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Chronic Depolarization Prevents Programmed Death of Sympathetic Neurons *in vitro* but Does Not Support Growth: Requirement for Ca^{2+} Influx but Not Trk Activation

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Continuous exposure of many types of neurons in cell culture to elevated concentrations of K^+ greatly enhances their survival. This effect has been reported to be mediated by a sustained rise of cytoplasmic free Ca^{2+} concentration caused by influx of Ca^{2+} through voltage-gated channels activated by K^+-induced chronic depolarization. In this report we investigate the effects of elevated K^+ on the programmed death that embryonic rat sympathetic neurons undergo in culture when deprived of NGF. Elevated K^+ in the culture medium did not significantly prevent death of NGF-deprived cells until after the third day following plating of embryonic day 21 neurons. On the fifth day after plating, incrementally increasing K^+ concentrations in the culture medium from 5 to 100 mM caused chronic depolarization of neurons and had a biphasic effect on survival of NGF-deprived cells. Enhanced survival was steeply related to membrane potential, increasing from no enhanced survival in cells held at potentials between −51 and −34 mV to 90–100% of control survival at about −21 mV. At potentials positive to −21 mV, survival decreased. Associated with the chronic depolarization was a sustained rise of steady-state free Ca^{2+} concentration that showed a biphasic relationship to membrane potential roughly similar to that exhibited by survival. Steady-state Ca^{2+} concentration increased with increasingly lower membrane potentials to a peak at about −23 mV (to ≈240 nM from ≈40 nM at about −51 mV) and then decreased at more positive potentials. The elevation of intracellular Ca^{2+} was largely blocked by dihydropyridine and phenylalkylamine Ca^{2+} channel antagonists and was potentiated by a dihydropyridine Ca^{2+} channel agonist. Neither the rise of Ca^{2+}, or survival was affected by the Ca^{2+} channel antagonist, ω-conotoxin. Therefore, the Ca^{2+} elevation was probably caused by Ca^{2+} influx through L-type, but not N-type, channels. Antagonists of L channels blocked both survival and the sustained increase of steady-state free Ca^{2+} at similar concentrations, suggesting that the relevant factor determining survival of depolarized cells was Ca^{2+} influx rather than some other effect of depolarization. Surprisingly, however, there was no clear correlation between the sustained rise of Ca^{2+} and survival. Some membrane potentials that induced similar increases of Ca^{2+} concentration produced widely different levels of survival. While chronic depolarization promoted survival of neurons in the absence of NGF, cells supported in this manner showed little growth as measured by neurite extension, total cellular protein, and mean somal diameter.

Compounds commonly used as calmodulin antagonists blocked survival of depolarized cells at concentrations that did not affect survival of cells maintained in NGF. However, these antagonists appeared to block survival by inhibiting Ca^{2+} influx rather than through an effect on calmodulin. Exposure to NGF, but not depolarization without NGF, caused activation of the tyrosine kinase activity of Trk, suggesting that depolarization does not promote survival by activating Trk. Both NGF and depolarization caused tyrosine phosphorylation of a protein with a molecular weight of about 44 kDa that may be an extracellular signal-regulated protein kinase (ERK).

These data show that increased Ca^{2+} influx induced by chronic depolarization can substitute for trophic factors in promoting survival of sympathetic neurons that would otherwise undergo programmed death. The data also demonstrate that the relationship between intracellular Ca^{2+} concentration and survival in depolarized neurons is not as straightforward as previously supposed. Additionally, these results suggest that Ca^{2+} may promote neuronal survival by activating tyrosine kinases downstream from receptor tyrosine kinases and that the signal transduction pathways for growth and survival are separate.

[Key words: programmed cell death, NGF, neuronal calcium, apoptosis, tyrosine kinases, Trk]

Massive cell death occurs as a part of the normal development of the vertebrate nervous system (Oppenheim, 1991). Depending on the neuronal population, approximately 20–80% of the neurons produced during neurogenesis die before or shortly after birth. A primary purpose of this death is thought to be attainment of an appropriate match between the amount of innervation of a neuronal target and the target size. Availability of neurotrophic substances provided by target and other tissues

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appears to be a major determinant of which cells survive this developmental period. Those neurons receiving a sufficient quantity of trophic factor live while those that do not die. Such developmental death is physiologically appropriate and is called programmed cell death (PCD) to distinguish it from inappropriate death caused by pathological processes. An extensively studied neuronal PCD is that occurring in rat sympathetic neurons deprived of neurotrophic support. These cells require continued presence of NGF to survive both *in vivo* and *in vitro*. When deprived of NGF, sympathetic neurons undergo PCD characterized by atrophy, neurite fragmentation, condensation of chromatin, and degradation of DNA into oligonucleosomal fragments (Martin et al., 1988; Edwards et al., 1991; Deckwerth and Johnson, 1993). The PCD of these neurons also requires macromolecular synthesis, suggesting that the death may be active process in which proteins are synthesized that are responsible for killing the cells (Martin et al., 1988, 1992). These characteristics identify the PCD of these neurons as a form of death known as apoptosis that occurs in many types of cells during embryogenesis, metamorphosis, and tissue turnover (Duvall and Wyllie, 1986).

While neurotrophic factors are major determinants of neuronal survival during the period of PCD, other factors also make important contributions. Electrical activity is thought to be one of these factors (Franklin and Johnson, 1992; Schmidt and Kater, 1993). This is suggested by experiments showing that removal of afferent input or pharmacological blockade of electrical activity or neurotransmission causes the death of some types of developing neurons (Lipton, 1986; Maderdrut et al., 1988; Ruitjer et al., 1991; Catsicas et al., 1992; Galli-Resta et al., 1993). In 1970, Scott and Fisher showed that maintaining chicken dorsal root ganglion neurons in cell culture in medium containing elevated concentrations of K^+ greatly enhances their survival. Since that time, high extracellular K^+ concentration ($[K^+]_o$) has been found to promote *in vitro* survival of many other types of developing neurons (Scott, 1971, 1980; Nishi and Berg, 1981; Collins and Lile, 1989; Koike et al., 1989; Eichler et al., 1992; for review, see Franklin and Johnson, 1992). Increased $[K^+]_o$ appears to enhance survival by causing a chronic depolarization of cells that may promote survival by mimicking effects of naturally occurring electrical activity. Sustained depolarization apparently induces prolonged activation of voltage-gated Ca^{2+} channels (Nishi and Berg, 1981; Collins and Lile, 1989; Koike et al., 1989). Influx of Ca^{2+} through these channels has been reported to cause a sustained increase of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) that is thought to mediate the effects of the depolarization on survival (Collins et al., 1991; Koike and Tanaka, 1991). The mechanisms by which increased Ca^{2+} influx prevents PCD are not known. In this report we analyze the effect of elevated $[K^+]_o$ on the membrane potential (V_m), survival, $[Ca^{2+}]_i$, and growth of NGF-deprived rat sympathetic neurons. Additionally, we begin to address the question of how depolarization and Ca^{2+} influx prevents PCD, investigating a possible role for calmodulin and tyrosine kinases.

Some of these results were reported in abstracts (Franklin et al., 1992, 1993).

Materials and Methods

Cell culture. Sympathetic neurons were dissociated from superior cervical ganglia (SCG) of Sprague–Dawley (Harlan) rat fetuses on embryonic day 21 (E21) and maintained in cell culture by a modification of the method of Johnson and Argiro (1983). Briefly, after dissection ganglia were treated at 35°C for 30–60 min with collagenase (1 mg/ml) or 30 min with collagenase followed by 30 min with trypsin (2.5 mg/ml) in L-15 medium. The latter method of dissociation was used to obtain cells for most experiments because it yielded more neurons. After enzymatic treatment, ganglia were triturated and debris was separated from dissociated cells by filtration through a size 3-20/14 Nitex filter (Tetko). Deviations from this method are indicated. Cells were plated on air-dried ammoniated collagen in two-well glass chamber slides (Nunc, Inc.) for cell survival assays, 35 mm plastic tissue culture dishes (Corning or Falcon) for electrophysiology experiments, and, for $[Ca^{2+}]_i$ measurements, glass coverslips glued with silicon adhesive over holes cut in the bottom of 35 mm culture dishes. For measurements of neurite outgrowth, neurons were plated at one end of a thin strip of air-dried ammoniated collagen (≈1 mm diameter and 10–15 mm long) applied with a P20 pipetman to the bottom of 35 mm culture dishes or 24 well plates (Costar). Five thousand cells were plated per condition, resulting in 1000–2000 neurons in 9 d old control cultures. The standard culture medium consisted of Eagle medium with Earle's modified salts supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 20 μM fluorodeoxyuridine, 20 μM uridine, 1.4 mM L-glutamine, and 50 ng/ml 2.5S NGF (from mouse submaxillary gland). In one series of experiments (see Fig. 14) the medium was also supplemented with 3.3 μg/ml aphidicolin. For experiments involving chronic depolarization by elevated $[K^+]_o$, NaCl in the culture medium was replaced with equimolar amounts of KCl to maintain osmolarity. Atomic absorption spectrophotometry showed that K^+ concentration in the culture medium was within 2 mM of the desired concentration in all instances. For experiments involving elevated extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$), NaH_2PO_4 concentration in the culture medium was lowered 10-fold (to 14 mg/liter) to prevent precipitation of Ca^{2+} salts and NaCl was replaced by $CaCl_2$ in equimolar amounts. This treatment did not alter the pH of the medium and had no apparent adverse effects on neurons. Details of this method will be reported elsewhere (P. A. Lampe, E. B. Cornbrooks, A. Juhasz, E. M. Johnson, Jr., and J. L. Franklin, unpublished observations). Cultures were maintained in 0.5–2 ml of medium depending on the size of the culture dish. Cultures were deprived of NGF by incubating in medium with no added NGF and an excess of an NGF-neutralizing antibody (an anti-mouse NGF antibody raised in goat).

Cell survival and protein assays. For assay of survival neurons were fixed with 4% paraformaldehyde and were stained for Nissl substances with crystal violet (EM Diagnostic Systems, Inc.). Cells were then destained with water, dehydrated with ethanol, and transferred to toluene. Coverslips were mounted on the slides with a toluene-based medium (Pro-Texx mounting medium, Baxter Diagnostics) and the slides were coded for blinded cell counting. All neurons on each slide were counted and each experiment was counted by a single person. Neurons were easily recognized because of their rounded morphology, extensive neurites, and dense Nissl staining. Non-neuronal cells showed little or no Nissl staining. Neurons were scored as viable at the time of fixation if they had well-defined cellular outlines and stained strongly for Nissl substances (see Fig. 2*A*). Dead neurons disintegrated into fragments that remained as amorphous debris in the culture dish. These fragments consisted of small enucleate remnants of neurons that could not be confused with intact cells. A few neurons that did not clearly fragment were considered dead because of loss of neurites, massive atrophy, loss of Nissl staining, and lack of an obvious nucleus. Deckwerth and Johnson (1993) have done a detailed temporal analysis of various morphological and biochemical changes occurring in rat SCG neurons in culture after NGF deprivation. They report that the time course of loss of Nissl stained neurons after NGF deprivation corresponds relatively closely to loss of the ability of cultures to reduce the tetrazolium dye 1-(4,5-dimethylthiozol-2yl)-2,5-diphenyltetrazolium bromide (MTT), a commonly used measure of cellular viability (Mosmann, 1983). However, they also report that MTT reduction can be affected by metabolic state in viable cells. Since some of the treatments used in this report might alter metabolic rate, MTT reduction was not used as a measure of survival. Several other possible indicators of neuronal viability including 2-deoxyglucose uptake, protein synthesis, RNA synthesis, and cytochrome oxidase activity all decreased after NGF deprivation, but over very different time courses than loss of MTT reduction and Nissl staining. Deckwerth and Johnson also report that the time course of loss of the ability of NGF to promote survival (and hypertrophy) when added to NGF-deprived cultures corresponds quite closely to loss of Nissl staining. Taken together, these data suggested to us that the best avail-

able assay for determining viability of these neurons was counting of Nissl stained cells. All survival experiments were performed first on neurons maintained in 24 well plastic tissue culture dishes before cells were plated on chamber slides for quantification of results. By visual inspection all screening experiments had outcomes similar to those obtained by cell counting. Non-neuronal cells were quantified by blinded counting. Cells were scored as non-neuronal based on their morphology (see text). Total protein content was determined by the BCA method with a kit from Pierce.

Electrophysiology. The tight-seal whole-cell recording technique was used to measure V_m (Hamill et al., 1981). Patch pipettes were pulled from Kimax-51 glass capillary tubing (Fisher) and their tips were fire polished to a diameter that gave resistances between 1–2 MΩ when filled with recording solutions. A Dagan 8900 patch-clamp amplifier in current-clamp mode was used to measure V_m. Neurons were maintained at 35°C with a heated stage and viewed with phase-contrast optics on an inverted microscope (Nikon Diaphot). Control cultures were bathed in a solution containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM glucose, and 5 mM HEPES-Na^+-HEPES (pH 7.4). For experiments in which $[K^+]_o$ was increased in the bathing medium, KCl replaced NaCl in equimolar amounts. Pipette solution contained 150 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA-NaOH, and 5 mM HEPES-Na^+-HEPES (pH 7.4).

Measurement of $[Ca^{2+}]_i$. Cultures were prepared for $[Ca^{2+}]_i$ measurements by incubating them for 45–60 min at 35°C in culture medium appropriate for the experiment and containing the acetomethoxy ester of the Ca^{2+}-sensitive dye fura-2 (6 μM in 0.6% dimethyl sulfoxide; Molecular Probes; Grynkiewicz et al., 1985). After neurons were loaded with fura-2, cultures were rinsed several times with the same medium (equilibrated with 5% CO_2 at 35°C in an incubator) without fura-2. The culture dishes were then filled with this medium, dead-air space was eliminated, and lids were sealed onto the dishes with vacuum grease. Cultures were placed on the heated stage of an inverted microscope and viewed through a 40× oil immersion objective (CF Fluor, 1.30 N.A.). Measurements were made at 35°C. This method allowed measurement of $[Ca^{2+}]_i$ under the same conditions (temperature, pH, ionic composition, etc.) in which the neurons had been maintained in culture. Deviations from this method, done in some early experiments, are indicated.

A 75 W xenon-arc lamp and an optical chopper provided alternating excitation at wavelengths of 340 ± 10 nm and 380 ± 10 nm. A Nikon P1 photomultiplier with a Hammamatsu R1104 photodiode was used to measure intensity of light emitted at 515–560 nm. The emitted light was restricted to that coming from the soma of single cells by an adjustable aperture in the light path. The output of the photomultiplier was filtered at 100 Hz, digitized by an analog to digital converter (Modular Instruments, Inc.), and collected by a specially written computer program that was also used to control the optical chopper and a shutter which allowed light to pass only during the sampling period. Each 340/380 measurement was made over a period of 1 sec. In early experiments four or five different determinations of $[Ca^{2+}]_i$ taken over a 10–12 sec period for each neuron were averaged. Little variation of $[Ca^{2+}]_i$ occurred in single neurons over this period; therefore, to expedite measurement of $[Ca^{2+}]_i$ in many neurons, a single measurement per cell was made in later experiments. All measurements were corrected for background fluorescence by subtracting the 515–560 emission intensity of the collagen substrate when it was excited at wavelengths of 340 and 380 nm from that of cells excited at 340 and 380 nm.

