PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Block of Neuronal Apoptosis by a Sustained Increase of Steady-State Free Ca<latex>$^{2+}$</latex> Concentration

James L. Franklin and Eugene M. Johnson

Phil. Trans. R. Soc. Lond. B 1994 **345**, 251-256
doi: 10.1098/rstb.1994.0102

Block of neuronal apoptosis by a sustained increase of steady-state free Ca^{2+} concentration

JAMES L. FRANKLIN AND EUGENE M. JOHNSON JR

Department of Molecular Biology and Pharmacology, Washington University School of Medicine, 660 South Euclid Avenue, St Louis, Missouri 63110, U.S.A.

SUMMARY

Programmed death is a ubiquitous feature of the development of the vertebrate nervous system. This death is prevented *in vivo* by trophic factors and by afferent input. Death of neurons can also be prevented in culture models of programmed death by trophic factors and by chronic depolarization with elevated concentrations of K^+ in the culture medium. The latter effect is mediated by Ca^{2+} influx through voltage-gated channels and may prevent death by mimicking survival-promoting effects of naturally occurring electrical activity. Little is currently known about the mechanism by which either trophic factors or increased cytoplasmic Ca^{2+} promote survival.

1. INTRODUCTION

During the neurogenesis of the vertebrate nervous system, about fifty percent of neurons produced die at about the time functional connections to target tissues are being made (Oppenheim 1991). As this death is a normal component of development rather than a pathological process, it is called naturally occurring or programmed cell death (PCD). The apparent purpose of this death is to sculpt the developing nervous system by matching the number of neurons innervating a target tissue with the target size. Neurotrophic factors, released by target or other cells, are thought to be the principal agents controlling neuronal survival during and after PCD. Secreted in minute amounts, these factors bind to receptors on neuronal processes where they are internalized and retrogradely transported with the receptors to the neuronal soma. A retrogradely transported signal(s), which may be conveyed via the ligand–receptor complex, initiates mechanisms that maintain cellular survival and stimulates a variety of trophic responses. It is believed that neurons obtaining a sufficient amount of trophic factor survive, whereas those that are inadequately supplied undergo PCD.

Neuronal PCD resulting from insufficient availability of trophic factors can be mimicked *in vivo* by separating neurons from their targets via axotomy or target removal. As with naturally occuring PCD, the death caused by these insults can be averted by systemic administration of the appropriate trophic factor (Hendry & Campbell 1976; Hamburger *et al.* 1981) or, after axotomy, by administration of trophic factor at the lesion site (Rich *et al.* 1987). Systemic treatment of developing animals with neutralizing antibodies to growth factors also mimics PCD. Thus, neuronal PCD caused by inadequate amounts of trophic factors can be modelled *in vivo* via antibody administration, axotomy or target removal.

The physiological role of the prototypical neurotrophic factor, nerve growth factor (NGF), is the basis for the most studied neuronal death caused by insufficient availability of trophic factor. Depriving immature sympathetic (Levi-Montalcini & Booker 1960) or certain sensory neurons (Johnson *et al.* 1980) of NGF produces massive cell death *in vivo* and also in cell culture. NGF deprivation causes extensive sympathetic neuronal death even in adult animals indicating a continued requirement for its availability, although the rate of this death in older animals is considerably slower than in immature ones (Gorin & Johnson 1980). *In vivo*, NGF also blocks death caused by axotomy, target removal, and even certain chemical (Johnson & Aloe 1974) insults to these neurons. Thus, death of sympathetic and sensory neurons caused by NGF deprivation provides an appropriate physiological model for studying neuronal PCD. Dissociated embryonic rat superior cervical ganglion neurons (SCG) maintained in cell culture for 5–7 days in the presence of NGF hypertrophy and develop extensive neurites. Most of these neurons die within 48 h after being deprived of NGF. This death is preceded by condensation of chromatin, membrane blebbing, fragmentation of neurites, and atrophy of the cell soma. Prior to demise the DNA of NGF-deprived SCG neurons becomes fragmented into oligonucleosomes (Edwards *et al.* 1991; Deckwerth & Johnson 1993). Descriptions of morphological changes after trophic factor deprivation of other types of neurons are similar (see Pilar & Landmesser 1976). The morphological changes and DNA fragmentation that occur after neurotrophic factor deprivation

Phil. Trans. R. Soc. Lond. B (1994) **345,** 251–256
Printed in Great Britain

suggests that the death of these cells is apoptosis, a type of death first identified in non-neuronal cells (Duvall & Wyllie 1986). Further evidence that the death of sympathetic neurons after trophic factor deprivation is apoptotic comes from experiments with inhibitors of macromolecular synthesis. In some types of cells, apoptosis appears to require production of new proteins since it is blocked by inhibitors of RNA and protein synthesis. Sympathetic neuronal PCD also exhibits this characteristic; block of macromolecular synthesis with inhibitors such as actinomycin-D and cycloheximide completely prevents death of these cells after NGF deprivation *in vitro* (Martin *et al.* 1988). Block of PCD by inhibition of macromolecular synthesis has also been demonstrated *in vivo* in motor neurons of chicken embryos (Oppenheim *et al.* 1990).

