65 antibody shows that p65 is overexpressed in cultures transfected with CMV-p65.
Middle: Western blot using Akt-phospho-Ser473 antibody. Akt phosphorylation is induced in cultures transfected with CMV-p65. No signal is seen in cultures transfected with CMV- IκB-α.
Right: Coomassie blue staining of membrane showing similar amounts of proteins in each lane.

Based on our observation that pharmacological inhibitors of NF-κB also reduced IGF-1-induced Akt activation, we had proposed to more directly investigate whether NF-κB regulated Akt activity. We have performed a preliminary experiment involving overexpression of the p65 subunit of NF-κB into the HEK293 cell-line, as proposed in our request for a supplement. We find that cultures overexpressing p65 display higher Akt phosphorylation than control cultures expressing lacZ (Fig. 5). Furthermore, expression of IκB-α (which would be expected to reduce activity) reduces Akt phosphorylation to a level below those expressing lacZ. These results are consistent with a role for NF-κB in the activation of Akt (Fig. 5).

Other neurotoxins

We proposed to examine if the different molecules chosen for study in our proposal were affected by 3 neurotoxic stimuli - treatment with methylmercury, β-amyloid protein, and high concentrations of glutamate. As a step towards this goal, we have started to establish conditiosn for the induction of apoptosis in neuronal cultures using methylmercury. We have found that methylmercury treatment causes a significant amount of cell death at doses of ≥1 uM. In an experiment that we have just performed, we find that β-amyloid protein is toxic at doses of ≥10 uM.

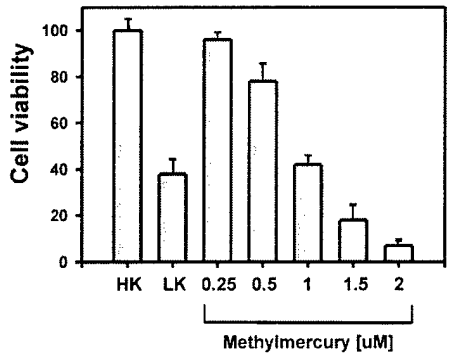


Fig. 6: Methylmercury induces apoptosis in granule neurons. Granule neurons were switched to medium containing HK, LK, or HK medium containing various doses of methylmercury chloride. Viability was determined using the MTT assay and expressed as percent of the viability displayed in HK cultures (which is adjusted to 100%).

KEY RESEARCH ACCOMPLISHMENTS

- NF-κB is required for the survival effects of HK as well as other survival-promoting factors.
- p38 MAP kinases regulate granule neuron survival in a complex manner; while p38-α may be proapoptotic, p38-β appears to be capable of inhibiting LK-induced apoptosis.
- NF-κB may be required to maintain Akt expression (and therefore activity)

REPORTABLE OUTCOMES

- Manuscript:

(a) Koulich E, T. Nguyen T, Johnson K, Giardina, C; D'Mello SR. (2001) NF-κB is involved in the survival of cerebellar granule neurons: Association of IkB-β phosphorylation with cell survival. J. Neurochem. 76:1188-98.
(b) Distinct phosphorylation patterns underlie Akt activation by different survival factors in neurons Rajaram S, Liu X, Nguyen T, Zhang X, D'Mello SR. Dev. Brain Research (in press).

- Abstracts:

(a) Koulich E, Nguyen T, D'Mello, SR. (2000) NF-κB maintains survival of cerebellar granule neurons. Soc. Neurosci. Absts. 27, 596.
(b) Koulich E and D'Mello, SR. Apoptosis in cerebellar granule neurons is associated with CBP hyperphosphorylation and reduced interaction between CBP and NF-κB Soc.Neuroscience Annual Mtg., New Orleans, LA (Nov, 2001, Abstract accepted).

- Funding: Based on the results obtained from the project, we submitted a R01 grant application to the NIH. The grant to the NIH shared no overlap with the grant funded by the USAMRMC. Although it received a good score (32 percentile ranking), it was not funded. The application has been revised and resubmitted to the NIH.

- Training: Funds from the grant were initially used for the salary of a postdoctoral fellow, Sunitha Kumari, who joined my lab in April 2000. Sunitha, however, left the lab in December for personal reasons (pregnancy). She was replaced in February, 2001, by Salah Mohamad, a postdoctoral fellow with 3 years experience. Funds from the grant were also used to support a graduate student, Kyle Johnson, as a Graduate Research Assistant in the Fall semester, 2000. Funding was also provided in the summer to an undergraduate, Elena Koulich. Both students and the postdoctoral fellow have gained tremendously from the training made possible by the grant and are making substantial research contributions. Funds from the supplement request have been used to suppoirt Fanyin Meng, who joined the lab in August 2001.

CONCLUSIONS

Our results have led further insight into the mechanisms underlying apoptosis in neurons from the brain. We hope to identify molecules that are affected by different neurotoxic stimuli and have viable candidates. Identification of such important molecules would be an important step in the development of therapeutic strategies aimed at preventing neuronal loss after exposure to neurotoxins.

APPENDIX

Journal of Neurochemistry, 2001, **76**, 1188–1198

NF-κB is involved in the survival of cerebellar granule neurons: association of Iκβ phosphorylation with cell survival

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Abstract

The NF-κB transcription factor consists of dimeric complexes belonging to the Rel family, which include p50, p52, p65 (RelA), RelB and c-Rel. NF-κB activity is tightly controlled by IκB proteins which bind to NF-κB preventing its translocation to the nucleus. Activation of NF-κB is most often mediated by IκB degradation, which permits NF-κB to enter the nucleus. We investigated the role of NF-κB in the survival of cerebellar granule neurons. We found that survival of these neurons in high potassium medium is blocked by three separate inhibitors of NF-κB activity: SN-50, *N*-tosyl-L-phenylalanine chloromethyl ketone and pyrrolidinedithiocarbamate, indicating that NF-κB is required for neuronal survival. Gel-shift assays reveal three complexes that bind to the NF-κB binding site in high potassium medium. Switching these cultures to low potassium medium, a stimulus that leads to apoptotic death, causes a reduction in the level of the largest complex, which contains p65. Overexpression of p65 by transfection inhibits low potassium-induced apoptosis, whereas overexpression of IκBα promotes apoptosis even in high potassium medium. Surprisingly, however, neither the level of endogenous p65 nor that of IκBα and IκBβ is altered by low potassium treatment. Similarly, no changes are seen in the nuclear or cytoplasmic levels of p50, p52, RelB and c-Rel. Phosphorylation of p65, which can lead to its activation, is unchanged. Phosphorylation of IκBβ is, however, reduced by low potassium treatment. Besides being necessary for high potassium-mediated neuronal survival, NF-κB is also involved in the survival-promoting effects of IGF-1 and cAMP as judged by the ability of SN-50 to inhibit the actions of these survival factors and the ability of these factors to inhibit the low potassium-induced alterations in the DNA-binding activity of NF-κB. Taken together, our results show that NF-κB may represent a point of convergence in the signaling pathways activated by different survival factors and that uncommon mechanisms might be involved in NF-κB-mediated survival of cerebellar granule neurons.

Keywords: apoptosis, cerebellar granule neurons, IκB, neuronal survival, NF-κB.

J. Neurochem. (2001) **76**, 1188–1198.

The NF-κB family of proteins are ubiquitously expressed and are inducible transcription factors that regulate the expression of genes involved in disparate processes such as immunity and inflammation, growth, development, viral-gene transcription and cell-death regulation (Ghosh *et al.* 1998; Karin and Ben-Neriah 2000; Mattson *et al.* 2000). In mammalian cells, there are five NF-κB proteins, p50, p52, p65 (RelA), RelB and c-Rel. NF-κB is composed of homodimers and heterodimers of these proteins, typically p65 : p50, which are held in the cytoplasm by the inhibitory IκB proteins, which bind NF-κB and mask its nuclear localization signal.