Calibration. "*In situ*" methods (McCormack and Cobbold, 1991) for calibrating fura-2 340/380 ratios to $[Ca^{2+}]_i$ proved inadequate. The standard method for obtaining this type of calibration is to treat dye-loaded cells with Ca^{2+} ionophores in the presence or absence of extracellular Ca^{2+} to obtain 340/380 ratios with fura-2 maximally and minimally saturated with Ca^{2+}. In combination with the K_d of fura-2 and the ratio of the fluorescence intensities of cells measured in maximally and minimally saturating $[Ca^{2+}]_i$ at an excitation wavelength of 380 nm, these values are used in Equation 5 of Grynkiewicz et al. (1985) to estimate $[Ca^{2+}]_i$. We found considerable variation in ratios measured from different cells when the ionophore ionomycin was used. This was especially true with, presumably, saturating $[Ca^{2+}]_i$, perhaps because of dye leakage or differences in the ability of individual neurons to regulate Ca^{2+} challenges. Since measurements from many cells over a protracted period were necessary, we considered it essential to utilize a method of calibration that was independent of the properties of the cells and that would reveal artifacts caused by changes in the measuring equipment. For these reasons, "*in situ*" calibrations were not done. We chose, instead, to estimate $[Ca^{2+}]_i$ from standard curves generated by determining fura-2 340/380 ratios in buffers containing known concentrations of Ca^{2+}. The calibrations were done with a commercially available kit having 11 different buffers containing concentrations of Ca^{2+} between 0–37.6 μM (Molecular Probes). Drops of these buffers containing fura-2 pentapotassium salt (6 μM) were placed on glass slides and ratios determined. All fura-2 340/380 ratios recorded from neurons were within the linear portion of these standard curves. The equations derived from linear regression ($R > 0.99$) of these curves were used to estimate $[Ca^{2+}]_i$. The purpose of these experiments was to determine relative $[Ca^{2+}]_i$ under different experimental conditions rather than absolute values. Therefore, the values reported herein may vary from the actual $[Ca^{2+}]_i$ in the cells since the calibration buffers are unlikely to adequately reflect cytoplasmic conditions that may significantly alter fura-2 characteristics (Grynkiewicz et al., 1985; McCormack and Cobbold, 1991). Although these concentrations should be considered estimates rather than true values, they are shown rather than fura-2 340/380 ratios to facilitate comprehension and allow easier comparisons of our results to those of others who have reported $[Ca^{2+}]_i$ values in these neurons.

Tyrosine phosphorylation assay. Approximately 10^6 cells were plated on a collagen substrate in 100 mm tissue culture dishes for each treatment (i.e., lane on a gel). After experiments, cells were rinsed rapidly in ice-cold PBS (pH 7.2) and solubilized at 4°C in 0.5 ml of Tris/NP-40 lysis buffer containing 10 mM Tris (pH 6.8), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, and 5 μg/ml leupeptin. After a 30 min incubation on ice, the samples were spun in a microcentrifuge for 15 min at 4°C to remove nuclei and cellular debris. To determine the level of Trk tyrosine phosphorylation, lysates were immunoprecipitated for 1 hr at 4°C with rabbit antibody G1086 prepared against a synthetic 15 amino acid peptide corresponding to the C-terminus of human Trk (QALAQAPPVYLDVLG) kindly provided by Dr. William Mobley and colleagues. This sequence is the same as that for all rodent full-length Trk proteins (Zhou et al., 1994). The resulting immunocomplexes were precipitated for 1 hr at 4°C with protein A–Sepharose beads (Sigma), separated by SDS-PAGE on 7.5% gels, transferred to Immobilon-P transfer membrane filters (Millipore), and blotted with anti-phosphotyrosine-specific monoclonal antibody IgG2bk (Upstate Biotechnologies, Inc.). An enhanced-chemiluminescence Western-blotting detection system (Amersham) was used to detect tyrosine-phosphorylated proteins on the membrane. To determine tyrosine phosphorylation of proteins in total cell lysates, proteins were separated, transferred, and blotted in a similar manner. The level of Trk tyrosine phosphorylation was determined by laser densitometry.

Chemicals. Calmidazolium, W7, and ω-conotoxin GVIA were obtained from CalBiochem. ω-Conotoxin MVIIA was a gift from Brenda Shivers at Parke-Davis. Thapsigargin was from L. C. Services. All other chemicals were obtained from Sigma. Chemicals were dissolved in either dimethyl sulfoxide or ethanol, both of which did not affect survival at the concentrations used.

Curve fitting and statistical analysis. Equations were fit to data by using a Marquardt-Levenberg nonlinear least-squares minimization routine (SIGMAPLOT version 5.0, Jandel Scientific). Statistics were done either with SIGMAPLOT or with INSTAT 2 (GraphPad Software). All means are presented ± SD unless otherwise indicated. The significance of differences was tested with two-tailed Mann–Whitney U tests, t tests, or by ANOVA. Differences were considered significant when $p < 0.05$.

Results

Development of PCD suppression by elevated $[K^+]_o$

Withdrawal of NGF from young (≈1 week postplating) cultures of embryonic rat SCG neurons causes PCD of most cells within 48–72 hr (Martin et al., 1988; Deckwerth and Johnson, 1993). As these neurons age *in vitro* (and *in vivo*) they become increasingly less acutely dependent upon NGF for survival taking much longer to die when deprived of NGF after they have been in culture for several weeks (Lazarus et al., 1976). Therefore, the effects of increased $[K^+]_o$ on PCD was investigated with young cultures of these cells. Previous reports concerning the effects of elevated $[K^+]_o$ on PCD of rat SCG neurons deprived of NGF

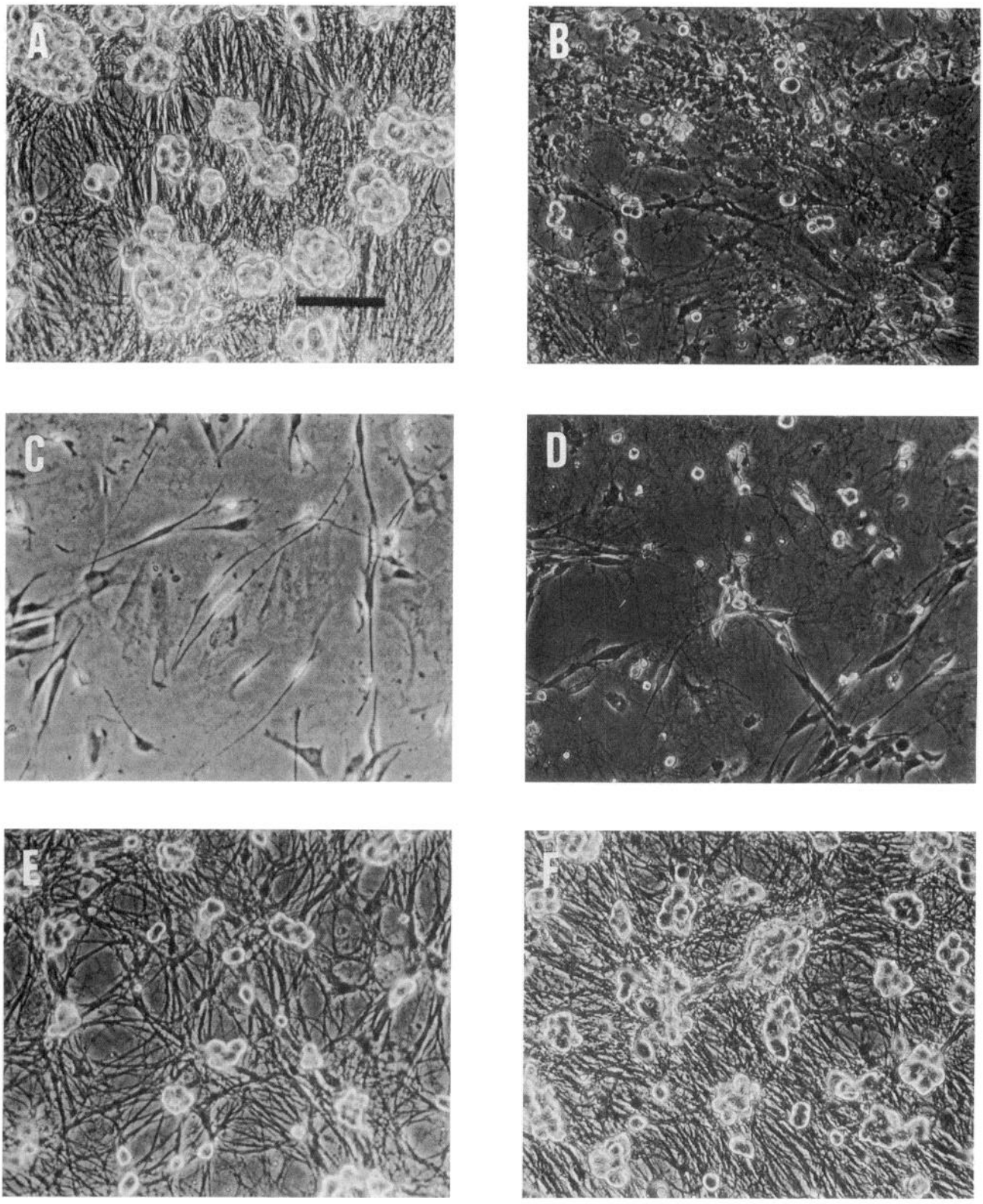
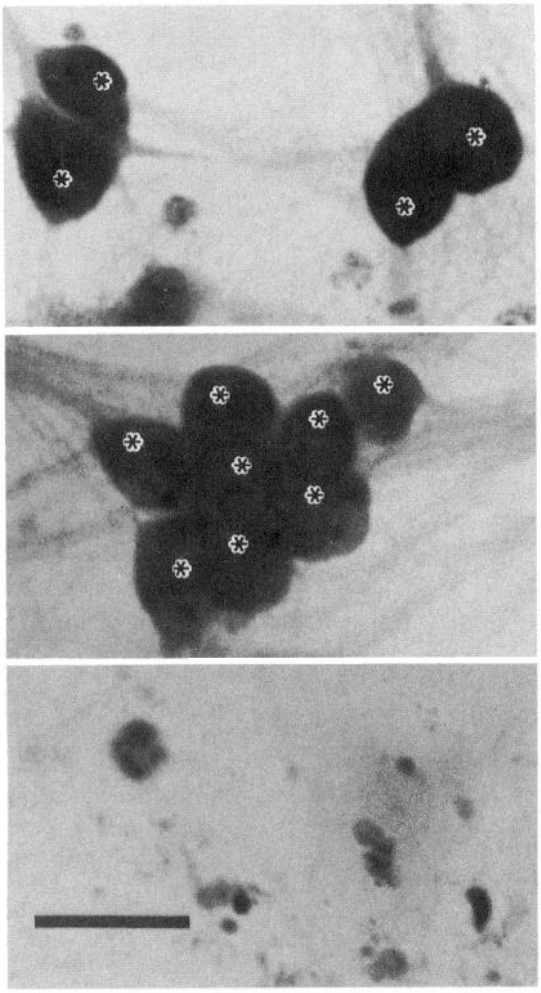


Figure 1. Phase-contrast photomicrographs showing the effects of NGF withdrawal and the ability of elevated $[K^+]_o$ to promote survival of SCG neurons deprived of NGF at different times after plating. *A,* Neurons maintained in the presence of NGF. *B,* Culture maintained in NGF for 5 d after plating and then deprived of NGF for another 7 d. Extensive somatic atrophy, neurite degeneration, and death have occurred. *C,* Culture in which neurons were plated in medium with 5K without NGF. No neurons survived. *D,* Culture in which neurons were plated without NGF in 50K medium. Elevated $[K^+]_o$ at the time of plating did not enhance survival; however, 4 (*E*) or 6 (*F*) d after plating, 50K medium suppressed PCD caused by NGF deprivation. Photographs were taken 12 d after plating. Scale bar, 215 μm.

in cell culture (Martin et al., 1988; Koike et al., 1989; Koike and Tanaka; 1991) have investigated the effects of high $[K^+]_o$ on neurons maintained for the first week after plating in the presence of NGF before they were deprived of NGF and exposed to increased $[K^+]_o$. The influence of elevated $[K^+]_o$ on PCD of younger neurons has not been investigated. Figure 1 illustrates the effect of NGF withdrawal and elevated $[K^+]_o$ on the morphology and survival of SCG neurons during the first week after



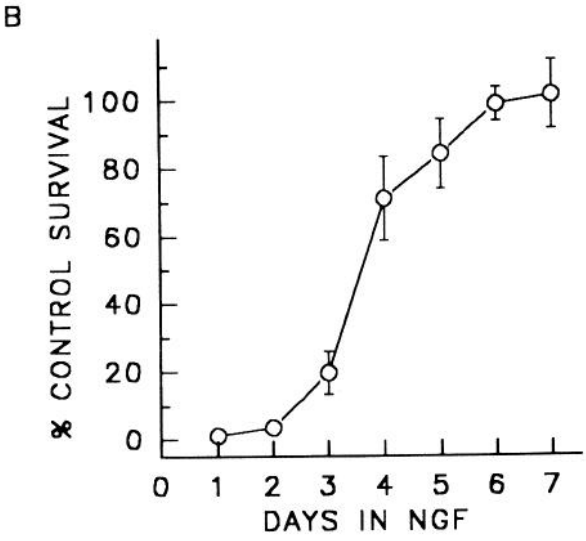


Figure 2. Development of the ability of 50K medium to support survival after NGF deprivation. *A,* Scoring of neuronal survival in Nissl-stained neurons. Top to bottom: culture maintained in NGF and 5K medium for 9 d after plating, 9 d old culture maintained without NGF in 50K medium for 4 d, and 9 d old culture deprived of NGF for 4 d. Note debris from cells that have died in the bottom photograph. *Stars* indicate cells scored as alive. Scale bar 30 μm. *B,* Development of high $[K^+]_o$-promoted survival. Cells were maintained in medium containing 5K with NGF for the indicated time before being switched to medium having 50K and no NGF. Twelve days after plating, neuronal survival was assayed. Survival is shown as percentage of the average number of neurons in cultures maintained for 12 d after plating in NGF-containing medium. $N = 10$ from two separate platings for each data point. Error bars are SD in all figures unless otherwise indicated. Lack of error bars in figures indicates bars smaller than symbols.

plating. Within 24 hr after dissociation, neurites were visible on neurons maintained in medium with NGF and became extensive during the first week in culture. A small number of non-neuronal cells were visible in cultures. These consisted of broad, flat cells that were probably fibroblasts and spindle-shaped cells that may have been Schwann cells. Neuronal somas supported by NGF were phase bright (Fig. 1*A*). Withdrawal of NGF caused loss of a phase-bright soma, neurite degeneration, somatic atrophy and fragmentation, and death of most neurons in less than 72 hr (data not shown; Fig. 1*B*). To determine whether elevated $[K^+]_o$ can prevent PCD during the first week in culture, cells were deprived of NGF at various times after plating and maintained in medium containing 50 mM K^+ (50K). When cells were plated in medium containing 5K (normal $[K^+]_o$) or 50K and no NGF, few neurons survived (Fig. 1*C,D*). However, by 4–6 d after plating 50K appeared to support survival of most NGF-deprived neurons (Fig. 1*E,F*). The cells supported by elevated $[K^+]_o$ were phase bright and, by inspection, appeared morphologically very similar to cells supported by NGF. Cells in cultures maintained in NGF for 9 d after plating were 9.04 $\pm$ 3 ($N = 8$) non-neuronal while those maintained from the fifth to the ninth day after plating in 50K without NGF were 9.27 $\pm$ 2% ($N = 8$) non-neuronal. The two values are not significantly different ($p > 0.05$). More neurons survived 4 d of NGF withdrawal in cultures dissociated with trypsin in addition to collagenase than in those cultures dissociated only with collagenase (see Fig. 6*B*). Enhanced basal survival in collagenase/trypsin-dissociated cultures might be because of dissociation of an additional population of neurons that were less acutely dependent upon NGF for survival. Another possibility is that collagenase/trypsin resulted in a more gentle dissociation than collagenase alone causing less damage to neurons.