2. ELEVATED POTASSIUM BLOCKS NEURONAL PCD *IN VITRO*

Scott & Fisher (1970) found that elevated extracellular K^+ concentrations ($[K^+]_o$) can maintain the viability of embryonic chicken dorsal root ganglion neurons *in vitro* that would die in normal $[K^+]_o$ (about 5 mM in vertebrates). Subsequent work from several laboratories demonstrated a similar effect of high $[K^+]_o$ on the survival of many other types of neurons from both the peripheral and central nervous systems (Franklin & Johnson 1992). Figure 1 illustrates the ability of increased $[K^+]_o$ to promote the survival of rat SCG neurons in the absence of NGF. When these cells are maintained in culture by high $[K^+]_o$ they appear morphologically very similar to neurons maintained by NGF and will live and remain healthy for weeks or months without NGF. While other agents (such as those increasing cellular cAMP, for example) are capable of supporting survival of sympathetic neurons in the absence of NGF (Martin *et al.* 1992), none have as potent an effect on survival as high $[K^+]_o$. In fact, it appears that high $[K^+]_o$ can almost exactly substitute for NGF in supporting survival of sympathetic neurons in culture (Franklin *et al.* 1994).

Intracellular and extracellular K^+ concentrations are the primary determinants of the resting membrane potential of neurons. Increasing $[K^+]_o$ *in vitro* causes depolarization to a new resting membrane potential at which cells remain for the duration of the exposure to the elevated $[K^+]_o$ (Chalazonitis & Fischbach 1980). Such sustained depolarization may promote survival by imitating the effects of naturally occurring electrical activity, i.e. afferent input (Franklin & Johnson 1992). This idea is supported by numerous studies demonstrating that pharmacological blockade of electrical activity or removal of afferent input can increase the death of some populations of developing neurons (for review, see Oppenheim 1991). Nishi & Berg (1981) demonstrated that enhancement of the survival of chicken ciliary ganglion neurons by high $[K^+]_o$ is blocked by the relatively non-selective Ca^{2+} channel antagonists D-600 and Mg^{2+}, suggesting that influx of Ca^{2+} through voltage-gated channels is responsible for mediating the survival-promoting effects of depolarization. More recent work has shown that promotion of the survival of several types of neurons by high $[K^+]_o$ can be prevented by the more specific dihydropyridine (DHP) Ca^{2+} channel antagonists (Gallo *et al.* 1987; Collins & Lile 1989; Koike *et al.* 1989). Conversely, at least some neurons are made more responsive to increased $[K^+]_o$ by DHP Ca^{2+}-channel agonists. These data suggest that, in these types of neurons, chronic depolarization causes activation of DHP-sensitive or L-type Ca^{2+} channels (Fox *et al.* 1987) and that influx of Ca^{2+} through these channels is responsible for the enhanced survival.

In addition to the pharmacological evidence, a role for voltage-gated Ca^{2+} channels in high $[K^+]_o$-promoted survival is supported by measurements of intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) with the Ca^{2+}-sensitive dye fura-2 (Grynkiewicz *et al.* 1985). Acute exposure of neurons to elevated $[K^+]_o$ causes a rapid elevation of $[Ca^{2+}]_i$. After the initial increase, $[Ca^{2+}]_i$ falls, in at least some types of neurons, to a new steady-state concentation that remains elevated above baseline levels for hours or days with continued exposure to increased $[K^+]_o$. A sustained rise of $[Ca^{2+}]_i$ in K^+-depolarized neurons has now been demonstrated in rat sympathetic neurons (Koike & Tanaka 1991; Franklin *et al.* 1994), chicken ciliary ganglion neurons (Collins *et al.* 1991), and rat cerebellar granule neurons (Bessho *et al.* 1994). Treatment of neurons with agents that block promotion of survival by chronic depolarization, such as DHP Ca^{2+}-channel antagonists, return the sustained rise of $[Ca^{2+}]_i$ to basal, or near basal, levels. Figure 2 illustrates the effects of elevated $[K^+]_o$ and the DHP antagonist, nifedipine, on steady-state $[Ca^{2+}]_i$ and survival of rat SCG neurons in culture. In these cells low concentrations of nifedipine block both the sustained rise of $[Ca^{2+}]_i$ caused by K^+ depolarization and also block survival. These data suggest that the sustained increase of $[Ca^{2+}]_i$ caused by influx of Ca^{2+} through DHP-sensitive, voltage-gated channels is responsible for promoting survival of these cells rather than some other effect of depolarization or high $[K^+]_o$.

In addition to chronic depolarization, we have found that NGF-deprived rat sympathetic neurons are saved from death by treatment with the sesquiterpene lactone, thapsigargin (Lampe *et al.* 1992), a potent and selective inhibitor of a Ca^{2+} pump responsible for sequestration of Ca^{2+} into a subset of intracellular Ca^{2+} stores (Thastrup *et al.* 1990). In many types of cells, exposure to thapsigargin causes a rapid rise of cytoplasmic $[Ca^{2+}]_i$ as Ca^{2+} leaks out of these stores after pump inhibiton. The plasma membrane Ca^{2+} pumps, which are unaffected by thapsigargin, then remove excess Ca^{2+} and reduce $[Ca^{2+}]_i$. However, $[Ca^{2+}]_i$ in some types of cells does not return to baseline concentrations after thapsigargin exposure but remains elevated at a new steady-state level. It is thought that a messenger molecule, released by depletion of the Ca^{2+} stores, activates a plasma membrane Ca^{2+} conductance and the resulting Ca^{2+} influx causes the sustained rise of $[Ca^{2+}]_i$