The role that NF-κB plays in the regulation of neuronal survival is complex. NF-κB has been found to be involved in the survival-promoting effects of NGF and cytokines (Maggirwar *et al.* 1998; Middleton *et al.* 2000) and its activation is associated with neuroprotection against death-inducing stimuli such as exposure to β-amyloid protein, oxidative stress and nitric oxide (Barger *et al.* 1995; Mattson *et al.* 1997; Lezoulac'h *et al.* 1998; Kaltschmidt *et al.* 1999; Glazner *et al.* 2000; for review,

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Abbreviations used: BME, basal Eagle's medium with Earles salts; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; HK/LK, high/low potassium; IKK, IκB kinase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; NGF, nerve growth factor; PBS, phosphate-buffered saline; PDTC, pyrrolidine-dithiocarbamate; SDS, sodium dodecyl sulfate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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Mattson *et al.* 2000). In some cases, however, NF-κB promotes neuronal death. NF-κB activation is detected *in vitro* following exposure to apoptotic stimuli (Lin *et al.* 1998; Cheema *et al.* 1999) and *in vivo* after global ischemia, kinate-induced seizures and traumatic spinal cord injury (Prasad *et al.* 1994; Grilli *et al.* 1996; Clemens *et al.* 1997; Bethea *et al.* 1998; for review, Mattson *et al.* 2000). Furthermore, increased levels of NF-κB activity have been detected in vulnerable regions of the CNS of Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis patients (Hunot *et al.* 1997; Kaltschmidt *et al.* 1997; Migheli *et al.* 1997), raising the possibility that NF-κB may play a causal role in neurological disorders (Kaltschmidt *et al.* 1993). Whether NF-κB inhibits or promotes apoptosis might depend on the cell type and the nature of the apoptosis-inducing stimulus. In addition, the composition of the NF-κB homo/heterodimer is likely to play a critical role in the regulation of apoptosis. In support of this idea are the observations that p65 activation increases survival in many cases, whereas c-Rel has been associated with apoptosis (Abbadie *et al.* 1993; Beg and Baltimore 1996; Wu *et al.* 1996). It is not clear precisely how NF-κB activation regulates cell survival although induction of the genes encoding the anti-apoptic bcl-2 homolog A1, and some members of the inhibitor of apoptosis proteins (IAPs) by NF-κB have been reported (Chu *et al.* 1997; Wang and Baldwin 1998; Wang *et al.* 1999).

The molecular mechanisms involved in the activation of NF-κB are beginning to be understood (Ghosh *et al.* 1998; Karin and Ben-Neriah 2000). In general, activation of NF-κB is the result of IκB phosphorylation by the multi-subunit IκB kinase (IKK) complex, which leads to IκB degradation. This permits NF-κB to translocate to the nucleus and activate transcription of target genes. A number of IκB proteins have been identified, including IκBα, IκBβ, IκBγ and IκBε proteins, of which IκBα and IκBβ are the best studied (Karin and Ben-Neriah 2000). IκBα and IκBβ share a characteristic ankyrin repeat motif required for their interaction with NF-κB. The IKK-mediated phosphorylation that leads to the degradation of IκBα and IκBβ occurs at two serine residues located at the N-termini of these proteins (Karin and Ben-Neriah 2000). In addition to these sites, IκBα and IκBβ can be phosphorylated by other cellular kinases such as DNA-dependent protein kinase and casein kinase II at the C-termini (McKinsey *et al.* 1996; Liu *et al.* 1998). The biological significance of phosphorylation at these other sites is poorly understood. Although activation of NF-κB proteins is generally regulated at the level of IκB-breakdown mediated translocation, the transcriptional activity of p65 can also be increased by phosphorylation (Wang and Baldwin 1998; Zhong *et al.* 1998). Furthermore, interaction with the coactivator CREB-binding protein (CBP)/p300 has been found to increase the activity of p65 in some cases (Perkins *et al.* 1997; Merika *et al.* 1998).

We examined the role of NF-κB in the regulation of survival/apoptosis using cultures of cerebellar granule neurons. These neurons can be cultured and maintained *in vitro* if depolarizing levels of potassium (K^+) are provided. Lowering the K^+ levels induces apoptosis (D'Mello *et al.* 1993; Yan *et al.* 1994). Apoptosis induced by K^+ deprivation can be prevented by IGF-1 as well as pharmacological agents such as cAMP (D'Mello *et al.* 1993). Using this paradigm, we show that NF-κB is necessary for neuronal survival and that overexpression of certain NF-κB members can prevent low K^+ (LK)-induced apoptosis. Interestingly, however, neither IκB levels within the cytoplasm nor nuclear expression of the individual NF-κB members are altered following K^+ deprivation. LK-induced death is, however, accompanied by a reduction in the phosphorylation of IκBβ.

Materials and methods

Reagents and plasmids

All reagents were from Sigma unless specified otherwise. SN-50 was purchased from Calbiochem. IGF-1 was from Roche Biochemicals. CMV-lacZ was purchased from Clonetech. FLAG-tagged CMV-p65 and CMV-p50 were kindly provided by Dr A. Baldwin (University of North Carolina, Chapel Hill), and myc-tagged IκBα by Dr R. Gaynor (University of Texas Southwestern Medical Center).

Cell culture and treatments

Granule neuron cultures were obtained from dissociated cerebella of 7–8-day-old rats as described previously (D'Mello *et al.* 1993). Cells were plated in basal Eagle's medium with Earles salts (BME) supplemented with 10% fetal calf serum, 25 mM KCl, 2 mM glutamine (Gibco-BRL), and 100 μg/mL gentamycin on dishes coated with poly-L-lysine in 24-well dishes at a density 0.5×10^6 cells/well or 2.5×10^7 cells/100-mm dish. Cytosine arabinofuranoside (10 μM) was added to the culture medium 18–22 h after plating to prevent replication of non-neuronal cells.

Unless indicated otherwise, cultures were maintained for 6–7 days prior to experimental treatments with IGF-1 or pharmacological agents. For this, the cells were rinsed once and then maintained in LK medium (serum-free BME, 5 mM KCl) with or without the agents, or in the case of control cultures, in high K^+ (HK) medium (serum-free BME, supplemented with 20 mM KCl). Treatment of cultures with NF-κB inhibitors was initiated 30 min prior to rinsing and was maintained through the subsequent incubation in LK medium.

Neuronal survival

Neuronal survival was quantified by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolim bromide (MTT) assay as described by Kubo *et al.* (1995). Briefly, the tetrazolium salt (MTT) was added to the cultures at a final concentration of 1 mg/mL, and incubation of the culture was continued in the CO_2 incubator for a further 30 min at 37°C. The assay was stopped by adding lysis buffer [20% sodium dodecyl sulfate (SDS) in 50% *N,N*-dimethyl

formamide, pH 4.7]. Absorbance was measured spectrophotometrically at 570 nm after an overnight incubation at room temperature. The absorbance of a well without cells was used as background and subtracted.

Results obtained using the MTT assays were confirmed using the fluorescein-diacetate method for quantification of cell viability, as described previously (D'Mello *et al.* 1993). Data are presented as mean ± SD. Statistical analysis was performed using ANOVA and Student-Neuman-Keuls' test.

Western blotting

For whole-cell lysates, the culture medium was discarded, the neurons washed twice with cold phosphate-buffered saline (PBS), and lysed in SDS–PAGE buffer (62.5 mM Tris–Cl, pH 6.8, 2% SDS, 5% glycerol, 1% 2-mercaptoethanol, and bromophenol blue). Following heating at 95°C for 5 min, proteins were subjected to SDS–PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore). After staining with Ponceau S (Sigma) to verify uniformity of protein loads/transfer, membranes were analyzed for immunoreactivity. Incubation with primary antibodies was performed overnight at 4°C and with secondary antibodies for 1 h at room temperature. Immunoreactivity was developed by enhanced chemiluminescence (ECL; Amersham) and visualized by autoradiography. Blots were reprobed with α-tubulin antibody to confirm that HK and LK lanes contained equal amounts of protein.

The following primary antibodies were used: p65 and p52 (Santa Cruz Biotech., 1 : 1000 dilution), p50 (Santa Cruz Biotech., 1 : 50), c-Rel and RelB (Santa Cruz Biotech., 1 : 500), IκBα and IκBβ (Santa Cruz Biotech., 1 : 500) and α-tubulin (Sigma, 1 : 10 000). Secondary antibodies were peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, 1 : 10 000) and donkey anti-goat IgG (Santa Cruz, 1 : 5000).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) analysis of DNA binding activity was performed as described previously (Schutze *et al.* 1992). The NF-κB oligonucleotide with the sequence TCGACAGAGGGGACTTTCCGAGAGGCTCGA was end-labeled using $\gamma[^{32}P]$ATP. For supershift experiments, 1 μL (0.2 μg) of antibody (Supershifting antibodies, Santa Cruz Biotech.) was pre-incubated with nuclear extract on ice for 30 min. After this pre-incubation, the binding reaction was performed normally. For probe competition experiments, unlabeled NF-κB or AP-1 (Promega) oligonucleotides were pre-incubated with nuclear extracts on ice for 30 min before performing binding reactions.

Nuclear and cytoplasmic extracts preparation

Preparation of nuclear extracts, with slight modifications, was performed as described by Latchman (1993). Briefly, cultures of 6–7-day-old neurons (≈1.5×10^8 cells) were lysed in buffer A (10 mM HEPES, pH 8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiolthreitol, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL aprotonin) and nuclei centrifuged at 1500 r.p.m. Supernatants were treated as cytoplasmic extracts. The nuclear pellet was resuspended in buffer B (10 mM HEPES, pH 8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiolthreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL aprotonin) and centrifuged at 3000 *g*. The pellet was incubated on ice in buffer C (20 mM HEPES, pH 8, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL aprotonin). Following lysis of the nuclei and centrifugation at 16 000 *g* for 20 min, the supernatants were treated as nuclear extracts.