The development of the ability of high $[K^+]_o$ to support survival of NGF-deprived cells is quantified in Figure 2. Elevated $[K^+]_o$ did not support survival of NGF-deprived cells during the first 2 d in culture (survival was <3%). By the third d after plating, 50K medium supported survival of 20 $\pm$ 6% of NGF-deprived neurons and, by the sixth d after plating, 50K supported survival of 99 $\pm$ 5% of the cells. Thus, while NGF can suppress PCD at the time of plating, the ability of elevated $[K^+]_o$ to do so develops over a period of several days.

Elevated $[K^+]_o$ supports survival but not growth

Campenot (1986) demonstrated that local exposure of the neurites of rat SCG neurons in culture to medium containing 50K causes the neurites to degenerate in the presence of NGF while exposure of entire neurons, including neurites, to medium containing 50K and NGF allowed continued neurite outgrowth. We confirmed that treatment of entire cells with elevated $[K^+]_o$ did not affect their ability to extend neurites (Fig. 3*A,B*). The effects of elevated $[K^+]_o$ on neurite outgrowth of NGF-deprived SCG neurons has not, to our knowledge, been investigated. To determine the effect of high $[K^+]_o$ on neurite growth in the absence of NGF, cells were plated at one end of thin strips of collagen.

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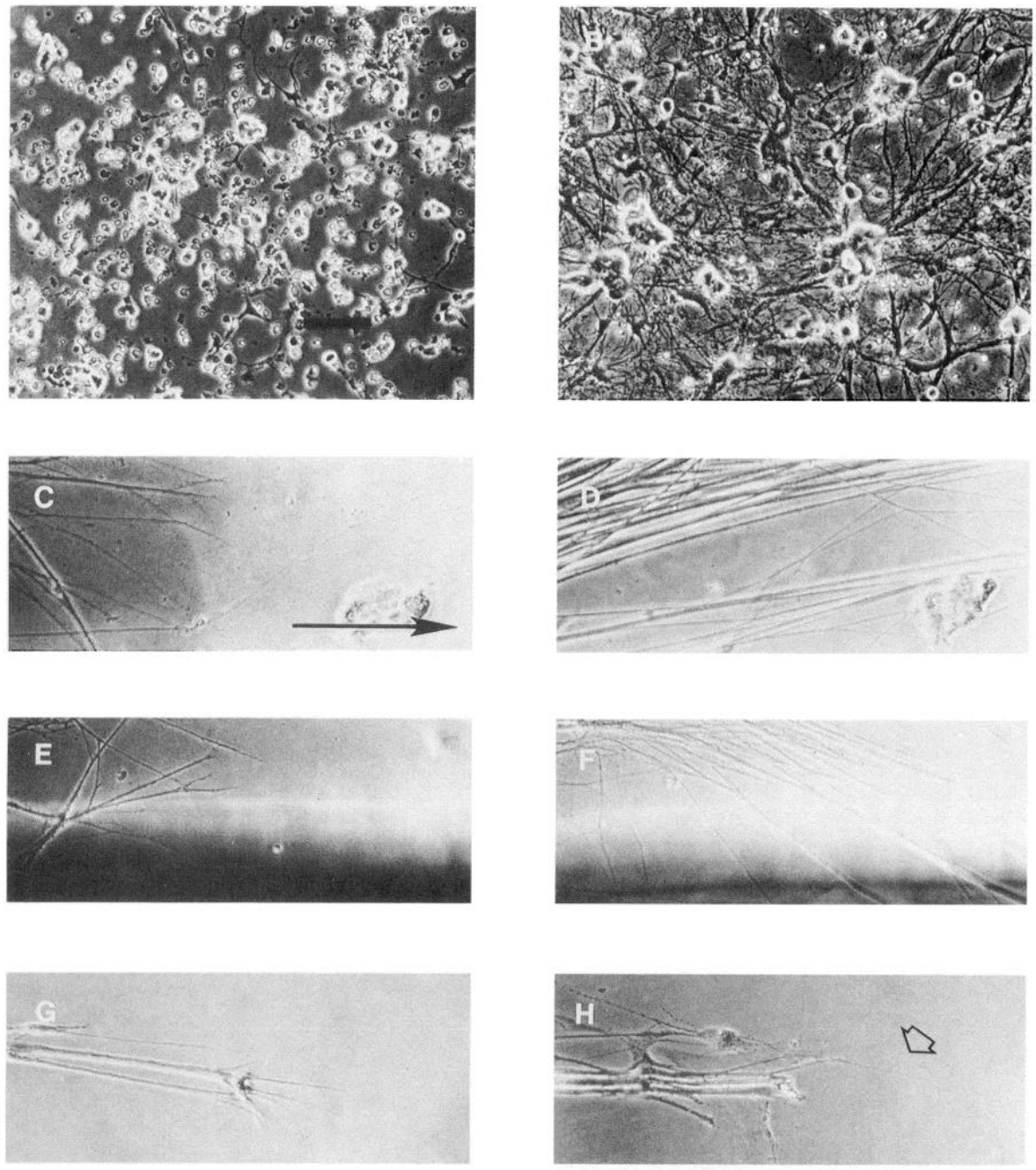

Figure 3. Effects of elevated $[K^+]_o$ and NGF on growth. *A*, Photomicrograph of E21 neurons plated for 1 d in NGF and 50K. *B*, Neurons maintained from the time of plating to the 8th day after plating in NGF and 50K. Somatic growth and extensive neurite outgrowth has occurred. *C*, Neurites of cells maintained for 6 d after plating in NGF and 5K. *D*, The same field of view as in *C* after cells were maintained in the same medium for 7 additional d. Neurites have grown far out of the field of view. *E*, Neurites of cells maintained for 5 d after plating in NGF and 5K and 1 d in NGF and 50K. *F*, The same field of view as in *E* after cells were maintained in the same medium for 7 additional days. Again, neurites have grown far out of the field of view. *G*, Neurites of cells maintained 6 d after plating in NGF and 1 d in media without NGF and 50K. *H*, The same field of view after cells were maintained in the same medium for 7 additional days. While some remodeling of neurites occurred, neurite outgrowth was far less extensive than when NGF was present. Note that one process has extended (*arrow*) for a short distance. The *arrow in C* shows the general direction of neurite growth along collagen strip. *Dark lines in E and F* are from a scratch on the bottom of the tissue culture dish that was used to orient the collagen strip. Scale bar, 100 μm. Scale is the same for *A–H*.

Neurites tended to grow straight along these strips and distance from origin was easily measured. Figure 3*C–F* shows that neurites continued to grow along these strips when the medium contained NGF and 5K or 50K. However, without NGF neurite outgrowth in the presence of 50K was greatly reduced (Fig. 3*G,H*). Some neurites grew short distances after the switch to 50K without NGF but most did not grow or retracted for short distances. Neurites in these cultures remained firmly attached to the substratum so that lack of attachment to collagen did not account for decreased growth, nor was there any apparent ten-

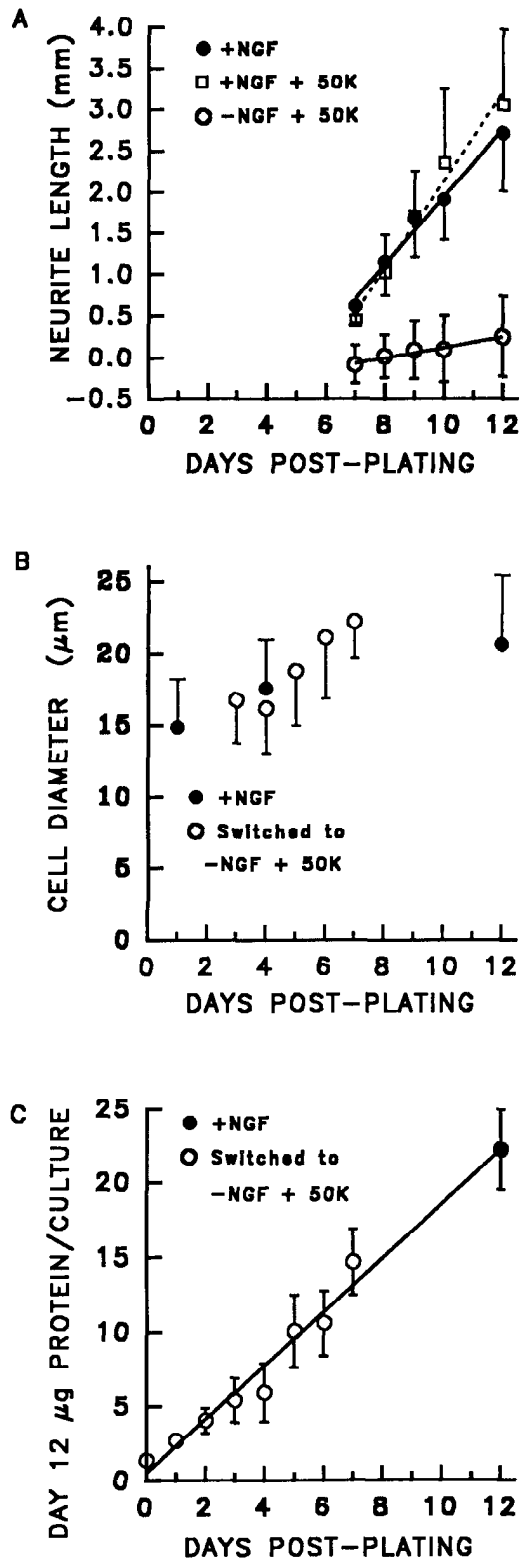

Figure 4. Effects of high $[K^+]_o$ and NGF on growth. *A,* Neurite outgrowth of cells exposed to NGF in 5K medium from the time of plating and of cells maintained for the first 5 d in culture in 5K medium and NGF, then switched to medium ± NGF and containing 50K. Neurites grew at about the same rate in 5K or 50K when NGF was present. Neurites of neurons maintained for the first 5 d in culture in 5K medium with NGF, and then switched to an NGF-free medium containing 50K, grew little. Lines are linear regressions of the three sets of data ($R > 0.98$ for each). $N = 9$–22 from three or four separate platings for each data point. Neurite length is neurite extension beyond the length of neurites measured 6 d after plating. *B,* Cell diameters of neurons maintained in NGF-containing medium with 5K before being switched to NGF-free medium containing 50K on the indicated days after plating (*open circles*). Diameter was measured on the twelfth day after plating. *Solid circles* show diameters of neurons maintained in NGF and 5K for 1, 4, and 12 d from the time of plating. $N = 23$–25 from a single plating for each data point. *C,* Total protein content of cultures maintained for the indicated period in NGF-containing medium before being switched to NGF-free medium with 50K and then maintained in this medium until the twelfth day after plating when protein content was determined (*open circles*). *Solid circle* is the protein content of cultures maintained for 12 d after plating in NGF and 5K. *Solid line* is linear regression of the data ($R > 0.99$). $N = 2$ cultures for day 0 and 3–4 cultures for each succeeding day. Data is from a single plating.

dency for neurites to grow back along themselves or on other neurites. Growth of neurites in media containing 5K + NGF and 50K ± NGF is quantified in Figure 4*A*. Cells were maintained for the first 5 d in culture in 5K + NGF. They were then switched to 50K ± NGF or continued in 5K + NGF. Neurite outgrowth was measured starting on the sixth day after plating. The length of the longest neurites were measured. From the sixth to the twelfth day after plating neurites grew linearly along the collagen strips. The average rate of this growth was about 0.42 mm/d when medium contained NGF and 5K, a rate that was maintained for at least 2 more weeks (data not shown). Neurons maintained in medium containing 50K and NGF grew at an average rate of about 0.52 mm/d. Even though cells remained viable in NGF-free medium with 50K, neuritic growth almost completely ceased. The mean rate of increase in the length of the longest measured neurites in 50K medium without NGF was 0.07 mm/d. Increase of the somal diameter of cells maintained in 50K medium without NGF was also inhibited (Fig. 4*B*). For example, the average somal diameters of neurons maintained in NGF for 12 d after plating was 21 ± 5 μm ($N = 25$), while the somas of neurons maintained in NGF for 4 d after plating before being deprived of NGF and switched to medium containing 50K were 16 ± 3 μm ($N = 25$) on the twelfth day after plating ($p < 0.001$). The average diameter of neurons maintained for the first 4 d in culture in NGF and 5K was 17.6 ± 3 μm ($N = 25$), not significantly different from cells switched to 50K medium without NGF and maintained until the twelfth day after plating in this medium ($p > 0.05$). Therefore, maintenance of cells in 50K without NGF does not appear to support growth of cell diameter.

The decreased growth of NGF-deprived neurons maintained in high $[K^+]_o$ was reflected in lower total protein content in these cells than in cells maintained in NGF. Neurons were deprived of NGF and exposed to 50K medium at various times after plating. The cells were then maintained in 50K without NGF until the twelfth day after plating when protein content was determined (Fig. 4*C*). The longer the cells had been in NGF and 5K before deprivation and switch to 50K, the greater the protein content measured on the twelfth day. Total protein content on the twelfth day after plating was a linear function of the amount of time the cells had been exposed to the NGF before deprivation and switch to 50K medium. In cultures switched to 50K without NGF before the fourth or fifth day after plating, decreased protein content compared to 12 d control can be accounted for at, least in part, by inability of elevated $[K^+]_o$ to promote survival (i.e., cells died). However, decreased protein in cells switched to 50K at later times, when most cells are saved

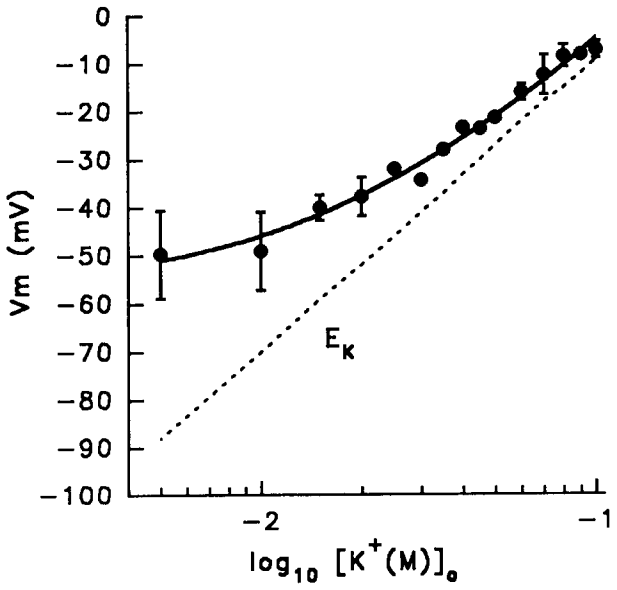

Figure 5. Effect of $[K^+]_o$ on V_m. Membrane potential was measured in cells exposed to different $[K^+]_o$ (5–50 mM in 5 mM increments and 50–100 mM in 10 mM increments) for up to 2 hr. The *solid line* is a quadratic regression of the data, and the dotted line represents E_K at 35°C calculated from the Nernst equation (Hille, 1992). $N = 8$ for 5K and 3 for all other data points. Potentials were measured in neurons 5–8 d after plating.

from death by 50K, likely reflects decreased growth. For example, cultures of neurons maintained in NGF and 5K for 6 day after plating and kept in 50K without NGF until the twelfth day after plating had a protein content of 10.6 ± 2 μg protein/culture while cultures maintained for the entire 12 d in NGF and 5K had a protein content of 22 ± 3 μg protein/well ($p < 0.01$). Taken together, these data indicate that NGF promoted both growth and survival of neurons, while high $[K^+]_o$ promoted survival in the absence of NGF but supported little growth.