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

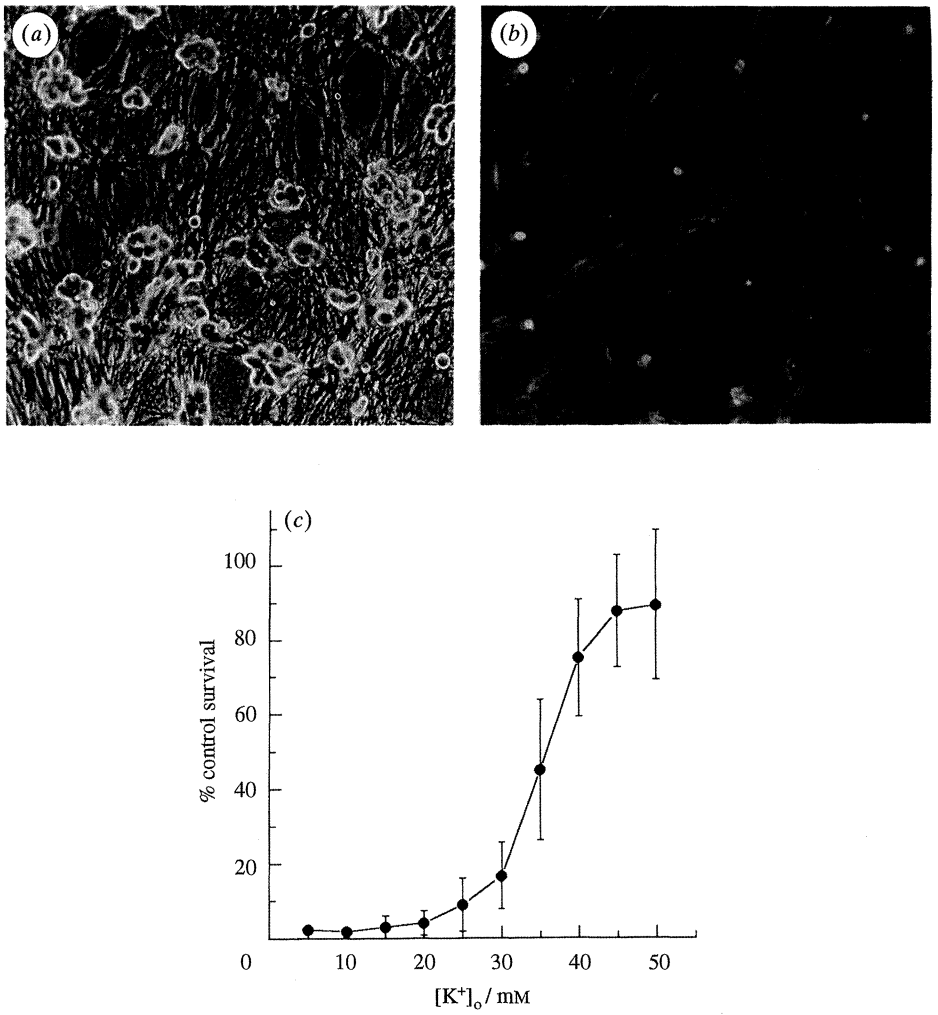


Figure 1. Effect of increasing $[K^+]_o$ on survival of NGF-deprived rat sympathetic neurons. (*a*) Culture of rat SCG neurons maintained for 6 days in medium containing an anti-NGF antibody and 50 mM extracellular K^+ with no NGF. Neurons are alive and healthy. (*b*) Culture maintained in medium with 5 mM extracellular K^+, but without NGF for 6 days. All neurons have died. (*c*) Quantification of the ability of different $[K^+]_o$ to maintain survival of these neurons in the absence of NGF. Cells were deprived of NGF 6 days after plating embryonic-day-21 cells and were exposed to the indicated $[K^+]_o$ for 4 days. Survival was assayed by blinded counting. Control survival is the number of cells maintained in NGF and 5 mM K^+ for the same period as the deprived cells. Error bars are s.d., $n = 8–12$ for each data point.

(Hoth & Penner 1992; Putney 1993). Our work shows that thapsigargin not only saves NGF-deprived sympathetic neurons from dying, but also causes a DHP-insensitive sustained increase of $[Ca^{2+}]_i$. Increasing extracellular Ca^{2+} in the presence, but not absence, of thapsigargin increases $[Ca^{2+}]_i$ to levels similar to those associated with optimal survival with $[K^+]_o$ but does not depolarize cells to membrane potentials that are associated with survival promotion by elevated $[K^+]_o$ (unpublished observation). Therefore, thapsigargin treatment provides a means for inducing a sustained rise of $[Ca^{2+}]_i$ that prevents PCD, is independent of depolarization, and independent of activation of L-type Ca^{2+} channels. This finding lends strong support to the notion that the elevation of steady-state $[Ca^{2+}]_i$ is responsible for promoting survival by high $[K^+]_o$.