Transfection

Granule neuron cultures were plated on glass coverslips in 24-well dishes and transfected 5 or 6 days later using the calcium-phosphate protocol of Dudek *et al.* (1997). Briefly, cells were washed twice with serum-free Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) and incubated at 37°C in a CO_2 incubator for 1 h. The conditioned medium containing serum and HK was kept aside. For each well a mixture was prepared as follows: 15 μL of HeBS buffer was combined with 15 μL of a 0.25-M $CaCl_2$ solution containing 3 μg of plasmid DNA, vortexed, and allowed to sit at room temperature for 30 min. The mixture was added to the cells dropwise and allowed to incubate for 30 min. After washing cells twice with DMEM, cultures were fed using the conditioned medium in which they had been maintained after plating. The following morning, the medium was replaced with HK or LK medium (as described in the text and legends) for 24 h, unless indicated otherwise.

The efficiency of transfection using this protocol ranged from 0.1 to 5% and based on morphological examination virtually all transfected cells were neurons. Only transfected cells with a clear neuronal morphology were used for quantification purposes. Viability of CMV-lacZ transfected cells was similar to that of untransfected cells, indicating that transfection itself did not affect cell viability.

Immunofluorescence analysis of transfected cultures

Immunofluorescence analysis of transfected cultures was performed as follows. Cells were fixed for 15 min in 4% paraformaldehyde. After blocking in 0.1 M phosphate buffer for 30 min, immunostaining was performed by incubation with the primary antibody for 1 h and the secondary antibody for 30 min at room temperature. The following mouse monoclonal primary antibodies were used: anti-FLAG (1 : 200, Sigma), anti-c-myelin (9E10, 1 : 200, Santa Cruz Biotech.) and anti-β-galactosidase (1 : 40, Gibco-BRL). The secondary antibody for all experiments was a Texas Red-conjugated goat anti-mouse antibody (1:200, Jackson ImmunoResearch Labs). During washing of the secondary antibody, nuclei were stained in 1-μg/ml-4,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. Coverslips were mounted with fluormount (Molecular Probes), viewed with a Olympus Optiphot-2 microscope under a 40× objective lens and images captured with a SPOT cooled CCD-camera. Apoptotic cells were recognized by bright staining and condensed/fragmented nuclear morphology when viewed under ultraviolet light (260 nm).

DNA fragmentation analysis

Fragmentation of DNA was analyzed as described previously (D'Mello *et al.* 1993). Soluble DNA was isolated from equal numbers of plated cells (2.5×10^7) by cell lysis and elimination of nuclei. After treatment with RNase A (50 ng/mL, 37°C for 30 min), samples were subjected to electrophoresis in a 1.5% agarose gel and the DNA was visualized by ethidium bromide staining.

Analysis of p65 and IκBβ phosphorylation

Sixty millimeter dishes of 6–7-day-old neurons were washed twice with warm, phosphate-free DMEM containing 25 mM KCl and incubated overnight in the same medium. The cultures were then incubated for 6 h in medium containing [^{32}P] orthophosphate (400 μCi; ICN) in either 25 mM (HK medium) or 5 mM KCl (LK medium). Following lysis in ice-cold RIPA buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 10 μL/ml protease inhibitor cocktail (Sigma)], the lysates were centrifuged for 10 min at 10 000 *g* at 4°C. Supernatants were incubated overnight with primary antibody (1.5–2 μg) and then for 2 h with 20 μL Protein A/G PLUS-Agarose (Santa Cruz Biotech.).

Immunoprecipitates were collected by centrifugation at 550 *g* for 5 min at 4°C and pellets resuspended in electrophoresis sample buffer and subjected to SDS–PAGE. The gel was dried and analyzed by autoradiography.

Nuclear extracts were also used to analyze p65 phosphorylation. For this, cultures were plated and labeled in 150 mm dishes and nuclear extract prepared. Immunoprecipitation of p65 and analysis of phosphorylation was performed as described above.

Results

Inhibitors of NF-κB activity induce neuronal death

As a first step towards examining whether NF-κB is involved in the regulation of neuronal survival, we examined the effect of SN-50, a cell-permeable recombinant peptide that blocks translocation of NF-κB to the nucleus.

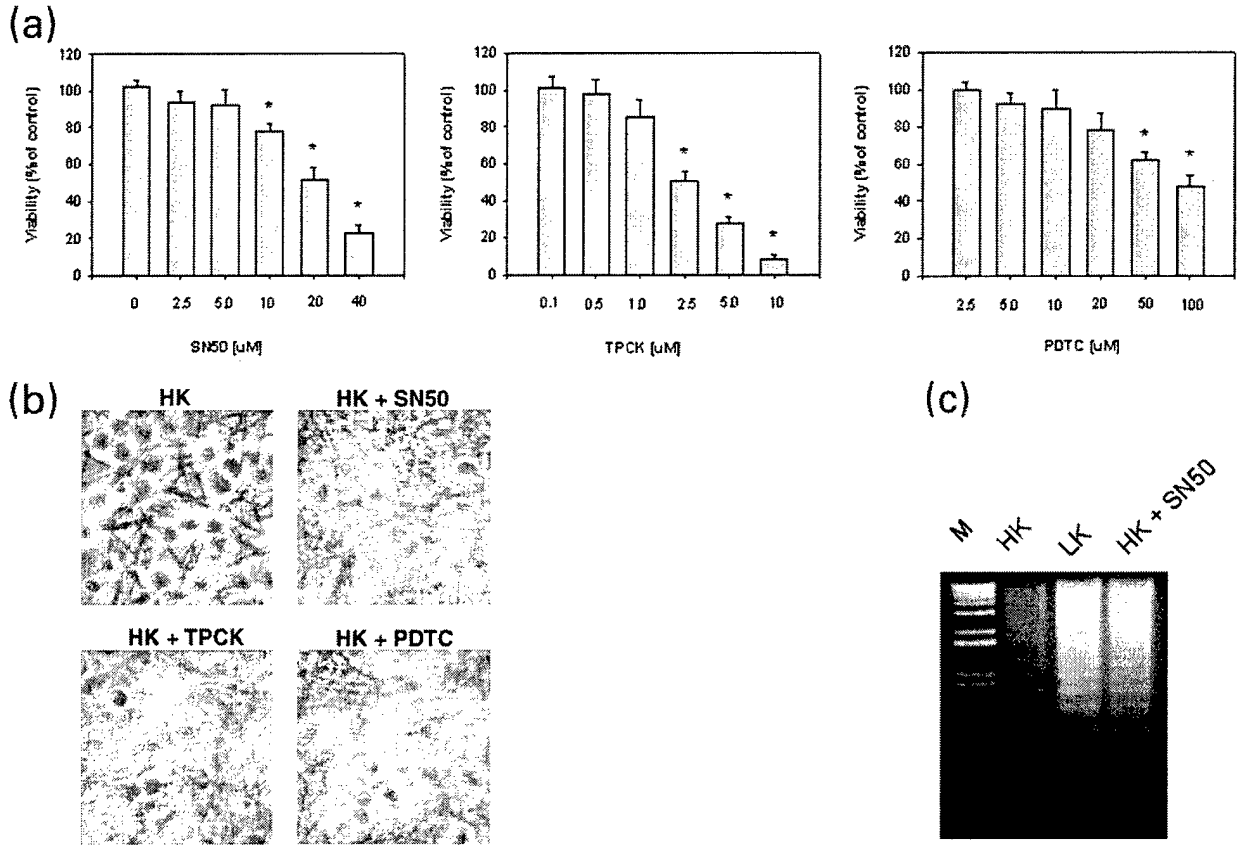

Fig. 1 NF-κB inhibitors induce apoptosis in the presence of HK. (a) Quantification of cell death induced by NF-κB inhibitors. Seven-day-old neuronal cultures were switched to serum-free culture medium containing HK supplemented with various doses of three NF-κB inhibitors: SN-50, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and PDTC. Viability was assayed 24 h later using the MTT assay. Survival is represented as the percentage of viability of control cells that received HK medium. Results shown come from three independent experiments performed in duplicate. $^*p < 0.01$ vs. viability of control cells. Because the MTT assay measures mitochondrial function rather than viability itself, the toxic effects of these drugs on neurons was confirmed by an independent assay for cell viability, FDA staining (D'Mello *et al.* 1993). Similar results were obtained using FDA staining (data not shown). (b) Phase-contrast micrographs showing the morphological appearance of cultures treated with NF-κB inhibitors. Seven-day-old cultures were switched to serum-free culture medium containing HK alone (HK) or HK plus SN-50 (20 μM, HK + SN50), TPCK (10 μM, HK + TPCK) or PDTC (100 μM, HK + PDTC). Pictures were taken 24 h later. (c) SN-50 treatment causes DNA fragmentation. Cultures were treated with HK, LK or HK medium containing 20 μM SN-50 (HK + SN50). Soluble DNA was isolated 24 h later and subjected to agarose gel electrophoresis. M is a molecular mass marker.