Effect of elevated $[K^+]_o$ on V_m

Elevated $[K^+]_o$ causes chronic depolarization of neurons in culture (Chalazonitis and Fischbach, 1980) and this depolarization, rather than another effect of increased $[K^+]_o$, is thought to enhance survival (Franklin and Johnson, 1992). To determine the membrane potentials (V_m) of rat SCG neurons in varying $[K^+]_o$, we exposed neurons to medium containing 5–100 mM K^+ and measured V_m by tight-seal whole-cell recording (Hamill et al., 1981). The mean V_m of neurons in medium containing 5K was −50 ± 9 mV ($N = 8$) with a range of −39 to −64 mV. These resting V_m values are similar to those reported previously for rat SCG neurons in culture with this recording method (Nerbonne and Gurney, 1989). Exposure of neurons to medium having elevated $[K^+]_o$ caused depolarization as illustrated in Figure 5. In medium containing 5K, mean V_m was about 40 mV positive to K^+ equilibrium potential (E_k), indicating that a substantial fraction of the resting V_m in these cells was determined by ions other than K^+. The measured V_m of neurons maintained in 45K medium for 4 d was not significantly different from that of neurons exposed for less than 2 hr to 45K (−23 ± 2 mV, $N = 4$ and −24 ± 0.6 mV, $N = 3$, respectively; $p > 0.05$), indicating that the depolarization was chronic. Three out of eight neurons in medium containing 5K fired spontaneous action potentials at a relatively slow rate (≈1 action potential/5 sec, e.g.) and one out of three cells in medium containing 10K was spontaneously active. In media having greater than 10K ($N = 39$ neurons), no electrical activity was evident in any neurons, probably because of voltage-dependent inactivation of ionic channels at more positive V_m values. The range of V_m values in media with K^+ greater than 10 mM was much narrower than that in medium with 5K and the V_m values were closer to E_K. The decrease in variability of V_m with increasing $[K^+]_o$ was probably a reflection of increased K^+ conductance at more positive V_m values which would have the effect of driving V_m closer to E_K.

The relationship between intracellular and extracellular K^+, Na^+, and Cl^- concentrations and the V_m of excitable cells is often modeled with the Goldman-Hodgkin-Katz (GHK) potential equation (Goldman, 1943; Hodgkin and Katz, 1949). However, the data were not adequately fit by this equation or by a modification of the constant field equation (Jan and Jan, 1976) that takes into account the relative permeabilities of all ions (K^+, Na^+, Mg^{2+}, Ca^{2+}, and Cl^-) in the extracellular and intracellular solutions (not shown). A monotonic relationship between $[K^+]_o$ and V_m values could, therefore, not be obtained with the GHK equation or its modifications. To obtain a "smooth" relationship between $[K^+]_o$, V_m values, and V_m-dependent variables, measured V_m values as a function of $[K^+]_o$ were fitted with a smooth curve by quadratic regression. This regression ($R > 0.99$) differed by less than 3 mV from the measured mean V_m values in all cases. In media with 5–10K, the measured V_m values of individual cells varied by 5–13 mV from the fitted regression values while, in media with greater than or equal to 15K, the measured V_m values in individual cells was 0–5 mV different from the fitted values. The V_m values in the remainder of this report were taken from the regression equation. The values stated imply a range of V_m values that at low $[K^+]_o$ (5–10K) is rather large and at higher $[K^+]_o$ is rather small.

Effects of varying V_m *on neuronal survival*

Figure 6*A* illustrates that survival of neurons deprived of NGF 5 d after plating and chronically depolarized for 4 d was steeply related to V_m. Potentials negative to −34 mV (25K) did not enhance survival. As mean V_m decreased from −34 mV to −21 mV (50K), mean survival increased from 4 ± 3% to 89 ± 20% of control. Positive to −21 mV, mean survival decreased steeply with decreasing V_m, dropping to a minimum of 12 ± 10% of control at −7 mV (90K). The decrease in survival at more positive V_m values was not the result of a toxic effect of the high $[K^+]_o$ that induced those potentials because neurons maintained in medium containing 70–100K remained healthy if NGF was present.

Prevention of PCD by chronic depolarization was long term. Approximately the same percentage of neurons that were depolarized for 10 d without NGF survived at a particular V_m as those depolarized for only 4 d (Fig. 6*B,C*). Mean survival in cultures depolarized for 10 d without NGF was enhanced as much as 119 ± 34% above the survival of NGF-deprived neurons maintained in 5K for 10 d. This level of survival was not significantly different from that of control cells maintained for the same period in NGF ($p > 0.05$). Cultures held at −21 mV for 1–2 months after NGF withdrawal were phase bright and appeared as healthy as cells maintained for the same period in the presence of NGF (not shown). Therefore, once the ability

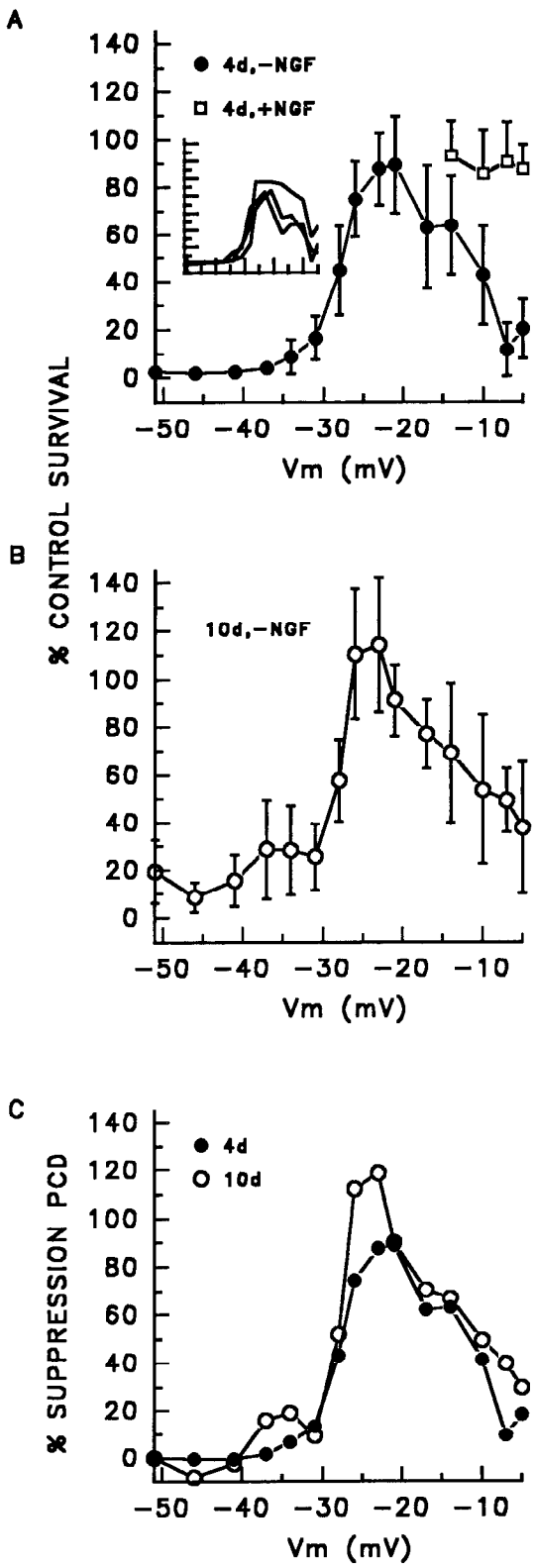

Figure 6. Relationship between V_m and survival. *A*, Survival of neurons maintained for 5 d after plating in medium containing NGF and 5K followed by 4 d in media ± NGF and containing various $[K^+]_o$ to induce different V_m values ($[K^+]_o$ was 5–50 mM K^+ in 5 mM increments and 50–100 mM K^+ in 10 mM increments). *Squares* show survival of neurons maintained in the presence of NGF for the same period. N = 8–12 from two or three platings for each data point. *Inset* shows mean survival of each of the three separate platings. Symbols and error bars are omitted in inset for clarity. *B*, Survival of neurons deprived of NGF 5 d after plating and held at different V_m values for 10 d as in *A*. Survival is similar to that 4 d after withdrawal. The baseline survival is greater in *B* than *A* because cells were dissociated with collagenase and trypsin while those in *A* were dissociated only with collagenase (see text). Control survival is average number of neurons in sibling cultures (usually four) maintained in NGF and 5K (−51 mV) for the same period. N = 6–12 from three platings for each data point. *C*, Data in *A* and *B* normalized to percentage suppression of PCD, defined as

$$\frac{\text{survival}_{-\text{NGF,at}V_m} - \text{survival}_{-\text{NGF,at}-51}}{\text{survival}_{+\text{NGF,at}-51} - \text{survival}_{-\text{NGF,at}-51}} \times 100,$$

where $\text{survival}_{-\text{NGF,at}V_m}$ is survival of cells at a particular V_m without NGF, $\text{survival}_{+\text{NGF,at}-51}$ is average survival of control cells in sister cultures with NGF, and $\text{survival}_{-\text{NGF,at}-51}$ is average survival of control cells in sister cultures without NGF.

of chronic depolarization to prevent PCD develops it can permanently substitute for NGF in promoting survival.

Effect of V_m *on* $[Ca^{2+}]_i$

Thayer et al. (1987) and Tolkovsky et al. (1990) have shown that acute depolarization of rat SCG neurons with elevated $[K^+]_o$ causes a biphasic increase of $[Ca^{2+}]_i$ consisting of a transient rise of $[Ca^{2+}]_i$ followed by a sustained increase lasting for at least several tens of minutes. Koike and Tanaka (1991) showed that long periods of exposure to elevated $[K^+]_o$ causes a sustained increase of $[Ca^{2+}]_i$ in these neurons that can last for several days. We have confirmed and extended these results. Figure 7*A* illustrates the effect of acute depolarization to −21 mV with 50K on the $[Ca^{2+}]_i$ of a single SCG neuron. Within 45 sec after initial depolarization, $[Ca^{2+}]_i$ in this cell rose from a resting concentration of 69 nM to a peak of 427 nM. During the next 5–10 min $[Ca^{2+}]_i$ fell to a sustained level of ≈160 nM. Figure 7*B–D* shows that $[Ca^{2+}]_i$ remained elevated above baseline with long periods of exposure to high $[K^+]_o$. In neurons held at −51 mV for 24–27 hr, mean $[Ca^{2+}]_i$ was 43 ± 13 nM. At all V_m values positive to −51 mV, mean $[Ca^{2+}]_i$ was elevated above control levels, increasing with lower V_m values to a peak of 239 ± 69 nM at −23 mV (45K). At V_m values lower than −23 mV, $[Ca^{2+}]_i$ decreased with lower V_m values to a minimum of 115 ± 37 nM at −7 mV. Neurons maintained at depolarized V_m values for 4 d (Fig. 7*C*) or 11 d (Fig. 7*D*) had mean $[Ca^{2+}]_i$ substantially elevated above that of cells maintained at −51 mV for the same period. For example, after 4 d of depolarization, mean $[Ca^{2+}]_i$ was 181 ± 35 nM in neurons held at −23 mV, while at −51 mV it was 58 ± 12 nM. After 11 d of depolarization mean $[Ca^{2+}]_i$ in cells held at −23 mV had dropped to 139 ± 38 nM, but remained elevated above the resting level of 51 ± 21 nM at −51 mV. These data suggest that chronic depolarization of SCG neurons causes continuous Ca^{2+} influx through voltage-gated channels. The decrease of $[Ca^{2+}]_i$ at potentials positive to −23 mV probably reflects increasing voltage-dependent inactivation of Ca^{2+} channels at more positive Vms.

Even though V_m was "clamped" within a relatively narrow range by $[K^+]_o$ greater than or equal to 15 mM, $[Ca^{2+}]_i$ varied widely from cell to cell at a specific V_m. This was particularly true of neurons that had been in culture for 6 d and depolarized for 24–27 hr when $[Ca^{2+}]_i$ measurements were made. For example, the range of $[Ca^{2+}]_i$ measured in cells depolarized to −23 mV for 24–27 hr was 113–406 nM, while the range in cells held at −23 mV for 4 d was 98–268 nM, and, for 11 d, the range was 81–177 nM. One possible explanation for such a wide variation

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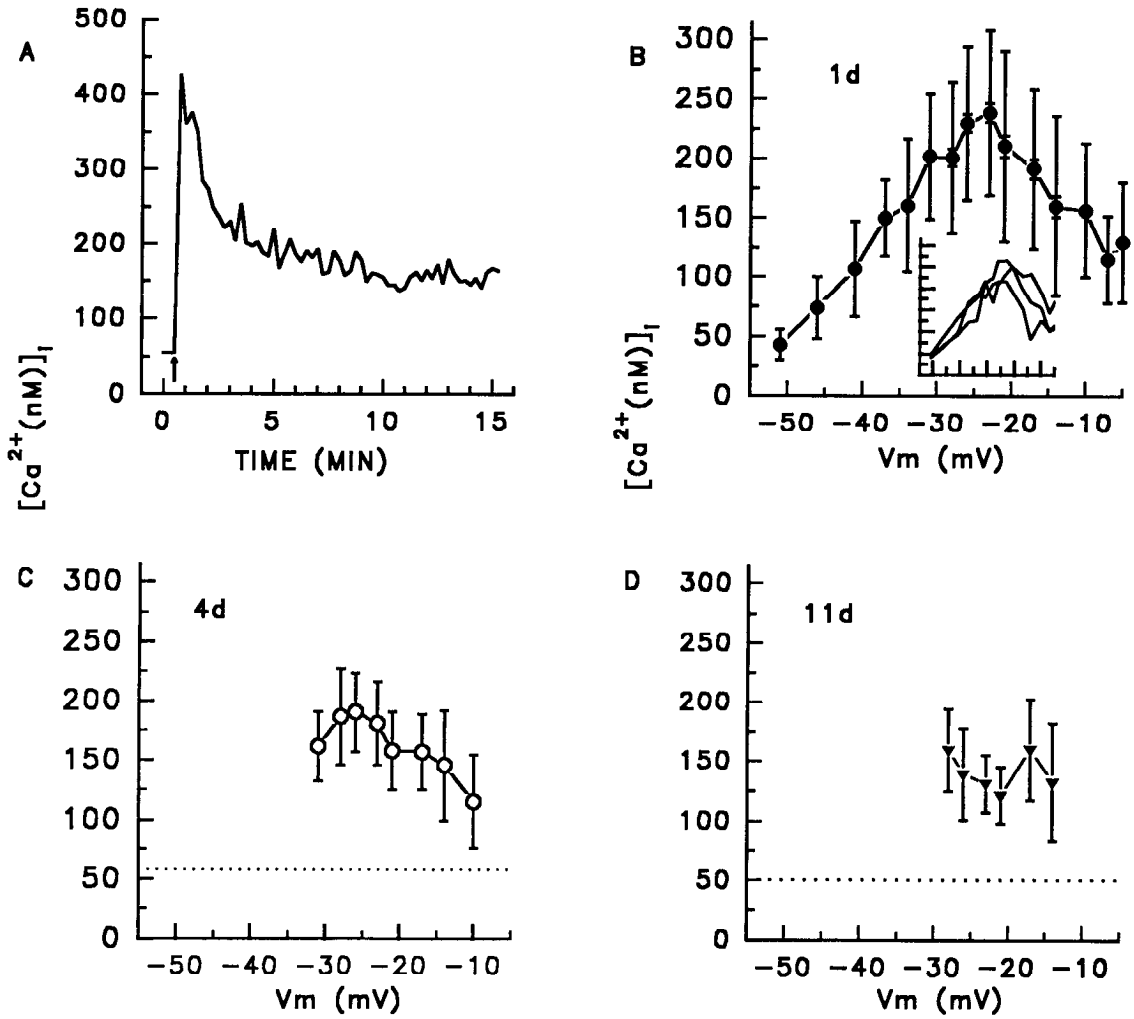