Based upon our work and that of others, we have suggested a 'Ca^{2+} set-point' hypothesis of neuronal survival and neurotrophic factor dependence (Koike *et al.* 1989; Franklin & Johnson 1992; Johnson *et al.* 1992). This hypothesis is similar to that of Kater *et al.* (1988) regarding the effects of $[Ca^{2+}]_i$ on neurite outgrowth. The survival set-point hypothesis posits four steady-state levels, or set-points, of $[Ca^{2+}]_i$ that affect neuronal survival. The first set-point encompases levels of $[Ca^{2+}]_i$ that are too low to support essential Ca^{2+}-dependent processes and cause neuronal death even in the presence of appropriate neurotrophic factors. The existence of this set-point is supported by experiments showing that very low levels of $[Ca^{2+}]_i$ are deleterious to neuronal growth and survival in cell culture (Tolkovsky *et al.* 1990; J. L. Franklin & E. M. Johnson, unpublished observations). Although neurons can be killed *in vitro* by reducing $[Ca^{2+}]_i$ to low levels by incubating them in medium containing low Ca^{2+} concentrations, *in vivo*, these levels of $[Ca^{2+}]_i$ probably never occur and this set-point is, therefore, of little relevance to physiological situations. In the presence of

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

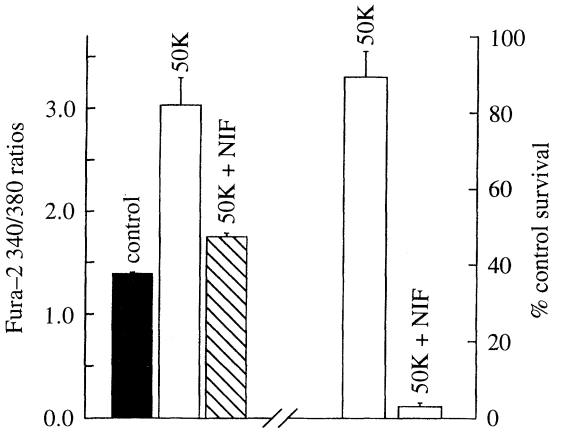


Figure 2. Effect of elevated $[K^+]_o$ on $[Ca^{2+}]_i$ and survival of SCG neurons maintained in culture medium containing 50 mM K^+ (50 K) and no NGF. Chronic depolarization with high $[K^+]_o$ caused a sustained increase of steady-state $[Ca^{2+}]_i$ (shown as higher Fura-2 340/380 ratios). The DHP Ca^{2+} channel antagonist nifedipine largely blocked this increase. Cells were depolarized with 50 K for 24 h and exposed to 100 nM nifedipine for 3–4 h before measurements were made. $n = 25–27$ for each bar. Survival enhancement of NGF-deprived neurons caused by growth in 50 K medium was also blocked by 100 nM nifedipine. Cells were deprived of NGF on the fifth day after plating and maintained four additional days in 50 K ± nifedipine. Survival was then assayed by blinded counting. $n = 9–12$ for each bar.

neurotrophic factors, resting $[Ca^{2+}]_i$ (about 100 nM in many cells; the second set-point) is adequate for the survival of trophic-factor-dependent neurons. A modest sustained elevation of $[Ca^{2+}]_i$, such as that induced by elevated $[K^+]_o$ or thapsigargin treatment (100 nM to several 100 nM), converts neurons to trophic-factor-independence. Thus, $[Ca^{2+}]_i$, over a limited range, determines the trophic factor dependence of neurons. These levels of $[Ca^{2+}]_i$ comprise the third set-point of our hypothesis. This set-point is of possible physiological relevance as the electrical activity of neurons resulting from afferent input can increase $[Ca^{2+}]_i$ and may reduce the requirement for neurotrophic support from target tissues (Schmidt & Kater 1993). Very high levels of $[Ca^{2+}]_i$ such as those occurring during excitotoxicity stimulate injurious processes within cells and are toxic to neurons (Choi 1987, 1988). These toxic levels of $[Ca^{2+}]_i$ comprise the fourth set-point of the hypothesis. Therefore, the near absence of $[Ca^{2+}]_i$ (the first set-point) or an excess of $[Ca^{2+}]_i$ (the fourth set-point) cause neuronal death; the second set-point allows neuronal survival with support by neurotrophic factor, while the third set-point promotes survival of neurons independently from neurotrophic factor.

3. MECHANISMS BY WHICH INCREASED $[Ca^{2+}]_i$ PROMOTES SURVIVAL

Little is known about the mechanisms underlying survival-promotion by elevated $[Ca^{2+}]_i$. However, because intracellular free Ca^{2+} is a major second-messenger molecule, it is likely that increased $[Ca^{2+}]_i$ promotes survival by stimulating a signal transduction pathway, possibly the same one stimulated by trophic factors. Identification of Ca^{2+} signalling pathways for survival may, therefore, also provide information about trophic-factor signalling. Calcium signals are translated into cellular events by intracellular Ca^{2+}-binding proteins. Of these proteins the most ubiquitous and abundant is calmodulin, the principal intracellular Ca^{2+} receptor of all non-muscle eukaryotic cells. When bound to Ca^{2+}, calmodulin modulates the activity of a number of Ca^{2+}-dependent molecules that are involved in a myriad of cellular events. Because of its abundance, ubiquity, and multiple effects, calmodulin is a reasonable candidate Ca^{2+}-binding protein for the initial signal transduction event that leads to Ca^{2+}-promoted survival. We have found that the calmodulin antagonists, calmidazolium and W7, prevent survival of depolarized NGF-deprived rat sympathetic neurons at concentrations that do not affect survival in the presence of NGF and normal $[K^+]_o$. However, these compounds appear to inhibit survival by blocking the sustained increase of Ca^{2+} caused by the chronic depolarization rather than through a specific effect on calmodulin (Franklin *et al.* 1992, 1994). The block of the sustained $[Ca^{2+}]_i$ increase is probably caused by antagonism of Ca^{2+} channels carrying the Ca^{2+} influx responsible for the increased $[Ca^{2+}]_i$ (Doroshenko *et al.* 1988). Recently, Hack *et al.* (1993) showed that calmodulin antagonists block depolarization-enhanced survival of embryonic rat cerebellar ganglion neurons at concentrations that do not significantly affect Ca^{2+} influx and that an antagonist of Ca^{2+}-calmodulin-dependent protein kinase (CAM-kinase) blocks depolarization-enhanced survival of these cells. These results suggest that a calmodulin-CAM-kinase pathway is responsible for the effect of Ca^{2+} on survival in these neurons. However, the effect on $[Ca^{2+}]_i$ of these compounds was not determined leaving open the possibility that the block of survival may be through an effect on $[Ca^{2+}]_i$ rather than on calmodulin. Indeed, preliminary experiments in our laboratory with fura-2 loaded granule cells have found that a survival-inhibiting concentration of calmidazolium (1 μM) blocks the sustained increase of $[Ca^{2+}]_i$ caused by 25 mM extracellular K^+ (T. Miller & E. M. Johnson, unpublished observations). We have not yet tested the effects of CAM-kinase inhibitors on $[Ca^{2+}]_i$ in these cells. Thus, while calmodulin may well be a mediator of Ca^{2+}-promoted neuronal survival, lack of specificity of available calmodulin inhibitors has hampered investigation of this possibility.