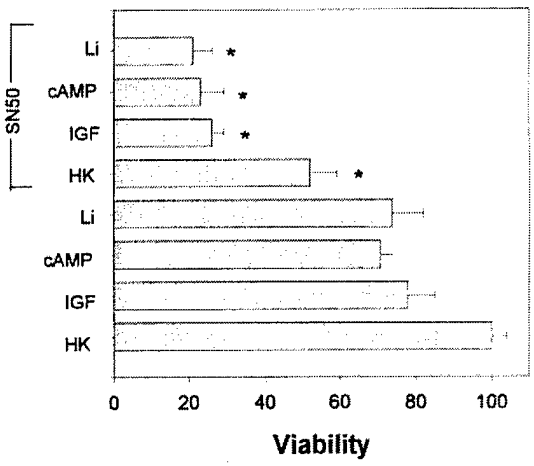

Fig. 2 SN-50 inhibits the survival-promoting effects of IGF-1, forskolin and lithium. Neuronal cultures were switched to LK medium containing IGF-1 (25 ng/mL), forskolin (cAMP; 10 μM) and lithium (Li, 10 mM) in the absence or presence of SN-50 (20 μM). Viability was assayed 24 h later using the MTT assay. $^*p < 0.01$ vs. viability of cells treated with the same survival factor in the absence of SN-50. Similar results were obtained using FDA staining (data not shown).

Previous work has shown that SN-50 has no effect on IκB-degradation (Lin *et al.* 1995) or on the activity of several other transcription factors including AP-1, CREB and OCT (Maggirwar *et al.* 1998). As shown in Fig. 1(a,b), SN-50 treatment caused a dose-dependent increase in cell death in cultures maintained in HK (Fig. 1a). Substantial loss of viability was observed at 10 μM, a dose at which this drug is known to significantly inhibit NF-κB translocation (Lin *et al.* 1995). To verify that SN-50 was not non-specifically toxic, we performed DNA fragmentation analysis of cells treated with the inhibitor. As shown in Fig. 1(c), cells treated with SN-50 in HK-medium displayed DNA fragmentation, a characteristic feature of apoptosis. In the absence of HK, survival of cerebellar granule neurons can be maintained by IGF-1, cAMP or lithium (D'Mello *et al.* 1993; Levick *et al.* 1995). As shown in Fig. 2, treatment with SN-50 also abolished the survival-promoting activity of these three factors, suggesting that NF-κB may represent a point of convergence in the signaling pathways activated by different survival factors.

While SN-50 has been shown to be a selective inhibitor of NF-κB translocation and has been widely used, an effect of this peptide on some other intracellular target cannot be completely ruled out. Two other commonly used inhibitors of NF-κB are the protease inhibitor, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Henkel *et al.* 1993; Wu *et al.* 1996; Phillips and Ghosh 1997) and the antioxidant pyrrolidinedithiocarbamate (PDTC; Schreck *et al.* 1992; Martinez-Martinez *et al.* 1997; Mahboubi *et al.* 1998). We used TPCK and PDTC to confirm the results obtained using SN-50. As shown in Fig. 1(a,b), both drugs inhibit HK-mediated neuronal survival at doses at which they inhibit NF-κB activity. The ability of NF-κB inhibitors to induce cell-death in the presence of HK was confirmed using FDA-staining to measure neuronal viability (not shown).

DNA-binding activity of NF-κB is altered in neurons primed to die

To examine whether K^+ deprivation altered the DNA-binding activity of NF-κB, we used EMSAs. Nuclear extracts were prepared from cultures switched to LK medium for 0–4 h and incubated with a radiolabeled NF-κB oligonucleotide probe. As shown in Fig. 3(a), three major bands are visible in control cells (0 h). Binding of all three complexes is eliminated in the presence of a 100-fold

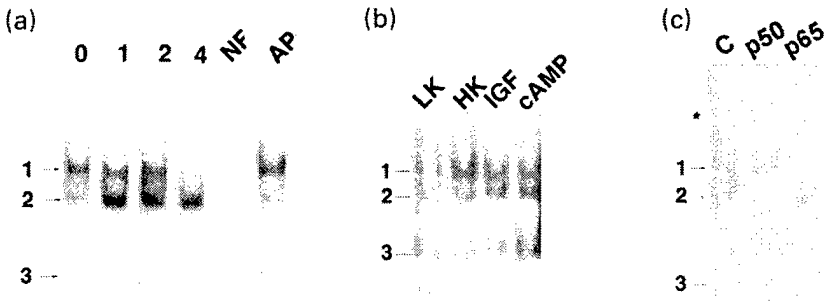

Fig. 3 EMSA assays of NF-κB binding. (a) Nuclear extracts were prepared from neurons switched to LK medium for 0, 1, 2 and 4 h. Extracts were used in EMSA assays using radiolabeled NF-κB probe. Three complexes (labeled 1, 2 and 3) can be observed. All three complexes are eliminated in the presence of a 100-fold excess of unlabeled NF-κB-oligo (lane NF) but not with an oligo containing an AP-1 binding site (lane AP), demonstrating specificity of binding. Complex 1 is reduced at 4 h. (b) Alterations in DNA-binding pattern of NF-κB proteins are inhibited by survival factors. Lysates were prepared from cells switched for 4 h to LK medium with no additives or supplemented with HK, IGF-1 (IGF, 25 ng/mL) or forskolin (cAMP, 10 μM) and subject to EMSA. (c) Complex 1 contains p65, whereas complex 2 contains p50. Nuclear extracts were pre-incubated with antibodies to either p50 or to p65 before binding reactions were performed. p50 Antibody reduces the intensity of complex 2 but does not affect complex 1 significantly. *Super-shifted complex. p65 antibody completely eliminates binding to complex 1 without affecting complex 2 or 3. Complex 3 is not affected.