Figure 7. Effect of V_m on $[Ca^{2+}]_i$. *A*, Representative response of $[Ca^{2+}]_i$ in a single SCG neuron to acute depolarization to −21 mV with 50K. *Arrow* indicates addition of 50K medium. $[Ca^{2+}]_i$ was sampled every 15 sec. *B*, $[Ca^{2+}]_i$ as a function of V_m. Neurons were depolarized for 24–27 hr before fura-2 measurements of $[Ca^{2+}]_i$ were made. N = 71–76 from three separate platings for each data point; inner error bars are SEM. *Inset* shows mean $[Ca^{2+}]_i$ of each of the three separate platings. *C*, Effect of 4 d of depolarization on $[Ca^{2+}]_i$. N = 49–75 from two or three platings for each data point. *D*, Effect of 11 d of depolarization on $[Ca^{2+}]_i$. N = 24–26 from a single plating for each data point. $[Ca^{2+}]_i$ in depolarized cells decreased with time in culture but remained substantially elevated above control levels. *Dotted lines* indicate $[Ca^{2+}]_i$ in neurons held at −51 mV in the presence of NGF for the same period. Neurons were depolarized to the indicated V_m with medium containing elevated $[K^+]_o$ (5–50 mM in 10 mM increments and 50–100 mM in 10 mM increments) and no NGF, 5 d after plating. Lack of a full range of V_m values at 4 d and 11 d indicates death of most cells at V_m values outside of the ranges shown (see Fig. 6).

of $[Ca^{2+}]_i$ at a particular V_m is a measurement artifact caused by the brief time that $[Ca^{2+}]_i$ was sampled in each cell (see Materials and Methods); that is, longer sampling periods might reveal that $[Ca^{2+}]_i$ in single neurons is not a true steady state but changes slowly with time. Thus, individual cells might fluctuate through a wide range of $[Ca^{2+}]_i$ and short sampling periods, which take brief $[Ca^{2+}]_i$ "snapshots," may not reveal the entire range of $[Ca^{2+}]_i$ that single cells have over longer periods. To test this possibility, $[Ca^{2+}]_i$ was measured over a period of 30 min in six neurons plated for 6 d and depolarized to −21 mV for 24–27 hr. Intracellular $[Ca^{2+}]$ in individual cells varied by only 5–35 nM from the mean value over this period. This range of $[Ca^{2+}]_i$ was equivalent to the $[Ca^{2+}]_i$ fluctuations of the acutely depolarized neuron shown in Figure 7*A* (28 nM maximal) and in other acutely depolarized cells. These small fluctuations appeared random and were probably caused by "noise" in the measuring system rather than actual changes of $[Ca^{2+}]_i$. Therefore, the measured $[Ca^{2+}]_i$ in single depolarized neurons remained at a steady state with only minor variations from a mean value, and the variation of $[Ca^{2+}]_i$ between cells was not a sampling artifact. Some of the variation in $[Ca^{2+}]_i$ between neurons "clamped" at the same V_m probably reflected real differences in the Ca^{2+} homeostatic properties of individual cells. However, part of the variation can probably also be accounted for by fura-2 artifacts such as differential compartmentalization of nonhydrolyzed fura-2 ester in different cells or differential fura-2 loading in individual cells. To what degree each of these possibilities contributed to the cell to cell $[Ca^{2+}]_i$ variations observed was not determined. Even though some neurons fired spontaneous action potentials at V_m values negative to about −41 mV (15K), individual cells did not show significant variations of $[Ca^{2+}]_i$ when held negative to this V_m. This lack of $[Ca^{2+}]_i$ change measured in electrically active cells may be because the low rate of electrical activity might not cause a significant global change of $[Ca^{2+}]_i$ that could be measured by the techniques used here. The slow sampling rate used might also

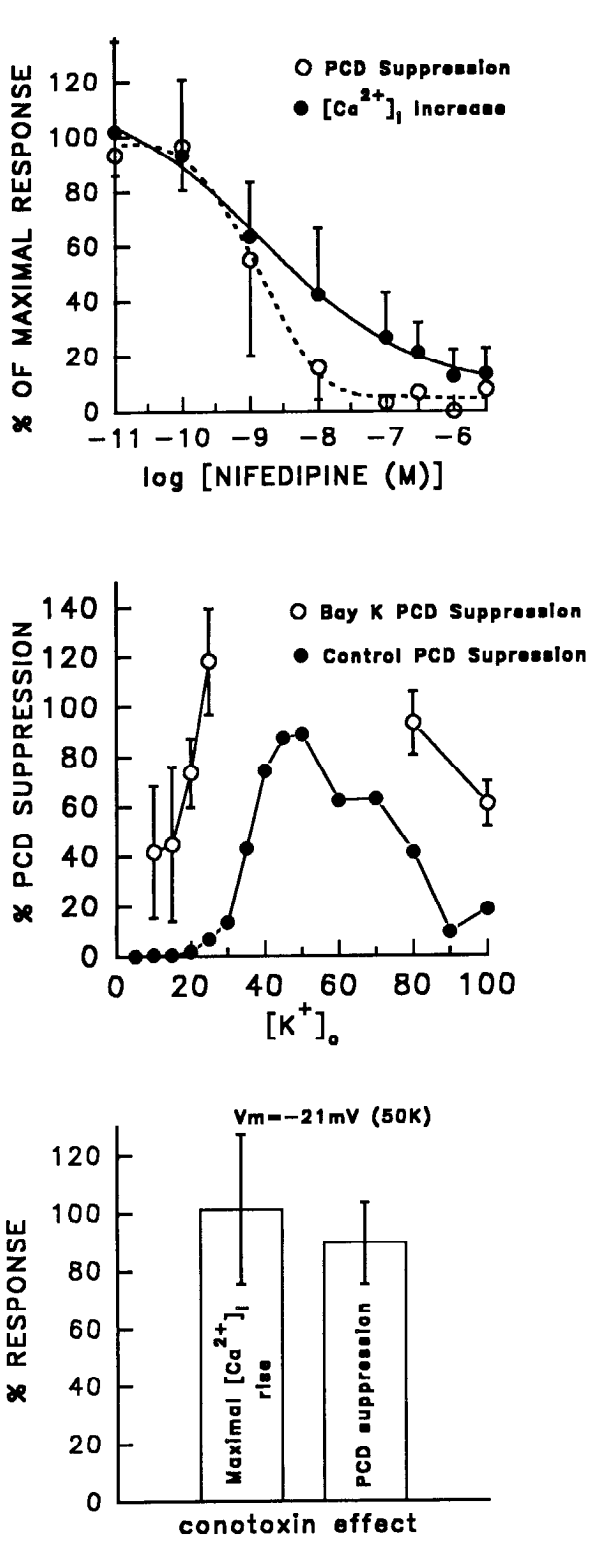


Figure 8. Effects of Ca^{2+} channel antagonists and agonists on survival and $[Ca^{2+}]_i$. *A*, Nifedipine blocked both survival and the sustained rise of $[Ca^{2+}]_i$ in neurons maintained in 50K. No additional block of survival or $[Ca^{2+}]_i$ increase was observed with nifedipine concentrations as high as 10 μM. For each survival data point, $N = 7$–14 from three or four platings for concentrations ≥1 nM; for concentrations <1 nM, $N = 3$–4 from one plating. For each $[Ca^{2+}]_i$ data point, $N = 50$–100 from two to four platings. The *smooth curves* are fits of the data to a logistic equation. *B*, The DHP agonist (±)Bay K 8644 (3 μM and 10 μM data combined) potentiated survival of NGF-deprived cells maintained in $[K^+]_o$ that did not promote survival in the absence of the agonist. *Solid circles* represent mean PCD suppression in neurons maintained in the same $[K^+]_o$ without (±)Bay K 8644 (from Fig. 6C). $N = 3$–7 from one or two platings for each data point. $[K^+]_o$ is shown rather than V_m because V_m of neurons exposed to (±)Bay K 8644 was not measured and the agonist may depolarize neurons by increasing Ca^{2+} channel activity. *C*, Conotoxin did not affect either suppression of PCD by chronic depolarization with 50K (10 μM fraction MVIIA; 1 μM GVIA was also tested, data not shown) or sustained elevation of $[Ca^{2+}]_i$ (fraction GVIA, 1 μM); $N = 4$ and 25, respectively, from one plating for each treatment. All survival was determined in cells depolarized for 4 d after NGF withdrawal and is shown as percentage PCD suppression caused by depolarization with 50K. Percent maximal response for $[Ca^{2+}]_i$ is percentage of increase of $[Ca^{2+}]_i$ caused by 50K without the drugs. $[Ca^{2+}]_i$ was measured 6 d after plating in neurons that had been depolarized for 24–28 hr. Drugs were added 2–5 hr before measurements were made.

miss rapid transient changes of $[Ca^{2+}]_i$ caused by brief electrical activity.

Pharmacology of depolarization-mediated PCD suppression and $[Ca^{2+}]_i$ increase

Depolarization-enhanced survival is blocked by Ca^{2+} channel antagonists in a number of types of neurons (Nishi and Berg, 1981; Gallo et al., 1987; Koike et al., 1989) suggesting that Ca^{2+} influx through voltage-gated channels mediates the effect. A Ca^{2+} channel antagonist, the dihydropyridine (DHP) antagonist isradipine (PN200-110), blocks depolarization-enhanced survival of chicken ciliary ganglion neurons in culture (Collins and Lile, 1989) and also reduces sustained elevation of $[Ca^{2+}]_i$ caused by depolarization of these cells (Collins et al., 1991). Similar experiments investigating the effects of DHP antagonists on sustained increases of $[Ca^{2+}]_i$ induced by chronic depolarization have not been done with other types of neurons. About 50% of the transient rise of $[Ca^{2+}]_i$ induced by acute depolarization of rat SCG neuronal somas with medium containing 50K is sensitive to block by the DHP Ca^{2+} channel antagonist nitrendipine (1 μM) while the more sustained portion of the $[Ca^{2+}]_i$ increase is reduced about 77% by the same nitrendipine concentration (Thayer et al., 1987). Three other DHP antagonists, nimodipine, nifedipine, and nicardipine, have been reported to block depolarization-enhanced survival of these neurons (1 μM, 5 μM, and 5 μM, respectively, were tested) while DHP agonists potentiate survival (Koike et al., 1989). However, we found that similar concentrations of at least some DHP antagonists (nifedipine and nicardipine were tested) can be toxic to SCG neurons maintained in culture in the presence of NGF (data not shown). Therefore, we tested the effects of lower concentrations of nifedipine, which did not affect control survival, on both $[Ca^{2+}]_i$ and survival of chronically depolarized, NGF-deprived cells (Fig. 8*A*). In neurons maintained in 50K, increasing concentrations of nifedipine caused a dose-dependent decrease of survival and of the sustained elevation of $[Ca^{2+}]_i$ induced by the depolarization. Maximal block of the $[Ca^{2+}]_i$ increase occurred in concentrations of nifedipine greater than or equal to 1 μM (87 ± 10% block in 1 μM). Maximal block of survival occurred in 10 nM nifedipine, a concentration that had no effect on the survival of control cells (data not shown). The IC_{50} values for block of the sustained rise of $[Ca^{2+}]_i$ and survival by nifedipine were ≈4 nM and ≈0.5 nM, respectively. These IC_{50} values are in the range of nifedipine concentrations that are highly selective for block of DHP-sensitive, or L-type, Ca^{2+} channels (Fox et al., 1987; Porzig, 1990; Triggle, 1990). These data suggest that

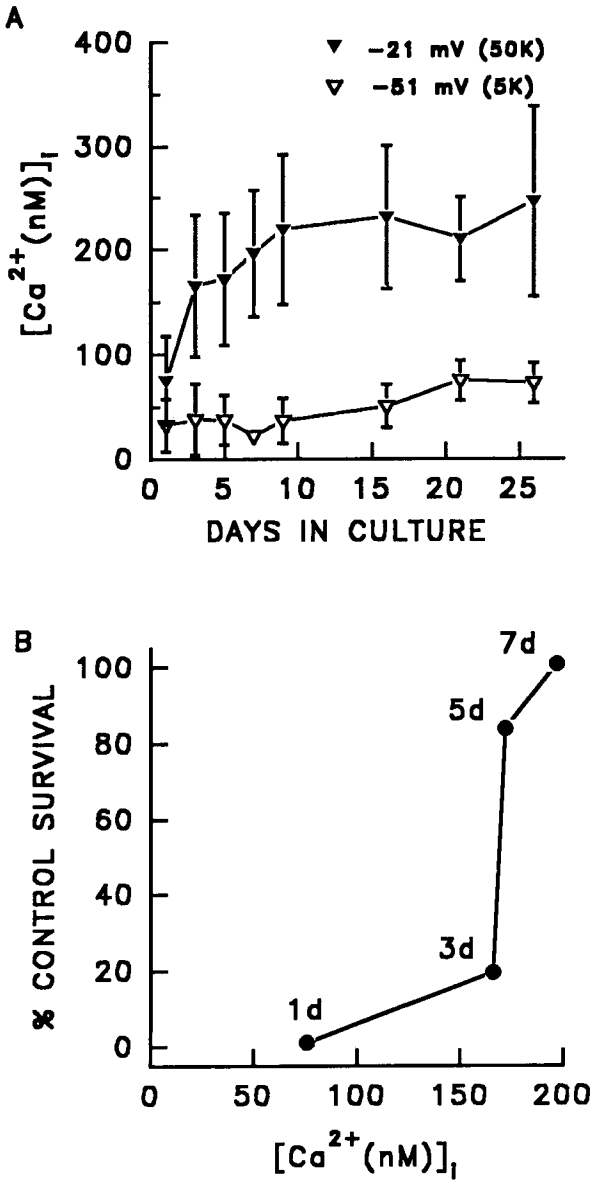


Figure 9. Development of the ability of chronic depolarization to cause a sustained increase of $[Ca^{2+}]_i$. *A*, Effects of depolarization with 50K on $[Ca^{2+}]_i$ of neurons maintained in the presence of NGF for 1–26 d after plating. While the ability of depolarization to cause a prolonged rise of steady state $[Ca^{2+}]_i$ increased substantially over this period, only a small increase in control $[Ca^{2+}]_i$ was seen. For measurement of $[Ca^{2+}]_i$, control neurons (5K) were maintained in a medium consisting of 140 mM NaCl, 3 mM KCl, 5 mM HEPES-Na-HEPES (pH 7.4), 2.4 mM $CaCl_2$, 0.72 gm/100 ml glucose, and 0.8 mM $MgCl_2$. Neurons were depolarized to −21 mV for 2–4 hr with culture medium containing 50 mM KCl and 90 mM NaCl. They were then placed in the above medium containing 50 mM KCl and 90 mM NaCl for measurement of $[Ca^{2+}]_i$. In one experiment neurons maintained in the standard culture medium with 50K during measurement (see Materials and Methods) gave similar results. N = 52–155 from one to three platings for each data point. *B*, Comparison of the ability of depolarization to promote survival of NGF-deprived neurons at different times after plating with its ability to cause a sustained elevation of $[Ca^{2+}]_i$. Survival data were taken from Figure 2*B* and $[Ca^{2+}]_i$ from Figure 9*A*.

block of PCD by depolarization was caused by increased $[Ca^{2+}]_i$ caused primarily by influx of Ca^{2+} through L-type channels.

Koike et al. (1989) reported that DHP Ca^{2+} channel agonists potentiate the effect of elevated $[K^+]_o$ on survival of NGF-deprived rat SCG neurons. We confirmed this with the DHP Ca^{2+} channel agonist (±)Bay K 8644, which increases L channel open-time and, subsequently, Ca^{2+} influx. This agonist increased survival of NGF-deprived neurons maintained in $[K^+]_o$ that had little or no effect on survival in its absence (Fig. 8*B*). Intracellular Ca^{2+} was also substantially increased above control levels by (±)Bay K 8644. In neurons maintained in 20K for 24–27 hr without the agonist, $[Ca^{2+}]_i$ was 64 ± 19% (N = 75) of the sustained rise in cells maintained in 50K for 24–27 hr, with (±)Bay K 8644, $[Ca^{2+}]_i$ was 98 ± 38% (N = 25) of the rise. These data provide further evidence that influx of Ca^{2+} through L-type channels was the relevant factor determining survival. Additional evidence was provided by the effects of the L-type Ca^{2+} channel antagonist, verapamil (Triggle, 1990). Verapamil caused a dose-dependent suppression of survival in NGF-deprived cells held at −21 mV with 50K. The IC_{50} of this block was about 2.5 μM (N = 7–10 for each concentration; data not shown). Verapamil blocked the sustained elevation of $[Ca^{2+}]_i$ induced by 50K with an IC_{50} of about 1.7 μM (N = 25 for each concentration; data not shown).