Another protein whose activity can be affected by increases of $[Ca^{2+}]_i$, protein kinase C (PKC), has been implicated in depolarization-promoted survival of embryonic chicken sympathetic neurons in culture. In these cells depolarization with elevated $[K^+]_o$ increases protein kinase C (PKC) activity and phorbol esters, which mimic PKC activation by diacyl glycerol, can substitute for chronic depolarization in promoting survival (Wakade *et al.* 1988). However, phorbol esters have no effect on survival of NGF-deprived rat sympathetic neurons (Martin *et al.* 1992); thus, PKC is probably not involved in depolarization-enhanced survival of these cells. It is

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

also unlikely that Trk, the high-affinity NGF receptor, is a part of the signal-transduction pathway for depolarization/Ca^{2+}-promoted survival of sympathetic neurons. We have found that, in SCG neurons, NGF causes constitutive phosphorylation of Trk on tyrosine residues, an indication of the activation of Trk tyrosine kinase activity (Franklin *et al.* 1992). Removal of NGF causes this phosphorylation to drop to undetectable levels. After deprivation, NGF readdition causes rapid rephosphorylation of Trk but depolarization has no effect. Therefore, it appears that Ca^{2+} and NGF signalling, if they converge on the same survival-signalling pathway do so downstream of Trk. A possible point of convergence of the two survival promoting signals is MAP kinases. In PC12 cells, MAP kinases show increased tyrosine phosphorylation when the cells are exposed to either NGF or elevated $[K^+]_o$ (Tsao *et al.* 1990). In total cell lysates of rat SCG neurons, we have found that a 44 kDa protein, probably a MAP kinase, is tyrosine phosphorylated by NGF and chronic depolarization (Franklin *et al.* 1994). It will be of interest to study further the effects of trophic factors and depolarization on the activity of these kinases and to characterize the downstream effects of their activation that may be involved in promotion of survival.

When embryonic rat SCG neurons are maintained in culture in the presence of NGF, they not only survive but exhibit sustained growth for at least several weeks after initial plating. The cell soma hypertrophies, neurite outgrowth occurs at a linear rate, and total protein content increases at a linear rate. When these cells are maintained in the absence of NGF with optimal survival-promoting $[K^+]_o$ little, if any, somatic hypertrophy, neurite outgrowth, or increase of protein content occurs. Therefore, although removal of NGF in the presence of optimal survival-promoting $[K^+]_o$ (50 mM) maintains these cells in a viable state, growth almost completely ceases. Thus, while NGF promotes both growth and survival of rat sympathetic neurons in culture, chronic depolarization supports only survival (Franklin *et al.* 1994). This finding strongly suggests that survival and growth are separable process mediated by different signalling pathways. There currently is considerable interest in utilizing neurotrophic factors for treatment of neurodegenerative diseases (Olson 1993). It is likely that a secondary consequences of such treatment is unwanted outgrowth of neurites. Because chronic depolarization stimulates survival-promotion, but not growth, it may be a useful means of teasing out the survival signal transduction pathway(s) from the signalling pathway(s) that induce growth. Such data would may aid in the development of agents that could be used clinically to enhance neuronal survival without causing ectopic growth.

REFERENCES

Bessho, Y., Nawa, H. & Nakanishi, S. 1994 Selective up-regulation of an NMDA receptor subunit mRNA in cultured cerebellar granule cells by K^+-induced depolarization and NMDA treatment. *Neuron* **12**, 87–95.

Chalazonitis, A. & Fischbach, G.D. 1980 Elevated potassium induces morphological differentiation of dorsal root ganglionic neurons in dissociated cell culture. *Devl Biol.* **78**, 172–183.

Choi, D.W. 1987 Ionic dependence of glutamate neurotoxicity. *J. Neurosci.* **7**, 369–379.

Choi, D.W. 1988 Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci.* **11**, 465–469.

Collins, F. & Lile, J.D. 1989 The role of dihydropyridine-sensitive voltage-gated calcium channels in potassium-mediated neuronal survival. *Brain Res.* **502**, 99–108.

Collins, F., Schmidt, M.F., Guthrie, P.B. & Kater, S.B. 1991 Sustained increase in intracellular calcium promotes neuronal survival. *J. Neurosci.* **11**, 2582–2587.

Deckwerth, T.L. & Johnson, E.M. Jr 1993 Temporal analysis of events associated with programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor. *J. Cell Biol.* **123**, 1207–1222.