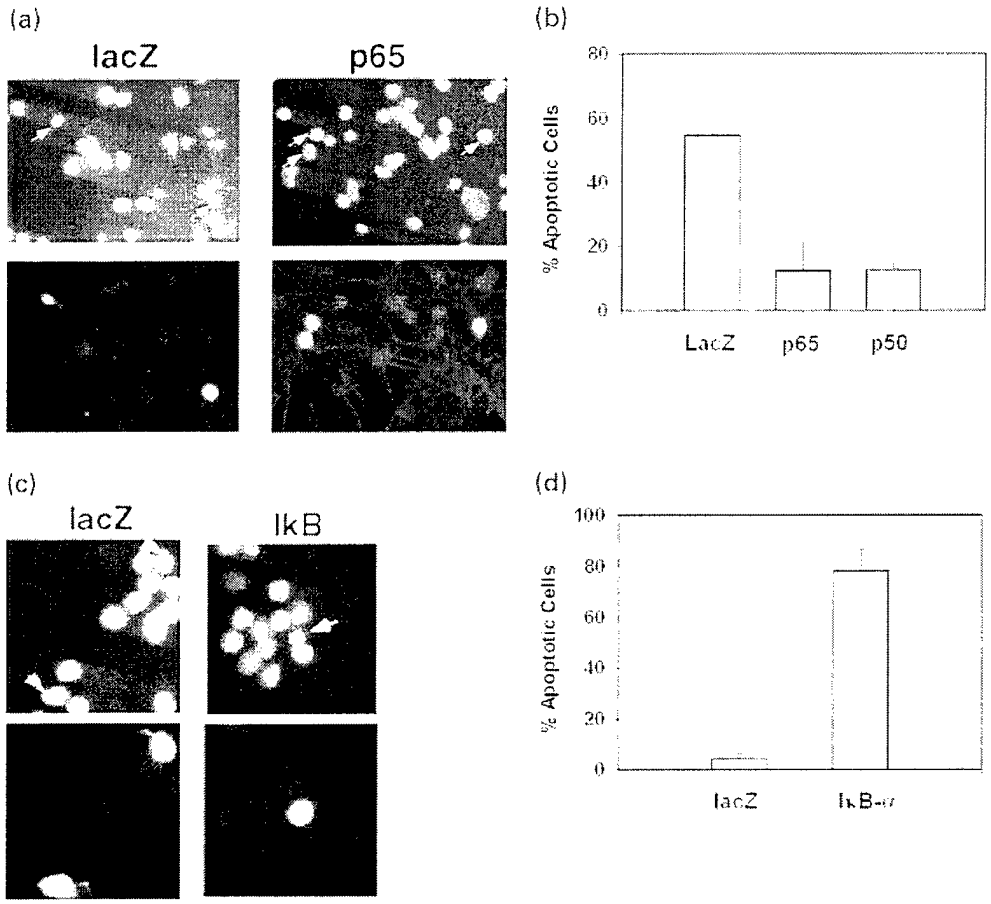

Fig. 4 Overexpression of p65 or p50 inhibits while that of IκBα induces cell death. Five-day-old granule neuron cultures were transfected with expression vectors using the calcium-phosphate method. (a) and (b) refer to p65 and p50 overexpression, whereas (c) and (d) refer to IκBα overexpression. (a) Neuronal cultures were transfected with expression vectors expressing lacZ, FLAG-tagged p65 or p50. The next morning the cultures were switched to LK medium and the viability of transfected cells was analyzed 24 h later. (Upper) Nuclei stained with DAPI, (lower) neurons overexpressing lacZ or p65 as judged by lacZ or FLAG-immunostaining. As seen in the upper panels, a significant proportion of the nuclei appear apoptotic (condensed). The proportion of lacZ-expressing neurons (arrow) that are apoptotic is similar to that of untransfected cells. Neurons expressing p65 (arrow) showed much higher viability than those expressing lacZ. (b) Quantification of results obtained from three experiments performed in duplicate. (c) Neuronal cultures were transfected with expression vectors expressing lacZ or myc-tagged IκBα. The next morning the cultures were switched to HK medium and viability of transfected cells analyzed 24 h later. (Upper) Nuclei stained with DAPI, (lower) neurons overexpressing lacZ or IκBα as judged by lacZ or myc-immunostaining. Nuclei of IκBα-transfected neurons (arrows) appear apoptotic as judged by DAPI staining. (d) Quantification of results obtained from three experiments performed in duplicate.

molar excess of unlabeled NF-κB probe (lane NF) but not an oligonucleotide containing an AP-1 consensus sequence (lane AP), indicating that binding is specific. The intensity of the band corresponding to complex 1 is reduced to $< 50\%$ of its original level at 4 h (Fig. 3a) and remains low even at 6 h (not shown). Because commitment to death in granule neurons occurs within the first 4–6 h after K^+ deprivation (Galli *et al.* 1995; Schulz *et al.* 1996; Borodezt and D'Mello 1997; Nardi *et al.* 1997), the reduction in complex 1 may be causally involved in the induction of apoptosis. Besides the change seen in complex 1, the intensity of the band corresponding to complex 2 is increased after K^+ deprivation and this occurs within 1 h. To examine whether the reduction of complex 1 was related to the induction of cell death rather than the lowering of K^+, we performed EMSAs on extracts prepared from neurons treated with IGF-1 and cAMP in LK medium. Both IGF-1 and cAMP can maintain granule neuron survival in the absence of HK (D'Mello *et al.* 1993). As shown in Fig. 3(b), in the presence of these survival factors, the reduction in binding of complex 1 seen after K^+ deprivation was inhibited.

To identify the components of the complexes that bind the NF-κB probe, nuclear extracts were pre-incubated with

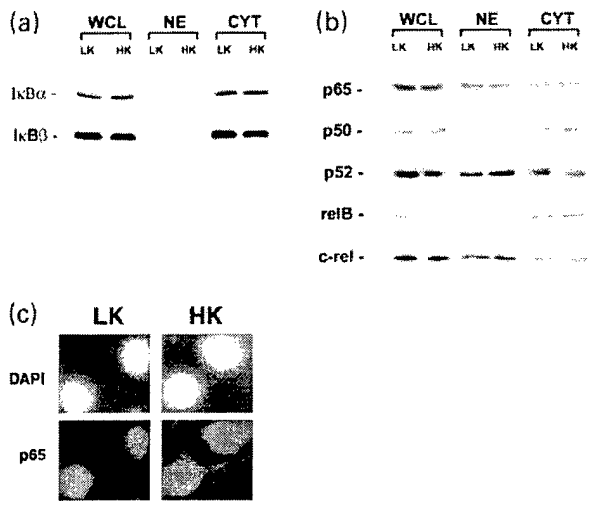

Fig. 5 Levels of IκB and NF-κB proteins are not altered in neurons primed to undergo apoptosis. Granule neurons (7–8-day-old cultures) were switched to HK or LK-medium for 6 h and subjected to western blotting (a and b) or switched for 24 h and subjected to immunofluorescence analysis (c). Approximately 10, 15 and 10 μg of total protein per lane from cytoplasmic and nuclear extracts, and whole cell lysates, respectively, were analyzed. That equal amounts of total proteins per HK or LK lane had been loaded was determined using Bradford assay of the proteins, Ponceau-staining of the membrane following electrophoretic transfer, and reprobing of the blots with a α-tubulin antibody (results not shown). Each blot was repeated at least three times using lysates from different cultures. (a) Analysis of IκBα and IκBβ expression. Whole cell lysates (WCL) or nuclear and cytoplasmic extracts (NE and CYT, respectively) were prepared and expression investigated using antibodies specific for IκBα and IκBβ. (b) Analysis of p50, p52, p65, c-Rel and RelB expression. Whole cell lysates (WCL) or nuclear and cytoplasmic extracts (NE and CYT, respectively) were prepared and expression analyzed. (c) Cultures that were switched to LK or HK medium for 24 h were fixed with 4% paraformaldehyde and cells subjected to immunofluorescence analysis using a polyclonal antibody against p65. (Upper) Nuclear staining with DAPI, (lower) p65 immunoreactivity. Only noncondensed cells are shown.

antibodies against p50 or p65. As shown in Fig. 3(c), incubation with the p50 antibody caused a supershift accompanied by a slight decrease in the intensity of complex 2 as well as complex 1. In contrast, the p65 antibody eliminated formation of complex 1 suggesting that the reduced activity of p65 is involved in the induction of apoptosis.

Overexpression of p65 inhibits while that of IκBα promotes apoptosis

To examine whether overexpression of p65 can prevent LK-induced neuronal death, neuronal cultures were transfected with a p65-expressing vector (CMV-p65) or a β-galactosidase-expressing vector (CMV-lacZ) and switched to LK medium. Whereas ≈ 50% of the untransfected cells and lacZ-expressing cells displayed condensed/fragmented nuclei after 24 h of K^+ deprivation, < 15% of the p65-expressing cells appeared apoptotic (Fig. 4a,b). As observed with p65, overexpression of p50 also protected granule neurons from LK-induced apoptosis (Fig. 4b).

Because NF-κB activation is normally regulated by IκB degradation and increased IκB expression would lead to a higher amount of NF-κB sequestration, we examined whether overexpression of IκBα would induce apoptosis under survival-promoting culture conditions. As shown in Fig. 4(c,d), > 75% of the neurons transfected with an IκBα-expressing vector appeared apoptotic in the presence of HK.

Steady-state levels of endogenous IκBα, IκBβ or p65 are not altered in neurons primed to die

Although our transfection experiments showed that p65 and p50 were protective against apoptosis and that IκBα overexpression induced neuronal apoptosis, they did not reveal whether LK-induced apoptosis was actually due to changes in endogenous IκB and NF-κB levels. To study this we performed western blot analysis of IκB and NF-κB expression 6 h after the switch to LK medium. We, and others, have shown that by 4–6 h of K^+ deprivation granule neurons are committed to dying and irreversible intracellular degenerative changes, such as DNA fragmentation, are underway (Yan *et al.* 1994; Galli *et al.* 1995; Schulz *et al.* 1996; Borodezt and D'Mello 1997; Nardi *et al.* 1997). More importantly, changes in the DNA-binding activity of NF-κB are clearly completed by 4 h of K^+ deprivation (Fig. 3). As expected, IκBα expression is confined largely to the cytoplasm in cultures maintained in HK (Fig. 5a). Surprisingly, no increase in IκBα level is discernible after K^+ deprivation (Fig. 5a). In addition to IκBα, translocation of NF-κB can be inhibited by IκBβ. Like IκBα, IκBβ levels remained unchanged following LK treatment (Fig. 5a).

Given that p65 has frequently been implicated in the promotion of cell-survival and because DNA-binding of p65 is reduced following K^+ deprivation, we examined its expression. No change was discernible in total cellular, nuclear or cytoplasmic p65-immunoreactivity following K^+ deprivation (Fig. 5b). To confirm that there were no alterations in the distribution of p65 following K^+ deprivation, we performed immunocytochemical analysis. In agreement with the data from Western blots, no difference in the intracellular localization of p65 was detectable between neurons treated with HK and LK medium (Fig. 5c). As observed for p65, no K^+ deprivation-associated changes were seen in the expression of p50, p52, c-Rel or RelB (Fig. 5b).