ω-Conotoxins GVIA (McClesky et al., 1987; Sher and Clementi, 1991) and MVIIA (Gray et al., 1988; Olivera et al., 1990) are antagonists of the non-DHP-sensitive, N-type (Fox et al., 1987; Plummer et al., 1989) Ca^{2+} channels of many types of neurons (Regan et al., 1991). ω-Contoxin does not affect L-type channels in rat SCG neurons. Since nifedipine maximally blocked 87 ± 10% of the sustained rise of $[Ca^{2+}]_i$ caused by depolarization in these cells, it appears that, little, or none, of the increase of $[Ca^{2+}]_i$ was caused by influx of Ca^{2+} through N-type channels. Application of ω-conotoxins to cultures deprived of NGF and depolarized with 50K had no effect on survival (1 μM GVIA and 10 μM MVIIA tested) or $[Ca^{2+}]_i$ (1 μM GVIA tested) confirming that N-type channels made no contribution to either depolarization-increased survival or elevation of $[Ca^{2+}]_i$ (Fig. 8*C*). Therefore, prevention of PCD by depolarization in rat SCG neurons appears to be entirely, or almost entirely, mediated by Ca^{2+} influx through DHP-sensitive channels.

Development of chronic depolarization-induced increase of steady-state $[Ca^{2+}]_i$

To determine whether the inability of elevated $[K^+]_o$ to block PCD during the first few days after plating (Figs. 1, 2) may reflect an inability to increase $[Ca^{2+}]_i$ during this period, neurons were depolarized with 50K at various times after plating and the effect on $[Ca^{2+}]_i$ measured (Fig. 9*A*). One day after plating, steady-state $[Ca^{2+}]_i$ in cells depolarized with 50K for 2–4 hr was considerably smaller (76 ± 42 nM, N = 150) than that of cells depolarized for the same period with 50K several days after plating. By the third day after plating, depolarization with 50K for 2–4 hr induced a sustained increase of mean steady-state $[Ca^{2+}]_i$ equal in magnitude to that produced after 5 d in culture by this concentration (166 ± 68 nM and 172 ± 63 nM, respectively; $p > 0.05$). The ability of depolarization to cause a sustained rise of $[Ca^{2+}]_i$ reached a maximum by 9 d after plating. In cells of this or greater age, the sustained rise of $[Ca^{2+}]_i$ caused by a 2–4 hr depolarization with 50K was not significantly different than that of cells depolarized with 50K 16, 21, and 26 d after plating (220 ± 72 for 9 d cells, e.g., compared to 211 ± 40 nM for 21 d cells; $p > 0.05$; N = 114 and 52, respectively).

Figure 9*B* shows the development of depolarization-enhanced survival as a function of increased steady-state $[Ca^{2+}]_i$ elicited by the same $[K^+]_o$ at the same time. Mean $[Ca^{2+}]_i$ in neurons

depolarized with 50K 1 d after plating was well below the mean $[Ca^{2+}]_i$ of neurons depolarized with 50K for the same period 5–7 d after plating. However, by the third day after plating, the mean $[Ca^{2+}]_i$ in cells treated with 50K was well within this range, yet most of these neurons did not survive NGF deprivation. These data suggest that while an increase in the ability of depolarization to increase $[Ca^{2+}]_i$ may be necessary for development of the ability of depolarization to promote survival, alone, it may not be sufficient.

Koike and Tanaka (1991) reported that $[Ca^{2+}]_i$ in rat SCG neurons maintained in control medium (5K) containing NGF for the first 3–4 weeks after plating increases to approximately the concentrations caused by chronic depolarization with optimal survival-promoting $[K^+]_o$. They suggested this increase may explain why NGF deprivation takes longer to kill older neurons *in vitro* and *in vivo*. In our hands, however, only a small increase of $[Ca^{2+}]_i$ was seen in cells maintained in NGF for this period (Fig. 9*A*). On the first day after plating, mean $[Ca^{2+}]_i$ was 32 ± 25 nM; by 26 d after plating, mean $[Ca^{2+}]_i$ had increased to 73 ± 19 nM ($p < 0.05$), a rise smaller than that associated with PCD prevention by depolarization. These data suggest it is unlikely that development of decreased requirement for trophic factor (for survival) with time can be explained on the basis of increased $[Ca^{2+}]_i$ as the cells age.

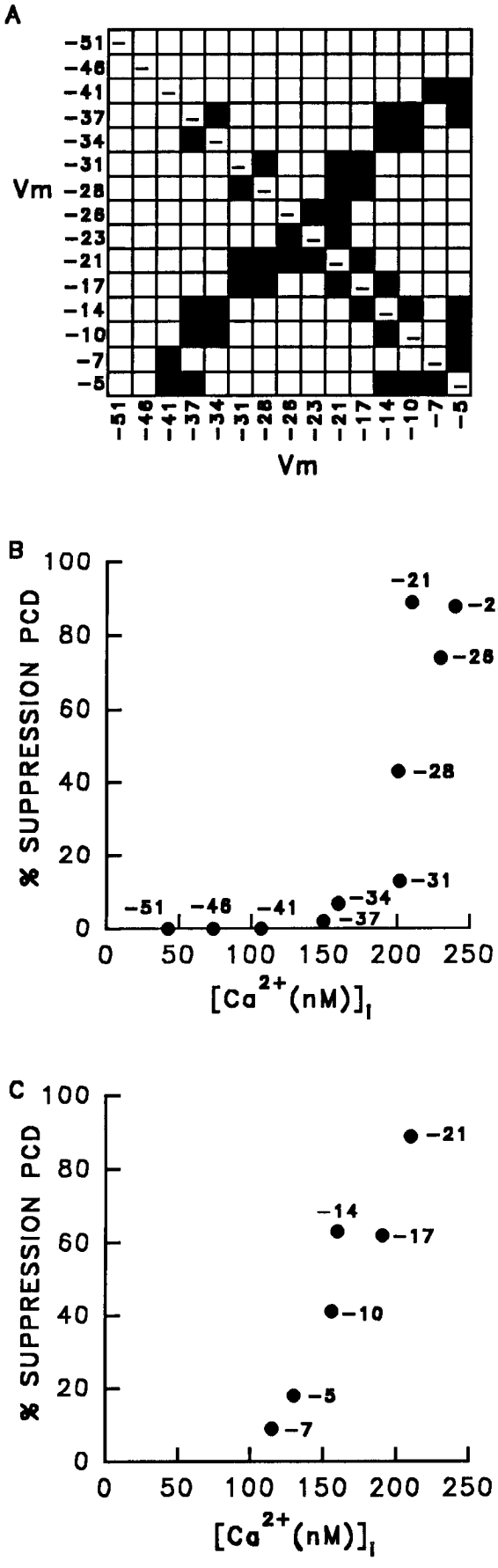
Relationship between survival and $[Ca^{2+}]_i$

The relationship between V_m and mean $[Ca^{2+}]_i$ was roughly similar to that between V_m and mean survival. Both mean $[Ca^{2+}]_i$ in cells depolarized for 24–27 hr (Fig. 7*B*) and mean survival after 4 d (Fig. 6*A*) and 10 d (Fig. 6*B*) of depolarization after NGF deprivation increased with increasingly lower V_m values to a maximum at about −21 to −23 mV and then declined at lower V_m values. Decreasing $[Ca^{2+}]_i$ in depolarized, NGF-deprived neurons with selective concentrations of Ca^{2+} channel antagonists blocked both survival and the sustained rise of $[Ca^{2+}]_i$. Koike et al. (1989) reported that reducing Ca^{2+} concentration in the culture medium also blocks the survival-promoting effects of elevated $[K^+]_o$ on SCG neurons. We confirmed this result. Calcium in the culture medium was reduced by adding 1 mM EGTA to medium in which the only added Ca^{2+} was that contained in the 10% fetal bovine serum. Neurons maintained in this medium in the presence of NGF lived while those maintained in 50K without NGF did not (data not shown). Taken together these data clearly indicate that a DHP-sensitive influx of Ca^{2+} is required for depolarization to prevent PCD of these neurons and suggest that the sustained rise of $[Ca^{2+}]_i$ in depolarized cells may mediate the effect of depolarization on survival. However, as illustrated in Figure 10, the relationship between survival and $[Ca^{2+}]_i$ in depolarized neurons was not clear-cut. Figure 10*A* shows which mean $[Ca^{2+}]_i$ of neurons held at V_m values between −51 and −5 mV for 24–27 hr are significantly different (by ANOVA). Figure 10*B* shows the relationship between survival of cells maintained for 4 d without NGF at V_m values between −51 and −21 mV and $[Ca^{2+}]_i$ in cells maintained for 24–27 hr at the same V_m values. It is apparent that survival varies widely at $[Ca^{2+}]_i$ values that are not significantly different from one another. For example, −21, −28, and −31 mV cause similar sustained rises of $[Ca^{2+}]_i$ ($p > 0.05$; from Fig. 10*A*) while percentage suppression of PCD at these V_m values was 89 ± 20, 45 ± 18, and 16 ± 9, respectively (all significantly different). Therefore, there was no obvious concentration–response relationship between PCD suppression and $[Ca^{2+}]_i$ at these V_m values. Figure 10*C* illustrates that at V_m values between −21 and −5 mV the relationship between survival and $[Ca^{2+}]_i$ more closely resembled a graded concentration–response relationship than it did at more negative V_m values, survival increasing to a maximum over about a 100 nM range of mean $[Ca^{2+}]_i$. Even here, however, different levels of survival are associated with $[Ca^{2+}]_i$ values that are not significantly different (for example at −10 and −14 mV). Some $[Ca^{2+}]_i$ values at V_m values negative to −21 mV (Fig. 10*B*) that are associated with no enhanced survival are also not significantly different from $[Ca^{2+}]_i$ associated with suppression of PCD at some V_m values positive to −21 mV (−37, −14, and −10 mV, for example). At least part of the greater spread of data at more positive V_m values may result from greater plating to plating variability for both survival and $[Ca^{2+}]_i$ at those V_m values than at more negative ones (see insets in Figs. 6*A* and 7*B*). These findings indicate that, while DHP-sensitive Ca^{2+} influx is required for depolarization-enhanced survival, the relationship between survival and $[Ca^{2+}]_i$ is more complex than previously suspected (Collins et al., 1991; Koike and Tanaka, 1991; Franklin and Johnson, 1992).

Effects of calmodulin antagonists on survival and steady-state $[Ca^{2+}]_i$

Gallo et al. (1987) report that survival of chronically depolarized rat cerebellar granule neurons in culture is blocked by calmodulin antagonists. A possible role for calmodulin in depolarization-enhanced survival of other types of neurons in culture has not been investigated. Application of the calmodulin antagonists calmidazolium and W7 to SCG neurons deprived of NGF and depolarized with 50K caused a dose-dependent block of survival at concentrations that had no effect on survival of control cells (Fig. 11*A,B*). The IC_{50} for block of survival was ≈400 nM for calmidazolium and ≈15–20 μM for W7. At concentrations of antagonists only slightly higher than those causing suppression of depolarization-enhanced survival both calmidazolium and W7 were toxic to control neurons maintained in NGF. Calmidazolium killed all neurons saved by depolarization at concentrations ≥750 nM and all control cell at ≥3 μM, including those neurons that were resistant to NGF deprivation. W7 killed all neurons saved by depolarization at concentrations ≥30 μM and all control neurons at concentrations ≥70 μM. Control neurons killed by the antagonists did not exhibit the atrophy characteristic of PCD and cells killed by calmidazolium were not "saved" by inhibition of protein synthesis by cycloheximide as were neurons deprived of NGF (data not shown) indicating that the death caused by the antagonists was not, by these criteria, similar to PCD.

These data appear to suggest a role for calmodulin in mediating the effect of elevated $[Ca^{2+}]_i$ on survival of NGF-deprived neurons. However, a caveat to this interpretation of the results is that inhibitors of calmodulin can block voltage-gated Ca^{2+} channels and reduce Ca^{2+} influx (Greenberg et al., 1987; Doroshenko et al., 1988). Because of this effect, block of depolarization-mediated effects by calmodulin antagonists should be interpreted with great caution. To determine whether calmodulin antagonists affected the sustained rise of $[Ca^{2+}]_i$ caused by depolarization in SCG neurons, cells treated with 50K were exposed to the antagonists. Calmidazolium caused a dose-dependent decrease of the sustained elevation of $[Ca^{2+}]_i$ (Fig. 11*C*) with an IC_{50} of ≈200 nM. Neurons depolarized with 50K for 26–27 hr and treated for 2–3 hr with 30 μM W7, a concentration



that completely blocked depolarization-enhanced survival without affecting control survival, also had $[Ca^{2+}]_i$ reduced to 17 ± 38% of the sustained rise caused by 50K alone ($N = 50$). These data suggest that these antagonists blocked Ca^{2+} influx into these cells and also suggest that this blockade was responsible for their effect on survival. Therefore, these calmodulin antagonists cannot be used to investigate a possible role for calmodulin in depolarization-enhanced survival.

Because of the apparent effect of the calmodulin antagonists on Ca^{2+} influx, an alternative means of chronically increasing $[Ca^{2+}]_i$ that is not affected by the antagonists is desirable. We have recently developed a technique that allows $[Ca^{2+}]_i$ to be increased in these neurons without exposure to elevated $[K^+]_o$. This method uses the potent and selective inhibitor of intracellular Ca^{2+} sequestration, thapsigargin. Treatment of cells with thapsigargin rapidly depletes intracellular Ca^{2+} stores. These stores are then thought to produce a second messenger that traverses the cytoplasm and activates a Ca^{2+} conductance in the plasma membrane (Hoth and Penner, 1992; Lückhoff and Clapham, 1992; Putney and Bird, 1993). Influx of Ca^{2+} through this conductance can cause a sustained increases of $[Ca^{2+}]_i$. We have found that thapsigargin induces a DHP-insensitive sustained rise of $[Ca^{2+}]_i$ in SCG neurons and, in combination with elevated $[Ca^{2+}]_o$ (to increase Ca^{2+} driving force), prevents PCD of NGF-deprived cells (Lampe et al., 1992; Lampe et al., unpublished observations). Figure 12*A* shows the sustained increase of $[Ca^{2+}]_i$ induced by thapsigargin and elevated $[Ca^{2+}]_o$. Calmidazolium had no effect on resting $[Ca^{2+}]_i$ in control cells and did not block the sustained increase of $[Ca^{2+}]_i$ caused by the thapsigargin and high $[Ca^{2+}]_o$ treatment at calmidazolium concentrations that almost completely blocked the sustained increase of $[Ca^{2+}]_i$ in depolarized neurons. These data suggest that calmidazolium does not interfere with Ca^{2+} influx resulting from thapsigargin/high $[Ca^{2+}]_o$ treatment. This lack of effect on the thapsigargin-induced $[Ca^{2+}]_i$ increase allowed NGF-deprived cells "saved" by thapsigargin and elevated $[Ca^{2+}]_o$ to be treated with calmodulin antagonists without affecting Ca^{2+} influx. Figure 12*B* shows that calmidazolium (e.g., 1 μM) did not block survival of NGF-deprived neurons supported by thapsigargin and high-$[Ca^{2+}]_o$ treatment at the same concentrations that completely blocked survival of depolarization-"saved" cells (Fig. 11*A*). Survival was affected by calmidazolium in the thapsigargin/high-$[Ca^{2+}]_o$ supported neurons only at concentrations that proved toxic (2–3 μM) to control cells. We did not determine whether this toxic effect was caused by block of calmodulin activity or by a nonspecific effect.