Doroshenko, P.A., Kostyuk, P.G. & Luk'yanetz, E.A. 1988 Modulation of calcium currents by calmodulin antagonists. *Neuroscience* **27**, 1073–1080.

Duvall, E. & Wyllie, A.H. 1986 Death and the cell. *Immunol. Today* **7**, 115–119.

Edwards, S.N., Buckmaster, A.E. & Tolkovsky, A.M. 1991 The death programme in cultured sympathetic neurones can be suppressed at the posttranslational level by nerve growth factor, cyclic AMP, and depolarization. *J. Neurochem.* **57**, 2140–2143.

Fox, A.P., Nowycky, M.C. & Tsien, R.W. 1987 Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. *J. Physiol.*, Lond. **394**, 149–172.

Franklin, J.L. & Johnson, E.M. Jr 1992 Suppression of programmed neuronal death by sustained elevation of cytoplasmic calcium. *Trends Neurosci.* **15**, 501–508.

Franklin, J.L., Juhasz, A., Cornbrooks, E.B., Lampe, P.A. & Johnson, E.M. Jr 1992 Promotion of sympathetic neuronal survival by chronic depolarization and increased $[Ca^{2+}]_i$ is a threshold phenomenon. *Soc. Neurosci. Abstr.* **18**, 30.4.

Franklin, J.L., Sanz-Rodriguez, C., Juhasz, A., Deckwerth, T.L. & Johnson, E.M. Jr 1994 Chronic depolarization prevents programmed death of sympathetic neurons *in vitro* but does not support growth: requirement for Ca^{2+} influx but not Trk activation. (Submitted.)

Gallo, V., Kingsbury, A., Balázs, R. & Jørgensen, O.S. 1987 The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. *J. Neurosci.* **7**, 2203–2213.

Gorin, P.D. & Johnson, E.M. Jr 1980 Effects of long-term nerve growth factor deprivation on the nervous system of the adult rat: an experimental autoimmune approach. *Brain Res.* **198**, 27–42.

Grynkiewicz, G., Poenie, M. & Tsien, R.T. 1985 A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. biol. Chem.* **260**, 3440–3450.

Hack, N., Hidaka, H., Wakefield, M.J. & Balázs, R. 1993 Promotion of granule cell survival by high K^+ or excitatory amino acid treatment and Ca^{2+}/calmodulin-dependent protein kinase activity. *Neuroscience* **57**, 9–20.

Hamburger, V., Brunso-Bechthold, J.K. & Yip, J.W. 1981 Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. *J. Neurosci.* **1**, 60–71.

Hendry, I.A. & Campbell, J. 1976 Morphometric analysis of rat superior cervical ganglion after axotomy and nerve growth factor treatment. *J. Neurocytol.* **5**, 351–360.

Hoth, M. & Penner, R. 1992 Depletion of intracellular

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

calcium stores activates a calcium current in mast cells. *Nature, Lond.* **355**, 353–355.

Johnson, E.M. Jr & Aloe, L. 1974 Suppression of the in vitro and in vivo cytotoxic effects of guanethidine in sympathetic neurons by nerve growth factor. *Brain Res.* **81**, 519–532.

Johnson, E.M. Jr, Gorin, P.D., Brandeis, L.D. & Pearson, J. 1980 Dorsal root ganglion neurons are destroyed by exposure in utero to maternal antibody to nerve growth factor. *Science, Wash.* **210**, 916–918.

Johnson, E.M. Jr, Koike, T. & Franklin, J. 1992 A "calcium set-point hypothesis" of neuronal dependence on neurotrophic factor. *Expl Neurol.* **115**, 163–166.

Kater, S.B., Mattson, M.P., Cohan, C. & Connor, J. 1988 Calcium regulation of the neuronal growth cone. *Trends Neurosci.* **11**, 315–321.

Koike, T. & Tanaka, S. 1991 Evidence that nerve growth factor dependence of sympathetic neurons for survival in vitro may be determined by levels of cytoplasmic free Ca^{2+}. *Proc. natn. Acad. Sci. U.S.A.* **88**, 3892–3896.

Koike, T., Martin, D.P. & Johnson, E.M. Jr 1989 Role of Ca^{2+} channels in the ability of membrane depolarization to prevent neuronal death induced by trophic-factor deprivation: Evidence that levels of internal Ca^{2+} determine nerve growth factor dependence of sympathetic ganglion cells. *Proc. natn. Acad. Sci. U.S.A.* **86**, 6421–6425.

Lampe, P.A., Cornbrooks, E.B., Juhasz, A., Franklin, J.L. & Johnson, E.M. Jr 1992 Thapsigargin enhances survival of sympathetic neurons by elevating intracellular calcium concentration ($[Ca^{2+}]_i$). *Soc. Neurosci. Abstr.* **18**, 30.5.

Levi-Montalcini, R. & Booker, B. 1960 Destruction of the sympathetic ganglia in mammals by an antiserum to nerve-growth protein. *Proc. natn. Acad. Sci. U.S.A.* **46**, 384–391.

Martin, D.P., Ito, A., Horigome, K., Lampe, P.A. & Johnson, E.M. Jr 1992 Biochemical characterization of programmed cell death in NGF-deprived sympathetic neurons. *J. Neurobiol.* **23**, 1205–1220.

Martin, D.P., Schmidt, R.E., DiStefano, P.S., Lowry, O.H., Carter, J.G. & Johnson, E.M. Jr 1988 Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.* **106**, 829–844.