The extent of IκBβ, but not p65, phosphorylation is changed by K^+ deprivation

Besides increased nuclear translocation, p65 activity can also be increased by its phosphorylation (Wang and Baldwin

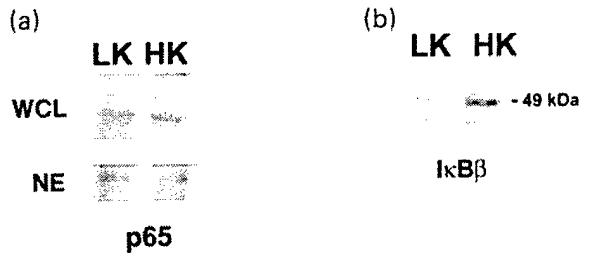

Fig. 6 IκBβ, but not p65 phosphorylation, is affected by K^+ deprivation. Neuronal cultures (7–8 days old) were switched to HK or LK medium. Phosphorylated proteins were labeled *in vivo* by the addition of [^{32}P] orthophosphate to cultures at the time of switching. Whole-cell lysates or nuclear extracts were prepared 6 h after the switch, p65 or IκBβ immunoprecipitated and analyzed by PAGE followed by autoradiography. (a) p65 phosphorylation in whole-cell lysates (WCL) and nuclear extracts (NE) prepared from cultures treated with HK and LK medium. (b) IκBβ phosphorylation in HK and LK whole-cell lysates (WCL). Similar results were obtained in two additional experiments using lysates from different cultures.

1998; Zhong *et al.* 1998). It was therefore conceivable that p65 was phosphorylated in healthy neurons and that the extent of its phosphorylation, and hence activity, was reduced upon K^+ deprivation. However, *in vivo* labeling and immunoprecipitation of p65 revealed that although phosphorylated to a small extent in healthy neurons, the level of phosphorylation is not changed by K^+ deprivation (Fig. 6a).

In vivo labeling revealed that IκBβ is also phosphorylated in HK (Fig. 6b). Interestingly, the extent of phosphorylation was reduced substantially following 6 h of K^+ deprivation. Similar analysis of IκBα showed no detectable phosphorylation in either LK or HK conditions (not shown).

Discussion

The survival effect of HK on cultured cerebellar granule neurons is inhibited by different pharmacological inhibitors of NF-κB, indicating that NF-κB is necessary for neuronal survival. Indeed, switching neurons from HK to LK medium causes alterations in the DNA-binding activity of various NF-κB complexes. Most significantly, a complex containing p65 is reduced at 4 h after K^+ deprivation. Given that these neurons become committed to death at 4–6 h after K^+ deprivation (Borodezt and D'Mello 1997), this finding suggests that LK-induced apoptosis may be due to reduced p65 activity. Consistent with this idea is the finding that overexpression of p65 prevents LK-induced apoptosis. Inhibition of apoptosis by p65 has been demonstrated in many cell types (Bellas *et al.* 1997; Levkau *et al.* 1999; Yang *et al.* 1999; Anto *et al.* 2000) and mice lacking p65 show increased apoptosis in the liver and die early during embryogenesis (Beg and Baltimore 1996).

The activity of p65 and other NF-κB proteins is normally regulated by IκB proteins by sequestration in the cytoplasm. Elevated expression of IκB thus inhibits the activity of NF-κB. Stimuli that activate NF-κB do so most often by inducing phosphorylation of IκB proteins, which triggers their degradation thus allowing NF-κB to translocate into the nucleus (Ghosh *et al.* 1998; Karin and Ben-Neriah 2000). Not unexpectedly, therefore, overexpression of IκBα promotes cell-death even in the presence of HK. Intriguingly, however, the level of endogenous IκBα, or that of IκBβ, is not reduced in HK-treated granule neurons. As observed for these IκB proteins, the level of endogenous p65 in nuclear and cytoplamic extracts is not altered by K^+ deprivation. To examine whether the decreased DNA-binding activity of p65 caused by LK treatment was due to an increase in the expression of some other NF-κB protein, which could then outcompete p65 for DNA-binding, we analyzed the levels of the other four members of the NF-κB family. No alterations in the expression of p50, p52, RelB and c-Rel were discernible. The absence of any alterations in the expression of NF-κB proteins is consistent with the finding that the cellular levels of IκB proteins are not affected by K^+ deprivation.

It is known that phosphorylation of p65 can increase its DNA-binding and transcriptional activity. It is therefore conceivable that LK-induced apoptosis was caused by decreased p65 phosphorylation, rather than by reduced translocation. *In vivo* labeling experiments, however, did not detect any change in the extent of phosphorylation following K^+ deprivation. Phosphorylation of p65 has been shown, in some cases, to promote interaction with the transcriptional coactivator CBP/p300 that leads to increased p65 transcriptional activity (Perkins *et al.* 1997; Merika *et al.* 1998). A recent report has shown that in PC12 cells, c-jun and p65 compete for binding to CBP (Maggirwar *et al.* 2000). Apoptosis in sympathetic neurons and granule neurons is preceded by an induction in c-jun expression and phosphorylation (Estus *et al.* 1994; Ham *et al.* 1995; Watson *et al.* 1998), raising the possibility that reduced p65 binding to CBP may contribute to apoptosis. Although we detected a modest interaction between CBP and p65, this was not reduced following K^+ deprivation (data not shown). Other novel mechanisms for the regulation of NF-κB activity have been reported. For example, it is known that p65 can dimerize with non-NF-κB proteins such as the Egr-1 transcriptional factor or the transcriptional corepressor AES, which then prevent p65 from interacting with target promoters (Chapman and Perkins 2000; Tetsuka *et al.* 2000). It is not known whether such mechanisms operate in granule neurons deprived of K^+.

NF-κB has also been shown to be involved in nerve growth factor (NGF)-mediated survival of sympathetic neurons (Maggirwar *et al.* 1998). In contrast to cerebellar granule neurons, degradation of IκBα was higher in

sympathetic neurons induced to die by NGF deprivation (Maggirwar *et al.* 1998). Overexpression of c-Rel was found to inhibit apoptosis caused by NGF deprivation (Maggirwar *et al.* 1998), although whether endogenous c-Rel expression or translocation is reduced by NGF deprivation was not examined directly. As observed in granule neurons, p65 expression was not affected by NGF deprivation in sympathetic neurons (Maggirwar *et al.* 1998).

One alteration that is associated with the induction of apoptosis in granule neurons is the reduced phosphorylation of IκBβ. It is not clear how higher IκBβ phosphorylation might enhance neuronal viability. Because the expression of IκBβ and NF-κB subunits are similar in HK and LK medium, it is unlikely that phosphorylation of IκBβ leads to its degradation. In addition to IKK, IκBα and IκBβ can be phosphorylated by other cellular kinases within the C-terminus. In the case of DNA-PK-induced phosphorylation, the stability of IκBβ is not affected (Liu *et al.* 1998). It is not known whether HK-induced IκBβ phosphorylation occurs at the two serine residues phosphorylated by IKK and which causes its degradation, or whether other kinases such as DNA-PK are involved in the HK-induced phosphorylation. Interestingly, DNA-PK has been implicated in the suppression of apoptosis in non-neuronal cell types (Nueda *et al.* 1999; for review, McConnell and Dynan 1996) and it is well established that this kinase is cleaved and inactivated by caspases during apoptosis (Casciola-Rosen *et al.* 1995; Han *et al.* 1996; Song *et al.* 1996). It is tempting to speculate that the decreased phosphorylation of IκBβ during LK-induced apoptosis may be the result of reduced activity of DNA-PK or a related kinase. In contrast to IκBβ, IκBα is not phosphorylated in healthy or apoptotic neuronal cultures.

In the absence of HK, the survival of granule neurons can be promoted by IGF-1 or cAMP. The survival-promoting effects of these factors are also inhibited by pharmacological inhibition of NF-κB. Furthermore, the alterations caused in the DNA-binding activity of NF-κB complexes seen after K^+ deprivation are prevented by IGF-1 and cAMP. Whereas HK causes membrane depolarization leading to Ca^{2+} influx through L-type voltage gated channels (Gallo *et al.* 1987; Galli *et al.* 1995), IGF-1 signals via the IGF-I receptor tyrosine kinase in a pathway involving phosphatidylinositol-3 kinase (D'Mello *et al.* 1997; Dudek *et al.* 1997; Miller *et al.* 1997). It is likely that cAMP activates a PKA-dependent pathway, which is independent of Ca^{2+} influx or phosphatidylinositol-3 kinase activation (Crowder and Freeman 1999; Moran *et al.* 1999). Our results suggest that NF-κB represents a point of convergence in the signaling pathways activated by these different survival factors.

In summary, although NF-κB activity is necessary for HK-mediated survival of cerebellar granule neurons, the mechanism by which it acts is unclear. In most cell types and in response to a variety of stimuli, NF-κB activity is regulated by the breakdown of IκB thus leading to increased levels of NF-κB proteins in the nucleus. Our finding that neither the levels of IκBα or IκBβ, nor those of the various NF-κB proteins are affected by K^+ deprivation argues against this mechanism of regulation. Similarly, no change in the extent of p65 phosphorylation or interaction of p65 with CBP/p300 is detectable after K^+ deprivation. It is possible that phosphorylation of IκBβ is protective for granule neurons and that the reduction of phosphorylation seen after the switch to LK is causally involved in the triggering of apoptosis. Taken together, our results suggest that uncommon and possibly novel mechanisms might be involved in NF-κB-mediated survival of granule neurons.