Effect of depolarization on tyrosine phosphorylation

The biologically active receptor for NGF is TrkA, a member of the tyrosine kinase family of growth factor receptors (TrkA, TrkB, TrkC; referred to here collectively as Trk) and the protein

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Figure 10. Suppression of PCD by depolarization is not clearly related to mean $[Ca^{2+}]_i$. *A*, Significance table comparing mean $[Ca^{2+}]_i$ of cells depolarized to different V_m values for 24–27 hr (from Fig. 7*B*). *Solid squares* indicate that mean $[Ca^{2+}]_i$ at the compared V_m values is not significantly different (by ANOVA). *B*, Relationship between $[Ca^{2+}]_i$ in cells depolarized for 24–27 hr and percentage suppression of PCD after 4 d of depolarization to V_m values between −51 and −21 mV. *C*, Relationship between $[Ca^{2+}]_i$ in cells depolarized for 24–27 hr and percentage suppression of PCD after 4 d of depolarization to V_m values between −21 and −5 mV. Survival data are taken from Figure 6*C*.

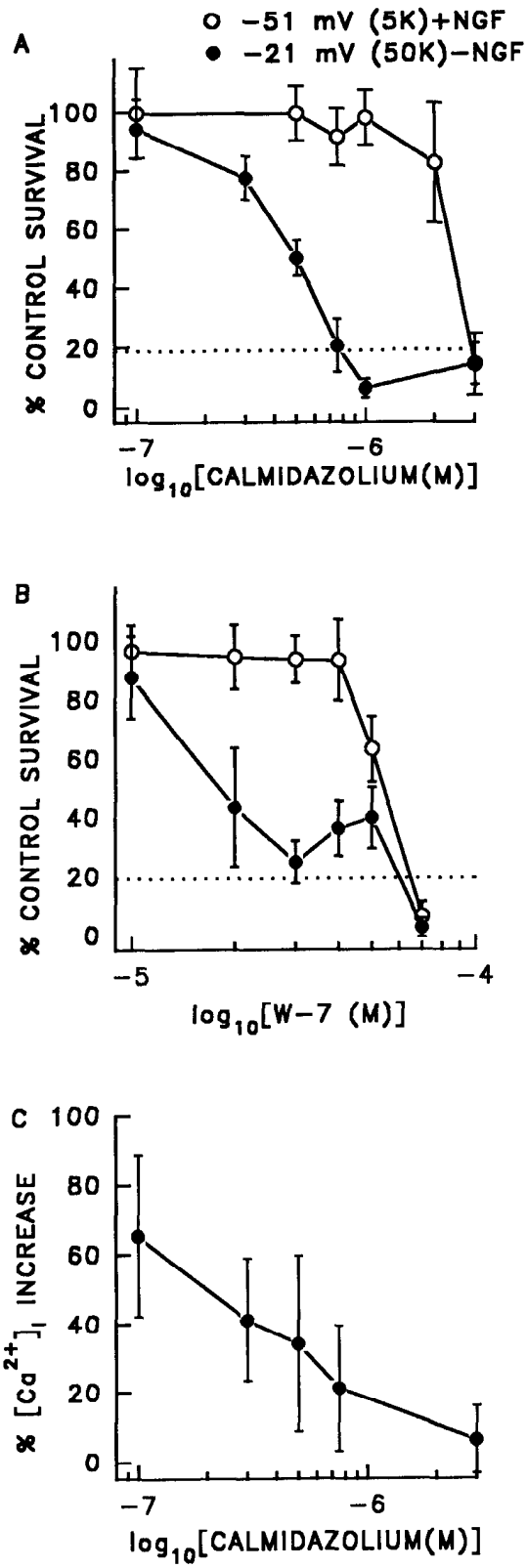

Figure 11. Effects of calmodulin antagonists on survival and $[Ca^{2+}]_i$. Survival of neurons deprived of NGF and depolarized with 50K for 4 d was blocked by calmidazolium (*A*) and W7 (*B*) at concentrations that did not affect survival of control cells. Control survival is average number of neurons in sibling cultures (usually four) maintained in NGF and 5K without drugs for the same period. $N \approx 5$–10 from two platings for each data point in *A* and *B*. *Dotted lines* show survival of cells in sibling 5K cultures deprived of NGF without the drugs. *C*, Calmidazolium caused a dose-dependent block of the sustained rise of $[Ca^{2+}]$ caused by chronic depolarization with 50K. Percent $[Ca^{2+}]_i$ increase is percentage of increase caused by 50K without the drug. Cells were depolarized for 23–28 hr and exposed to calmidazolium for 2–5 hr before $[Ca^{2+}]_i$ measurements were made. $N = 74$–75 from three platings for each data point.

product of the *trk* proto-oncogene (Kaplan et al., 1991). When bound to their ligands, tyrosine kinase receptors autophosphorylate on tyrosine residues. This phosphorylation leads to a cascade of events that mediates most, if not all, of the effects of the growth factors (Schlessinger and Ullrich, 1992). The effect of increased $[Ca^{2+}]_i$ on receptor tyrosine kinase activity has, to our knowledge, not been examined. One conceivable mechanism by which increased $[Ca^{2+}]_i$ may support survival is by activation of TrkA in the absence of NGF. Figure 13, *A* and *B*, shows the effects of NGF and chronic depolarization on Trk (TrkA, TrkB, and TrkC) tyrosine autophosphorylation. In neurons continuously exposed to NGF, Trk (presumably TrkA) was constitutively phosphorylated on tyrosine residues. One hour of NGF deprivation led to a 44% decrease of this phosphorylation. After 2--6 hr of deprivation, Trk phosphorylation was less than 3% of that in cells continuously exposed to NGF. When neurons were deprived of NGF for 2 hr and then exposed to NGF, Trk became rapidly phosphorylated on tyrosine residues. Five minutes after NGF addition, Trk phosphorylation had increased to 226% of the level found in cells continuously exposed to NGF. When NGF-deprived cells were exposed to NGF and depolarized to −21 mV with 50K, the increase in Trk phosphorylation was 217% of that in cells continuously exposed to NGF, similar to that of neurons exposed to NGF at control V_m (−51 mV). When cells that had been deprived of NGF for 1, 2, or 6 hr were exposed to 50K medium containing no NGF, Trk tyrosine phosphorylation was similar to that of cells deprived for the same period (80, 8, or 3% of control, respectively). Similar results were found in the rat pheochromocytoma cell line, PC12, when these cells were exposed to 50K (data not shown). Therefore, depolarization had little effect on Trk autophosphorylation and activation of Trk tyrosine kinase activity is probably not the mechanism by which depolarization promotes survival.

While Trk tyrosine phosphorylation was not substantially affected by depolarization, analysis of total cell lysates showed a single protein band with a molecular weight of about 44 kDa that had increased tyrosine phosphorylation when neurons were exposed to either NGF or 50K (Fig. 13*C*). The molecular weight of this protein suggests that it might be an extracellular signal-regulated protein kinase (ERK; Boulton et al., 1991). These kinases are important signaling molecules in growth factor signal transduction. If this protein is an ERK, the NGF and $[Ca^{2+}]_i$ signal transduction pathways for survival may converge at this point. The data shown in Figure 14 support the possibility that NGF and elevated $[K^+]_o$ may utilize similar transduction pathways to promote survival. These data demonstrate that both NGF and 50K "rescued" NGF-deprived neurons over the same time course. Other agents that can rescue NGF-deprived cells (e.g., cycloheximide) do not follow this time course (Deckwerth and Johnson, 1993). In addition to the 44 kDa protein, NGF caused tyrosine phosphorylation of two proteins with molecular weights of approximately 80 and 95 kDa, respectively. Since these proteins were not tyrosine phosphorylated by depolariza-

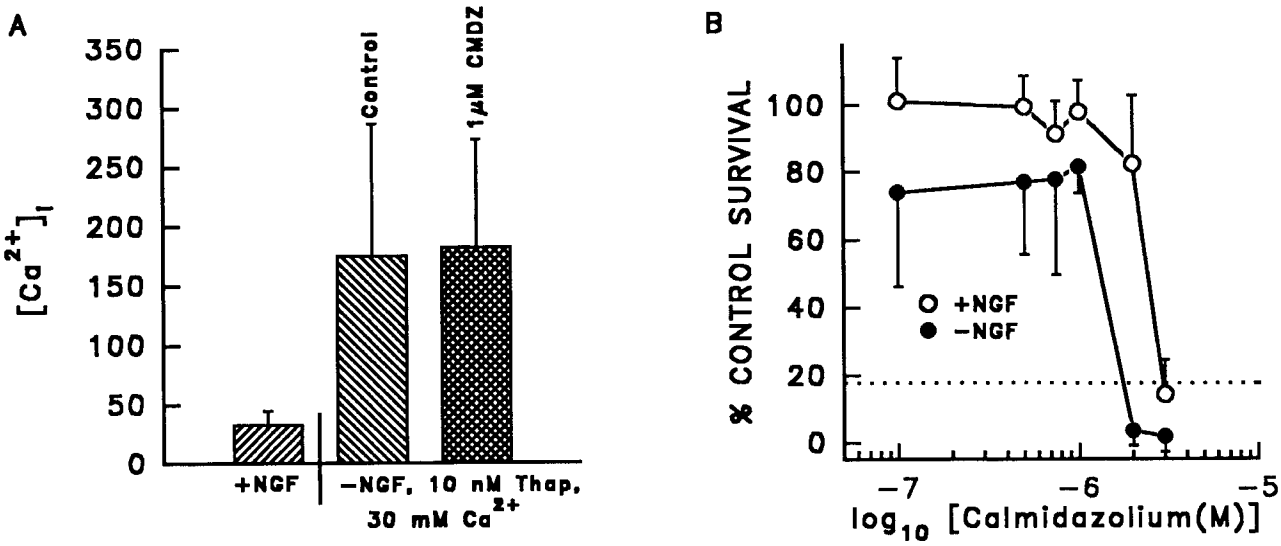

Figure 12. Effect of calmidazolium on survival and the sustained rise of $[Ca^{2+}]_i$ in cells maintained in thapsigargin and 30 mM Ca^{2+}. *A*, Calmidazolium (*CMDZ*) did not decrease a sustained rise of $[Ca^{2+}]_i$ induced by thapsigargin (*Thap*) and elevated $[Ca^{2+}]_o$. Deprived neurons were maintained in medium with 30 mM Ca^{2+} and 10 nM thapsigargin for 22–27 hr and in calmidazolium for 3–4 hr before measurements were made. N = 50–75 from two or three platings for each treatment. *B*, Calmidazolium killed neurons maintained in thapsigargin and elevated $[Ca^{2+}]_o$ (*solid circles*) at approximately the same concentrations that killed control neurons maintained in NGF and standard culture medium. NGF was withdrawn from neurons 5 d after plating and cells were exposed to 10 nM thapsigargin, 30 mM $[Ca^{2+}]_o$, and calmidazolium for 4 d. Survival was then assayed. Control survival is average number of neurons in sibling cultures maintained in NGF and 5K without drug for the same period. *Dotted lines* show survival of cells in sibling control cultures deprived of NGF without calmidazolium. N = 5–8 from two platings for each data point.

tion, they are unlikely to be part of the signal transduction pathway by which depolarization promotes survival.

Discussion

The purpose of this study was to provide insight into the mechanisms by which elevated $[K^+]_o$ prevents neuronal PCD. To this end we analyzed and compared the effects of elevated $[K^+]_o$ on the V_m, growth, $[Ca^{2+}]_i$, and survival of rat SCG neurons deprived of NGF in culture. Elevated $[K^+]_o$ caused chronic depolarization of neurons and a sustained increase of steady-state $[Ca^{2+}]_i$. This $[Ca^{2+}]_i$ rise appeared to be caused primarily by DHP-sensitive Ca^{2+} influx. By the fifth day after plating depolarization with medium containing 50 mM K^+ prevented death of most NGF-deprived cells. Small decreases of V_m were associated with large changes in survival. The relevant parameter suppressing PCD of NGF-deprived cells maintained in high $[K^+]_o$ appeared to be Ca^{2+} influx rather than depolarization since the DHP Ca^{2+} channel antagonist, nifedipine, blocked both depolarization-enhanced survival and increased $[Ca^{2+}]_i$ at similar low concentrations. Additionally, lowering $[Ca^{2+}]_o$ blocked the ability of depolarization to suppress PCD. While these data strongly suggest that increased $[Ca^{2+}]_i$ mediates depolarization-enhanced survival there was no clear correlation between measured $[Ca^{2+}]_i$ at different V_m values and the ability of those V_m values to promote survival. This lack of correlation suggests that the relationship between $[Ca^{2+}]_i$ and survival is more complex than we (Franklin and Johnson, 1992), and others (Collins et al., 1991; Koike and Tanaka, 1991), have previously supposed. Chronic depolarization with 50 mM K^+ was ineffective in preventing death of freshly plated neurons deprived of NGF. This may be, in part, because this treatment did not appear to cause a substantial Ca^{2+} influx in these cells. While survival in the absence of NGF was supported by chronic depolarization, growth of cells was greatly reduced when NGF was removed from the culture medium.

We also investigated possible signal transduction pathways by which depolarization-induced Ca^{2+} influx may be translated into enhanced survival. Calmodulin antagonists blocked the depolarization-enhanced survival of NGF-deprived cells at concentrations that did not affect control cells but apparently did so by blocking Ca^{2+} influx rather than through an effect on calmodulin. At slightly higher concentrations, calmodulin antagonists were toxic to control cells and to NGF-deprived cells "saved" by increased Ca^{2+} influx induced by thapsigargin/high $[Ca^{2+}]_o$; therefore, it was not possible to use these compounds to obtain insight into whether calmodulin was involved in the effect of increased $[Ca^{2+}]_i$ on survival. Depolarization to optimal survival-promoting V_m had little effect on autophosphorylation of Trk indicating that activation of the Trk tyrosine kinase was probably not the mechanism by which Ca^{2+} influx promoted survival. Both NGF and depolarization caused tyrosine phosphorylation of a protein with a molecular weight of about 44 kDa.