Nishi, R. & Berg, D.K. 1981 Effects of high K^+ concentrations on the growth and development of ciliary ganglion neurons in cell culture. *Devl Biol.* **87**, 301–307.

Olson, L. 1993 NGF and the treatment of Alzheimer's disease. *Expl Neurol.* **124**, 5–15.

Oppenheim, R.W. 1991 Cell death during development of the nervous system. *A. Rev. Neurosci.* **14**, 453–501.

Oppenheim, R.W., Prevette, D., Tytell, M. & Homma, S. 1990 Naturally occurring and induced neuronal death in the chick embryo in vivo requires protein and RNA synthesis: evidence for the role of cell death genes. *Devl Biol.* **138**, 104–113.

Pilar, G. & Landmesser, L. 1976 Ultrastructural differences during embryonic cell death in normal and peripherally deprived ciliary ganglia. *J. Cell Biol.* **68**, 339–356.

Putney, J.W. Jr 1993 The signal for capacitative calcium entry. *Cell* **75**, 199–201.

Rich, K.M., Luszczynski, J.R., Osborne, P.A. & Johnson, E.M. Jr 1987 Nerve growth factor protects adult sensory neurons from cell death and atrophy caused by nerve injury. *J. Neurocytol.* **16**, 261–268.

Schmidt, M.F. & Kater, S.B. 1993 Fibroblast growth factors, depolarization, and substratum interact in a combinatorial way to promote neuronal survival. *Devl Biol.* **158**. 228–237.

Scott, B.S. & Fisher, K.C. 1970 Potasssium concentration and number of neurons in cultures of dissociated ganglia. *Expl Neurol.* **27**, 16–22.

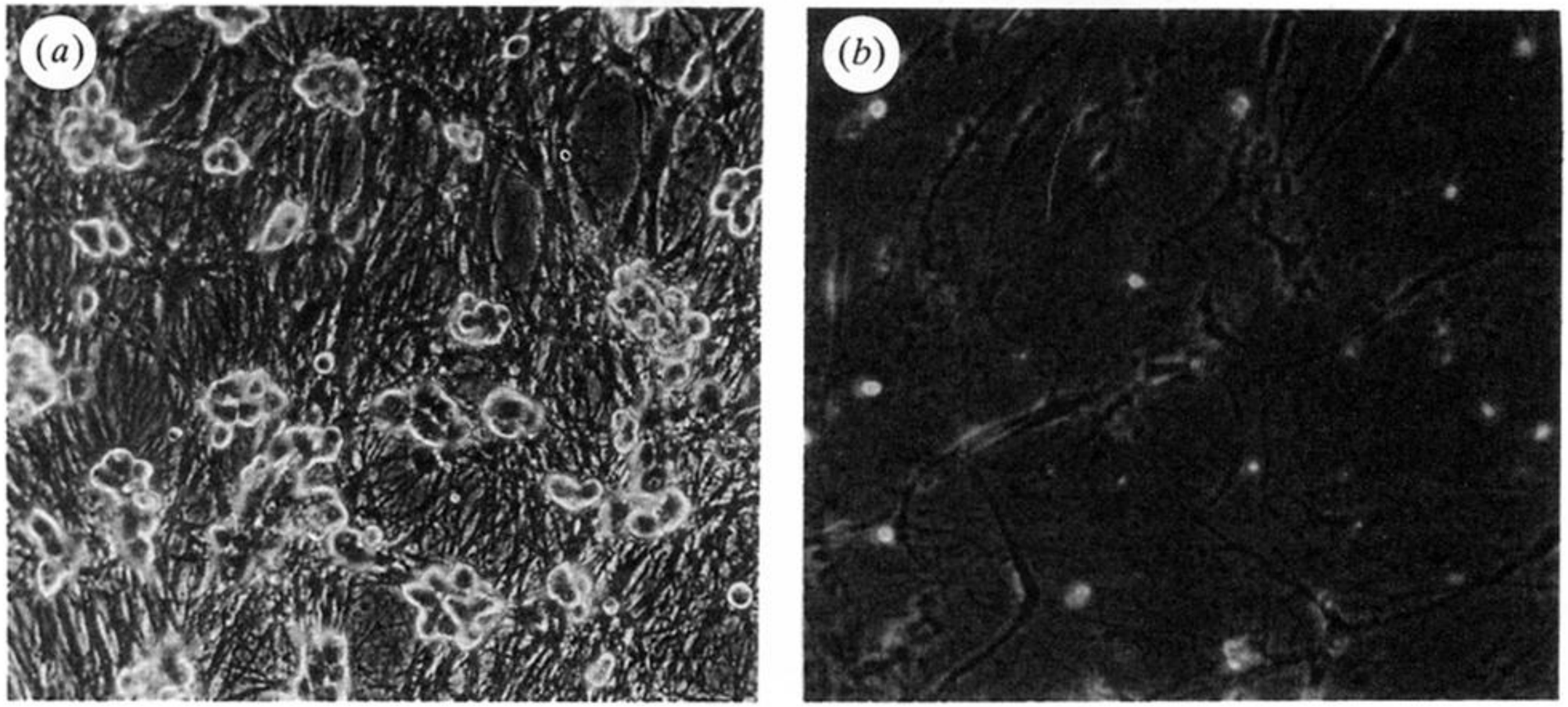
Thastrup, O., Cullen, P.J., Drøbak, B.K., Hanley, M.R. & Dawson, A.P. 1990 Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. natn. Acad. Sci. U.S.A.* **87**, 2466–2470.