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Distinct phosphorylation patterns underlie Akt activation by different survival factors in neurons

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RUNNING TITLE : AKT IN NEURONAL SURVIVAL

Abstract

The survival of cultured cerebellar granule neurons can be maintained by depolarizing levels of potassium (high K+, HK), insulin-like growth factor (IGF-1), cyclic AMP or lithium. We examined the possibility that the signaling pathways activated by these different factors converge and that Akt might represent such a point of convergence. Consistent with this possibility, we find that Akt is phosphorylated and activated by all four survival factors. The pattern of Akt phosphorylation induced by the four survival factors, however, shows differences. While IGF-1 induces phosphorylation of Akt at both Ser473 and Thr308, HK and cyclic AMP stimulate phosphorylation at Thr308 only. Lithium increases phosphorylation at Ser473 but not at Thr308. Our results are consistent with the possibility that Akt is a central component of different survival-promoting pathways in granule neurons. The different phosphorylation patterns, however, point to a previously unappreciated complexity in the regulation of Akt activity in neurons. Finally, we provide evidence indicating that SGK, a kinase that is structurally related to Akt, is also activated by the four survival factors.

Introduction

Apoptosis is a cell-suicide program that is necessary for the normal development of the nervous system [reviewed in 14,18,37,52]. Deregulation of the apoptotic program results in aberrant cell-death which is believed to occur in several neurodegenerative pathologies [reviewed in 37,40]. The intracellular mechanisms by which apoptosis is inhibited and neuronal survival maintained are presently unclear although much insight has been gained using cultures of primary neurons. Cultures of cerebellar granule neurons represent one such system. The survival of these neurons can be maintained by depolarizing levels of extracellular potassium [K+; 24]. Switching of granule neuron cultures from a high K+ (HK) medium to one with low K+ (LK) induces apoptosis [15]. In addition to HK, several other factors/agents have survival-promoting activity on these neurons including insulin-like growth factor, cyclic AMP, lithium and the calcium-ATPase inhibitor, thapsigargin [15,16,17, 34]. Interestingly, HK, cyclic AMP, and thapsigargin can also prevent apoptosis of peripheral sympathetic neurons which are normally dependent on nerve growth factor for their survival [21,33,42,44,45]. Similarly, the *in vitro* survival of other central and peripheral neuronal types and of differentiated neuronal PC12 cell-line can be supported by HK and cAMP, in addition to specific growth factors [25,29,45].

While growth factors such as IGF-1and NGF activate receptor tyrosine kinases and promote neuronal survival by a phosphotidylinositide-3 kinase (PI-3K)-dependent pathway [10,17,19,20,26,36], HK-treatment causes membrane depolarization resulting in the influx of calcium through voltage-gated channels which is necessary for HK-mediated survival [23,30]. In cerebellar granule neurons, survival by HK occurs via a PI-3K-independent pathway [17,20] although this conclusion has been contradicted by some studies [36,47]. Neuronal survival by cyclic AMP on the other hand, requires neither elevated intracellular calcium nor PI-3K activation [10,37]. Although affecting distinct cell-surface molecules, the signaling pathways activated by these different survival promoting factors may

converge on common downstream molecules. If so, such molecules would be ideal targets for the development of therapeutic approaches aimed at curing or preventing neuronal degeneration seen in a number of neuropathological conditions.

One molecule that may serve as a convergent point of different survival-promoting signaling pathways is Akt, a 60 kDa serine/threonine kinase which can be activated by a variety of growth factors including IGF-1 and NGF [reviewed in 1, 9,28]. Activation of Akt has been shown to inhibit apoptosis, and thus promote survival, in many different cell-types and in response to several different stimuli. Growth factor-mediated activation of Akt is dependent on PI-3K activity and occurs via phosphorylation of two residues – Ser473 and Thr308 [1,9,28]. Mutational analysis has shown that while Thr308 is sufficient to activate Akt, both residues are required for maximal activation. In contrast to growth factor-mediated stimulation, however, HK treatment of the NG108 cell-line activates Akt in a PI-3K-independent pathway involving calcium-calmodulin kinase kinase [CamKK; 51]. Similarly, cyclic AMP may activate Akt in a PI-3K-independent manner [22,46]. Once activated Akt is though to maintain cell-survival by inhibiting the actions of proapoptotic molecules such as the bcl-2-related protein, Bad [11,12], caspase-9 [7], the Forkhead transcription factor [5], and the IκB-phosphorylating kinase IKK [41,43].

In this study, we have used cultured cerebellar granule neurons to examine the role of Akt in the survival effects of four different factors - HK, IGF-1, cAMP, and lithium. We show that all four survival factors activate Akt. Interestingly, however, there are differences in the pattern by which these different survival factors phosphorylate Akt. Furthermore, SGK a kinase related to Akt is also phosphorylated by the different survival factors.

Unless specified otherwise, all chemicals and reagents were obtained from Sigma Chemical Co (St. Louis, Mo). Antibodies to Akt were obtained from Cell Signaling Technologies (Beverly, MA). Recombinant IGF-1 was purchased from Roche Biochemicals (Indianapolis, IN).

Granule neuron cultures were obtained from dissociated cerebella of 7-8 day old rats as described previously [15]. Cells were plated in Basal Eagle's Medium with Earles salts (BME) supplemented with 10% fetal calf serum (FCS), 25 mM KCl, 2 mM glutamine (Gibco-BRL), and 100 ug/ml gentamycin on dishes coated with poly-L-lysine in 24-well dishes at a density 0.5 x 10^6 cells/well or 2.5 x 10^7 cells/100 mm dish. Cytosine arabinofuranoside (10 uM) was added to the culture medium 18 - 22 h after plating to prevent replication of nonneuronal cells. Unless indicated otherwise, cultures were maintained for 6 - 7 days prior to experimental treatments with IGF-1 or pharmacological agents. For this, the cells were rinsed once and then maintained in low K^+ medium (serum-free BME medium, 5 mM KCl) with or without the agents, or in the case of control cultures, in high K^+ medium (serum-free BME medium, supplemented with 20 mM KCl). The final concentrations of various factors and agents were as follows : 25mM KCl in HK medium, 50 ng/ml IGF, 10 uM forslokin, and 10 mM lithium chloride.

For immunoprecipitation analysis, cells were placed on ice and harvested using non-denaturing lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM Sodium pyrophosphate, 1 mM Glycerophosphate, 1 mM Na_3Vo_4, 1 μg/ml leupeptin). The lysates were centrifuged for 10 min at 10,000 g and the protein Akt was immunoprecipitated from 200 ug of cell free extracts. The immune complexes were washed twice with lysis buffer. When in vitro kinase assay was carried out the immune complexes were washed with twice with kinase buffer (25 mM Tris (pH 7.5), 5 mM ß-glycerophosphate, 2 mM DTT, 0.1 mM Na_3Vo_4, 10 mM $MgCl_2$). In vitro kinase assays were performed for 30 min at 30°C in 40 μl of reaction volume containing the 30 μl of immunoprecipitates in kinase buffer, 200 μM ATP. GSK-3 fusion protein (Cell Signaling Technology) was used as a substrate for Akt kinase activity. The reactions were terminated with 20 μl 3-x SDS sample buffer.

For Western blot analysis, cell lysates from granule neuron cultures and immunoprecipates were resolved by 10% SDS-PAGE and transferred to Poly Vinyl Dipyrolidone Fluoride (BioRad). The membrane was blocked in 5% non-fat dry milk

for 30min Tris-buffered saline containing tween-20. The membrane was incubated with an antibody overnight, diluted 1000 fold in 5% BSA solution. The secondary antibody was a HRP conjugated to the rabbit IgG or Sheep IgG diluted 4000-fold in the blocking buffer. The detection and quantification of protein was carried out using the ECL kit from Amersham-Pharmacia

Cultured cerebellar granule neurons undergo apoptosis when switched from HK medium to one with LK. Although neuronal death begins after about 16 h, commitment to death takes place within the first 6h and characteristic features of apoptosis, such as chromatin condensation and DNA fragmentation can be observed after 8 hours [4,23,39,48]. Apoptosis caused by K+ deprivation can inhibited by IGF-1, lithium and forskolin [15,16]. To examine whether the signaling pathways activated by these factors converge at Akt, we examined whether Akt is activated by these four factors. As shown in Fig.1, treatment with all four factors resulted in Akt activation, consistent with the idea that it might represent a convergent point in survival promotion.

It has previously been shown that in granule neurons Akt is phosphorylated by IGF-1, although the sites of phosphorylation were not identified (Dudek et al., 1997). We have confirmed this finding using antibodies specific for Akt phosphorylated at Ser473 and Thr308. Both these antibodies are phospho-specific and do not recognize unphosphorylated Akt in our experience (not shown). As shown in Fig. 2 and consistent with growth-factor mediated Akt activation, treatment of granule neurons with IGF-1 results in increased phosphorylation at both Ser473 and Thr308. Increased phosphorylation at both sites is seen within 2 min and is maximal at 15 min after which the level is somewhat reduced but persists for at least 60 min (Fig. 2). While Akt phosphorylation at Thr308 is not detectable in unstimulated cells, Ser473 is phosphorylated even before IGF-1 treatment (Fig. 2). That Ser473 is phosphorylated under unstimulated conditions has been confirmed using a monoclonal antibody that is totally specific for phosphorylated Ser473 and which does not cross-react with unphosphorylated Akt.

Treatment with HK also results in increased phosphorylation at Ser 308 (Fig. 1). But in contrast to IGF-1, HK treatment does not increase phosphorylation of Ser473 beyond basal levels (Fig. 1), even when analysis was extended to 2 h following HK addition (unpublished observation). Basal levels of Ser473 phosphorylation does, however, appear to be necessary for neuronal survival as deprivation of all trophic support results in a reduction of phosphorylation to barely detectable levels within 6h (Fig. 3), which coincides with the time at which these neurons are committed to death.

In contrast to both IGF-1 and HK, treatment with lithium treatment causes phosphorylation at Ser473 but not at Thr308. Finally forskolin treatment, which increases intracellular cAMP levels, results in increased phosphorylation at Thr308 but not at Ser473, a pattern that is different from that elicited by IGF-1 and lithium, but which is similar to HK treatment. Although results of Akt phosphorylation are shown at 15 min only in Fig. 1, the failure of lithium to phosphorylate Akt at Ser308, and of forskolin to phosphorylate Ser473, was confirmed in analysis extending for time-points ranging from 5 min to 2 h following addition of these agents.

Work done in a number of laboratories has shown that Akt phosphorylation at Thr308 is mediated by PDK-1[1,2,28]. How Akt is phosphorylated at Ser473 is less clear but is believed to be a consequence of Thr308 phosphorylation. One hypothesis is that Ser473 may be phosphorylated by the integrin-linked kinase / PDK-2 [13]. More recent studies have found that Ser473 can also be phosphorylated by PDK-1 although this requires its interaction with a protein fragment, PIF [2]. It is also possible that Ser473 is phosphorylated by Akt itself following its phosphorylation at Thr308 [49]. Our finding that Ser473 phosphorylation is stimulated in the absence of Thr308 phosphorylation (following lithium treatment) argues against the Thr308-induced autophosphorylation model.

In addition to Akt, immunoprecipitation with Akt antibody pulls down a 49 kDa protein that is recognized by both the phospho-Ser473 and phospho-Thr308-specific antibody (Fig.1). We find that the 49kDa protein is also phosphorylated

following treatment with the four survival factors albeit with a slightly different profile; it is most strongly phosphorylated by forskolin at a site that is recognized by the phospho-Ser473 and the phospho-Thr308 antibody, whereas IGF-1 has the least stimulatory effect. Whether the 49kDa protein represents another Akt isoform is not known at this time. One possibility is that this protein is serum-glucocorticoid kinase (SGK) a 49kDa serine/threonine kinase that shares considerable structural similarity with Akt, and which like Akt, is rapidly phosphorylated by IGF-1 and serum [31,32,50]. While this manuscript was in preparation, Brunet et al. (2001) demonstrated the ability of IGF-1 to stimulate SGK phosphorylation in granule neurons. Furthermore, these authors and another group [35] reported the ability of SGK to inhibit apoptosis in cell-lines. Previous work has shown that activation of SGK by IGF-1 requires phosphorylation at two sites - Thr256 and Ser422. The residues around Thr256 and Ser422 are virtually identical to that of Thr308 and Ser473 of Akt, respectively, making cross-reactivity of the phospho-Akt antibodies a possibility. To test the possibility that the 49 kDa protein was SGK, immunoprecipitation was performed using Akt antibody and the immunoprecipitate subjected to immunoblotting using an SGK antibody. As shown in Fig. 4, the 49 kDa band is strongly recognized by the SGK antibody, which also cross-reacts to a small extent with Akt.

Previous studies have shown the ability of SGK to phosphorylate GSK-3 and other substrates commonly used to measure Akt activation, including the synthetic peptide Crosstide. Thus, although our activity assays using immunoprecipitated Akt and Gsk-3 substrate are likely to primarily reflect Akt activation, given the fact that Akt and SGK share structural similarities and that the Akt antibody crossreacts with SGK, a contribution of SGK activity in assays cannot be excluded in our studies (and in other studies using the same approach).

In summary - although HK, IGF-1, cAMP, and lithium phosphorylate Akt, they do so in three distinct patterns – while phosphorylation at both Ser473 and Thr308 is stimulated by IGF-1, HK and forskolin induce phosphorylation at Thr308 only, and lithium increases Akt phosphorylation at Ser473 alone. Therefore,

contrary to what is generally believed, phosphorylation at the two Akt phosphorylation sites is not coordinated as commonly believed [for review, 9]. Moreover, our results implicate SGK as another kinase that is activated by survival-promoting stimuli in neurons. Along with the two reports showing inhibition of apoptosis in cell-lines by SGK, our findings suggest that like Akt, SGK is a general inhibitor of apoptosis.

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Figure legends

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min later and immunoprecipitated using an Akt antibody. The immunoprecipitate was subjected to Western blotting using an SGK antibody.

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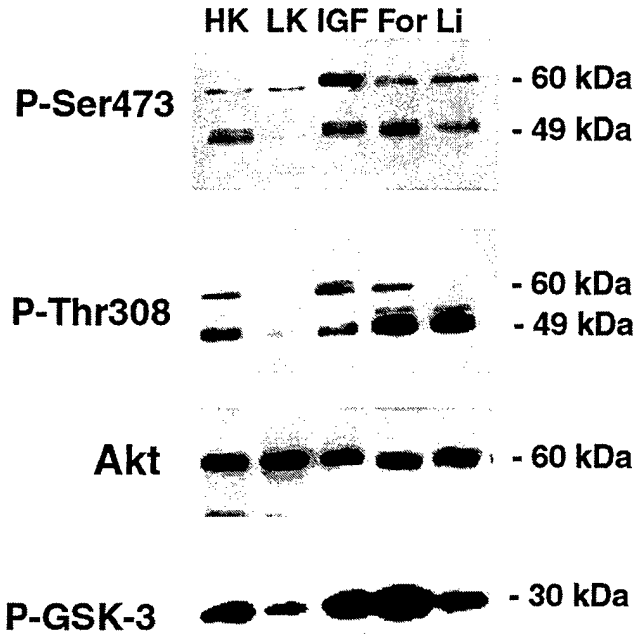
HK LK IGF For Li
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- 49 kDa
P-Thr308
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- 49 kDa
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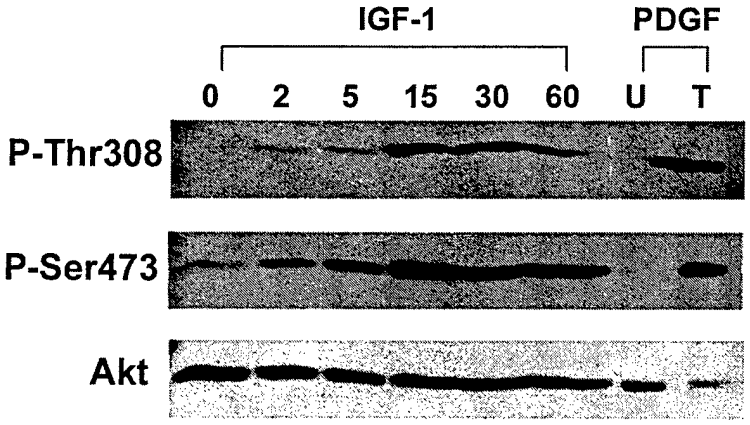
- 60 kDa
P-GSK-3
- 30 kDa

FIGURE 1



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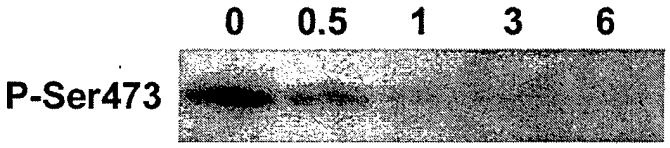

FIGURE 3

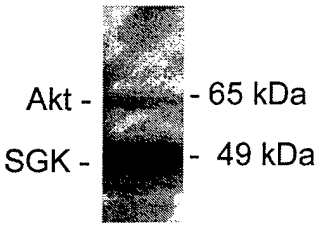

FIGURE 4