Tolkovsky, A.M., Walker, A.E., Murrell, R.D. & Suidan, H.S. 1990 Ca^{2+} transients are not required as signals for long-term neurite outgrowth from cultured sympathetic neurons. *J. Cell Biol.* **110**, 1295–1306.

Tsao, H., Aletta, J.M. & Greene, L.A. 1990 Nerve growth factor and fibroblast growth factor selectively activate a protein kinase that phosphorylates high molecular weight microtubule-associated proteins. *J. biol. Chem.* **265**, 15471–15480.

Wakade, A.R., Wakade, T.D., Malhotra, R.K. & Bhave, S.V. 1988 Excess K^+ and phorbol ester activate protein kinase C and support the survival of chick sympathetic neurons in culture. *J. Neurochem.* **51**, 975–983.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



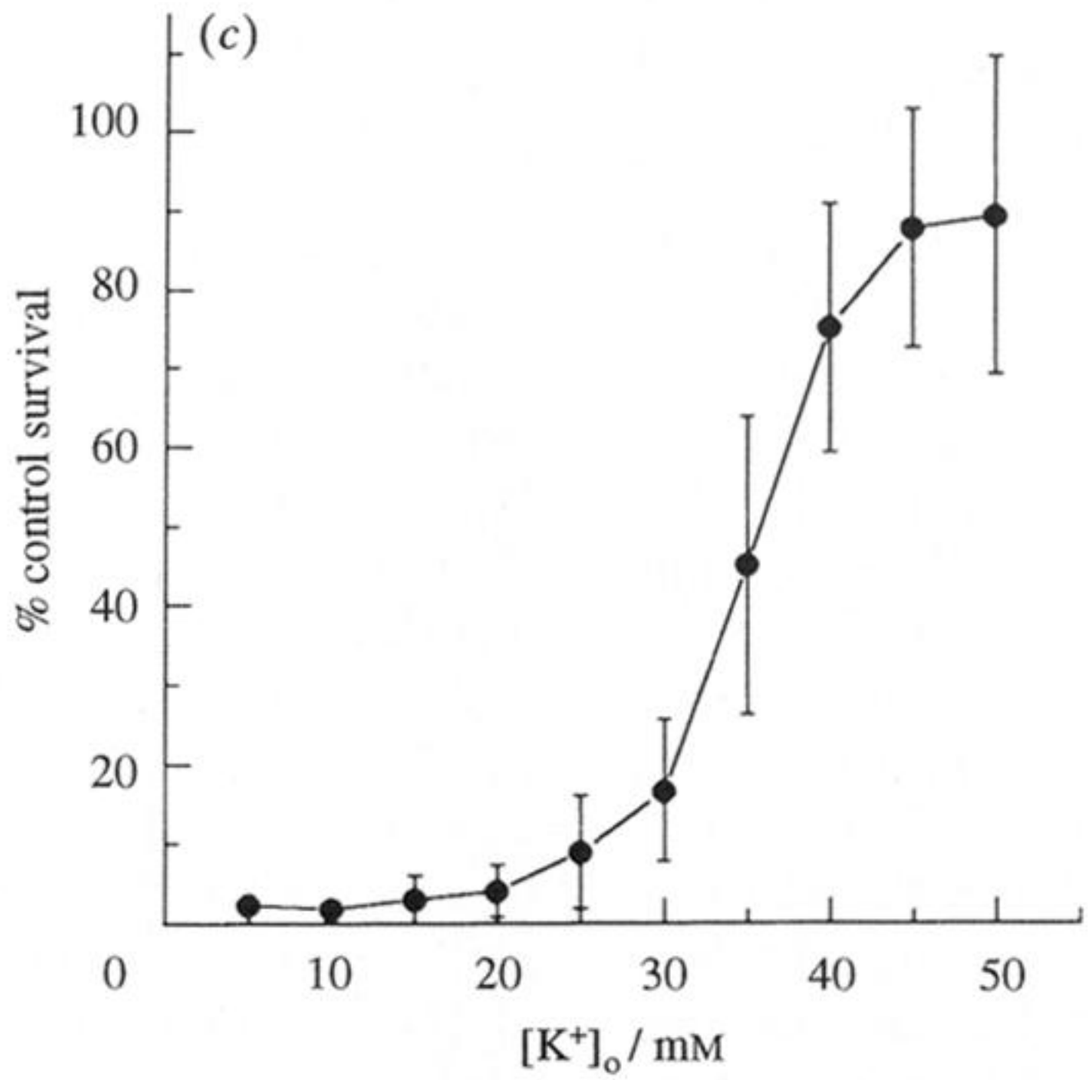


Figure 1. Effect of increasing $[K^+]_o$ on survival of NGF-deprived rat sympathetic neurons. (*a*) Culture of rat SCG neurons maintained for 6 days in medium containing an anti-NGF antibody and 50 mM extracellular K^+ with no NGF. Neurons are alive and healthy. (*b*) Culture maintained in medium with 5 mM extracellular K^+, but without NGF for 6 days. All neurons have died. (*c*) Quantification of the ability of different $[K^+]_o$ to maintain survival of these neurons in the absence of NGF. Cells were deprived of NGF 6 days after plating embryonic-day-21 cells and were exposed to the indicated $[K^+]_o$ for 4 days. Survival was assayed by blinded counting. Control survival is the number of cells maintained in NGF and 5 mM K^+ for the same period as the deprived cells. Error bars are s.d., $n = 8–12$ for each data point.