Relationship between V_m, *survival,* $[Ca^{2+}]_i$, *and growth*

Chalazonitis and Fischbach (1980) used intracellular recording techniques to demonstrate that prolonged exposure of embryonic chicken dorsal root ganglion (DRG) neurons in cell culture to a wide range of elevated $[K^+]_o$ (1–100 mM) causes these neurons to become chronically depolarized to a wide range of V_m values. They also found that prolonged exposure to high $[K^+]_o$ enhances survival of these neurons. However, only two K^+ concentrations were used for the survival experiments so the influence of a wide range of V_m values on survival of these cells was not determined. To ascertain the relationship between V_m and survival of NGF-deprived rat SCG neurons, we used the tight-

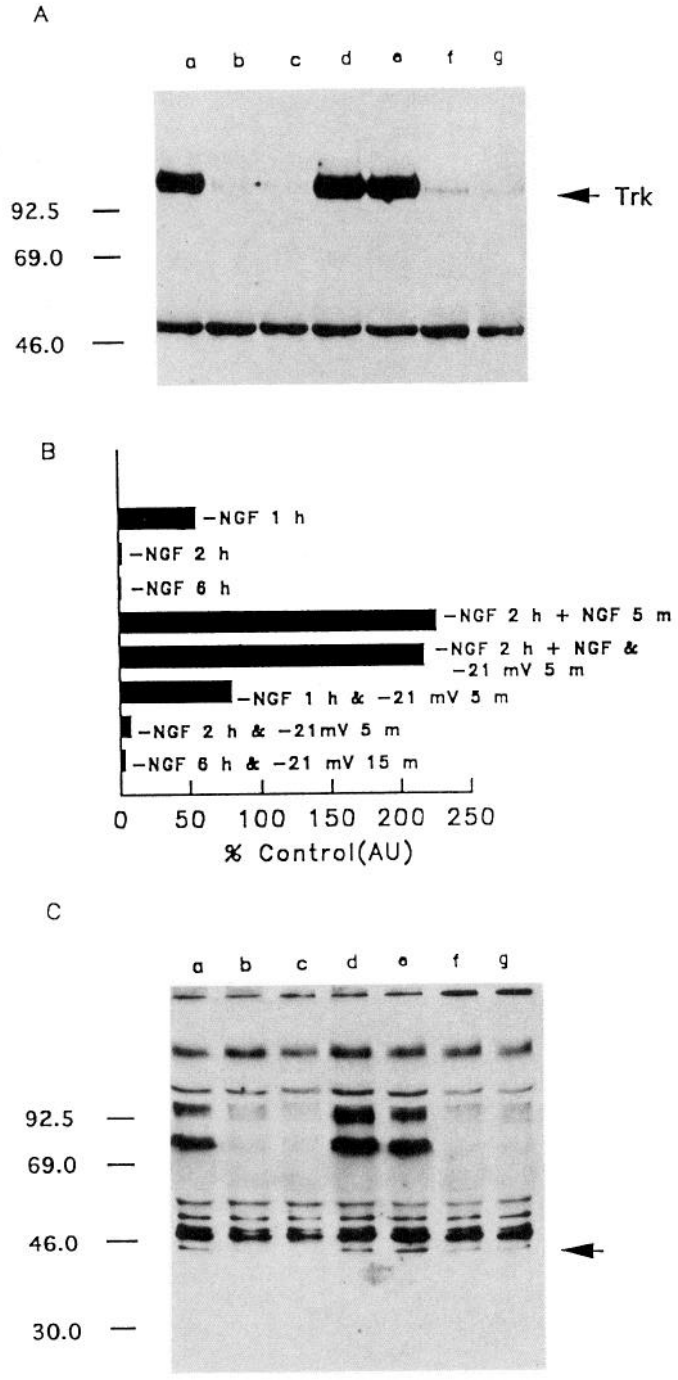

Figure 13. Effect of NGF and depolarization on tyrosine phosphorylation. *A*, Immunoblot illustrating the effects of NGF and depolarization with 50K (−21 mV) on Trk tyrosine autophosphorylation. Lanes: *a*, Control, tyrosine phosphorylation of Trk in SCG neurons continuously exposed to NGF; *b* and *c*, 2 or 6 hr after NGF deprivation in 5K; *d*, 2 hr of deprivation followed by 5 min exposure to NGF in 5K; *e*, 2 hr of deprivation followed by 5 min exposure to NGF and 50K; *f* and *g*, NGF deprivation for 2 hr followed by 5 or 15 min exposure to 50K without NGF. Depolarization had little effect on Trk autophosphorylation either in the absence or presence of NGF. The *lower bands* are IgGs. *B*, Quantification by densitometry of Trk tyrosine autophosphorylation in cells receiving the indicated treatments. Data is normalized to phosphorylation of Trk in cells continuously exposed to NGF (*AU*, absorbance units). Data was taken from the immunoblot in *A* except for the 1 hr data, which was taken from a different immunoblot. *C*, Immunoblot showing tyrosine phosphorylation of proteins in lysates of the cells used for the immunoprecipitation of Trk in *A*. The lanes are arranged in the same order. The *arrow* indicates a protein with a molecular weight of about 44 kDa that showed increased tyrosine phosphorylation with both NGF and high $[K^+]_o$ treatment.

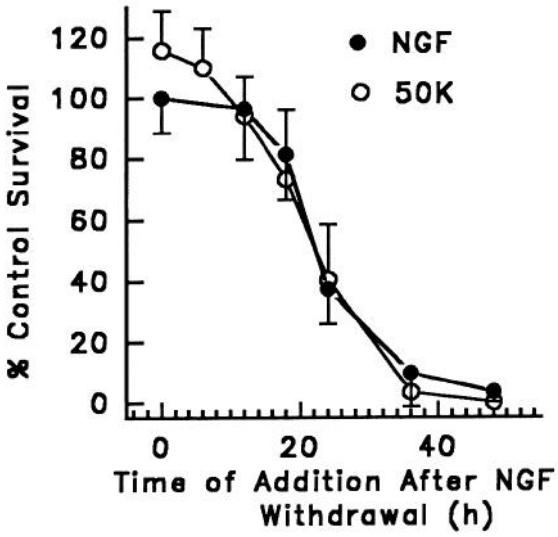

Figure 14. The time course of rescue by high $[K^+]_o$ and NGF are identical. Neurons were deprived of NGF 7 d after plating. 50K or NGF were added at the indicated time after deprivation and survival was assayed 1 week later. Cells were dissociated by treatment with 1 mg/ml dispase and 1 mg/ml collagenase. $N = 8$--12 for each 50K data point. NGF data is taken from Deckwerth and Johnson (1993).

seal whole-cell recording technique to measure V_m values of neurons in all $[K^+]_o$ in which the survival of NGF-deprived cells was examined. The data show that survival was steeply related to V_m, increasing from no enhanced survival to maximal enhancement over about a 13 mV decrease of V_m. While some SCG neurons held at mean V_m values greater than or equal to −46 mV fired action potentials, no neurons maintained at mean V_m values less than −46 mV exhibited any electrical activity. The lack of electrical activity at potentials that saved cells from dying when deprived of NGF indicates that the critical factor for survival was the sustained depolarization rather than action potentials or synaptic potentials induced by the elevated $[K^+]_o$. This was previously suggested by the work of Koike et al. (1989) showing that tetrodotoxin does not affect high $[K^+]_o$ promoted survival of these cells.

Steep voltage dependence of a cellular phenomenon can indicate mediation by voltage-gated channels in the plasma membrane that have V_m thresholds that must be exceeded for activation. This is probably not the mechanism underlying the steep relationship between survival of NGF-deprived rat SCG neurons and V_m. Intracellular Ca^{2+} concentration increased more gradually with increasingly lower V_m values (between −51 and −5 mV) than did survival. Thus, the steep relationship between survival and V_m and is not explained by a V_m-dependent threshold for activation of Ca^{2+} channels. Scott and Fisher (1970) and Scott (1971) showed that increasing $[K^+]_o$ incrementally from less than 5 mM to 81 mM gives an inverted U-shaped concentration–response curve for survival of dissociated embryonic chicken DRG neurons and human spinal ganglion neurons in culture; no explanation for this effect was given. The effect of such a wide range of $[K^+]_o$ on survival of other neuronal types

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has not been investigated. The inverted U-shaped V_m ($[K^+]_o$)-survival curve for NGF-deprived rat SCG neurons reported here is qualitatively similar to their findings. The relationship between survival and V_m was roughly similar to the relationship between V_m and $[Ca^{2+}]_i$, mean $[Ca^{2+}]_i$ and mean survival reaching a peak in cells held at about −23 to −21 mV and then decreasing at more positive V_m values. These data suggest that the inverted U-shaped V_m-survival curve was caused by the ability of the different V_m values to cause an adequate Ca^{2+} influx. The most likely explanation for decreasing $[Ca^{2+}]_i$ at increasingly lower V_m values is increasing steady-state inactivation of Ca^{2+} channels. This would result in fewer channels available to carry Ca^{2+} current and, subsequently, reduced Ca^{2+} influx.

Only after 3 d in culture (in NGF) did elevated $[K^+]_o$ support survival of a substantial number of NGF-deprived cells. While the effect of increased $[K^+]_o$ on $[Ca^{2+}]_i$ was blunted 1 d after plating as compared to that of cells that had been in culture for longer periods, by the third day after plating, $[Ca^{2+}]_i$ of cells in the same $[K^+]_o$ was well within the range of concentrations of neurons "saved" by this concentration of $[K^+]_o$ at later times in culture and yet few cells survived. These data suggest that factors other than the effect of depolarization on $[Ca^{2+}]_i$ were, at least partially, responsible for development of the ability of depolarization to prevent PCD. Since NGF appears to be necessary for significant growth of neurites, one possible explanation for the development of this response is that cells must extend neurites and become well attached to the substrate in the tissue culture dish before chronic depolarization can promote their survival. Without NGF, the cells might not attach and could float off the substrate and be lost to the survival assay that was used even though they may remain alive. While this possibility might explain why cells could not be saved by depolarization at the time of plating, it cannot explain the failure of depolarization to prevent death at later times. By the third day after plating, neurons had developed neurites and appeared well attached to the culture dishes even when they were dying after NGF deprivation (data not shown). Another possibility is that neurons require NGF to recover fully from damage caused by dissociation and only after this recovery can depolarization replace NGF in supporting survival. Since depolarization promotes little growth, damage to cells caused by dissociation may not be repaired in depolarized, NGF-deprived neurons and without this repair cells cannot live. Yet another possibility is that the appropriate signal transduction pathways or mechanisms by which Ca^{2+} influx promotes survival are not mature in freshly plated neurons and several days of exposure to NGF are required for these pathways to become fully functional. Acheson et al. (1987) found that the ability of elevated $[K^+]_o$ to support survival of embryonic chicken DRG neurons in culture is much greater with cells dissociated from day 12 than day 6 embryos. Determining whether the development of depolarization-enhanced survival of rat SCG neurons during the first week in culture is a true developmental change or an artifact of dissociation will require similar experiments examining survival of depolarized, NGF-deprived neurons dissociated from week-old rather than E21 rats. This issue will be addressed in future work.

Our results only partially confirm those of Koike and Tanaka (1991) concerning the effect of chronic depolarization on $[Ca^{2+}]_i$ and survival of NGF-deprived embryonic rat SCG neurons in culture. We extended their results by investigating the effect of a wider range of $[K^+]_o$ on survival and $[Ca^{2+}]_i$, determined the V_m values associated with the different $[K^+]_o$, and determined the effects on both survival and $[Ca^{2+}]_i$ of wide range of concentrations of a Ca^{2+} channel antagonist. We concur with Koike and Tanaka that Ca^{2+} influx is required for depolarization-enhanced survival of NGF-deprived SCG neurons. However, by doing a more complete analysis of the relationship between $[K^+]_o$, $[Ca^{2+}]_i$, and survival, we find that Koike and Tanaka's conclusion that there is a good correlation between the sustained rise of $[Ca^{2+}]_i$ and survival in depolarized SCG neurons requires reevaluation. Similarly, the conclusion of Collins et al. (1991) that their data show a good correlation between survival and a sustained rise of $[Ca^{2+}]_i$ induced by chronic depolarization ± DHP agonist treatment of chick embryo ciliary ganglion neurons may not be justified. In that work survival appeared to vary widely at $[Ca^{2+}]_i$ that, by close inspection, do not appear to be significantly different. The discrepancy between our results and those of Koike and Tanaka (1991) concerning increased $[Ca^{2+}]_i$ in control neurons as they age in culture is puzzling. However, due to a small sample number, it is not clear that the increase of $[Ca^{2+}]_i$ they measured in older cells is significant. The small increase of mean $[Ca^{2+}]_i$ that we measured in control cells maintained for about 4 weeks in culture was probably not enough to account for decreased trophic factor dependence. Therefore, our results are not consistent with Koike and Tanaka's suggestion that increased $[Ca^{2+}]_i$ with age causes decreased trophic-factor dependence.

It is clear from our data and that of others (Collins et al., 1991; Koike and Tanaka, 1991; see Franklin and Johnson, 1992, for review) that Ca^{2+} influx is required for depolarization to promote survival. It is, therefore, surprising that there is no obvious correlation between $[Ca^{2+}]_i$ and survival in chronically depolarized cells. There are at least two possible explanations for lack of an obvious relationship between survival and steady-state $[Ca^{2+}]_i$. First, perhaps the sustained elevation of $[Ca^{2+}]_i$ measured here is only loosely related to the relevant pool of Ca^{2+} that enhances survival. For example, the Ca^{2+} pool promoting survival may be highly localized near the point of Ca^{2+} entry into the cells. It is known that $[Ca^{2+}]_i$ can reach very high concentrations near the cytoplasmic side of Ca^{2+} channels and that this is important for neurotransmitter release (Augustine and Neher, 1992). These highly localized increases of $[Ca^{2+}]_i$ are not detectable by standard methods of $[Ca^{2+}]_i$ measurement such as those used here and may be only roughly reflected in measurements of global $[Ca^{2+}]_i$. Second, the relationship between survival and $[Ca^{2+}]_i$ may be a threshold effect with no PCD suppression occurring until the required concentration is attained. Once this concentration is exceeded, survival is promoted in a nearly all-or-none manner. If a threshold of $[Ca^{2+}]_i$ is required to promote survival, the lack of significant difference in $[Ca^{2+}]_i$ that promote widely different levels of survival may be explained, at least in part, by experimental error. That is, if very small increases in $[Ca^{2+}]_i$ (at the threshold) make the difference in all cells living or dying the technique used here might not be able to accurately distinguish these differences due to "noise" in the measurement process.

The role of L-type Ca^{2+} channels in depolarization-induced rise of $[Ca^{2+}]_i$ and suppression of PCD

Almost 90% of the sustained increase of steady-state $[Ca^{2+}]_i$ and 100% of the survival enhancement caused by chronic depolarization were blocked in a dose-dependent manner by the DHP

Ca^{2+} channel antagonist nifedipine. These data strongly suggest that Ca^{2+} current carried by L-type Ca^{2+} channels was responsible for the increased $[Ca^{2+}]_i$ and enhanced survival. The N-type channel antagonist ω-conotoxin, which has no effect on L-channels in rat SCG neurons (Plummer et al., 1989), also had no effect on steady-state $[Ca^{2+}]_i$ in depolarized cells or on survival of depolarized, NGF-deprived neurons. The lack of complete block of the sustained rise of $[Ca^{2+}]_i$ by a DHP antagonist might be because of Ca^{2+} influx through channels other than L-type or N-type or because of failure of L-channel antagonists to completely block the activity of L-channels even at saturating concentrations. Therefore, most, or all, of the $[Ca^{2+}]_i$ increase in chronically depolarized rat SCG neurons is probably caused by Ca^{2+} influx through L-type channels. This finding is rather surprising since ≈85–90% of the whole-cell Ca^{2+} current in the soma of these cells is carried by N-type channels, most of which are sensitive to block by ω-conotoxin (Plummer et al., 1989; Regan et al., 1991). Moreover, N-type channels in rat SCG neurons have a noninactivating or slowly inactivating component that lasts for at least several hundred ms during sustained depolarizations to some of the V_m values examined in this article. Such a noninactivating component would, seemingly, contribute to prolonged increases of $[Ca^{2+}]_i$ in chronically depolarized cells. Since ω-conotoxin–sensitive channels were clearly not involved in the depolarization-induced increase of $[Ca^{2+}]_i$ and survival reported here, it seems likely that during longer depolarizations these channels do undergo steady-state inactivation.

Although the whole-cell and single-channel Ca^{2+} currents of rat SCG neurons have been studied in considerable detail (Belluzzi and Sacchi, 1989; Nerbonne and Gurney, 1989; Plummer et al., 1989; Regan et al., 1991), an in-depth analysis of pharmacologically separated L-type and N-type whole-cell Ca^{2+} currents in these cells has not yet been done. The most likely explanation for how L-channels may cause a sustained elevation of $[Ca^{2+}]_i$ is that there is a range of V_m values in which the activation and inactivation curves of the channels cross resulting in a continuously active Ca^{2+} "window" current. It will be of interest to characterize more completely the L-type currents in these cells and correlate their properties with the effects of different V_m values on survival and $[Ca^{2+}]_i$. Because the prolonged depolarizations caused by elevated $[K^+]_o$ are unlikely to occur *in vivo*, determination of the effects of more physiological electrical activity on $[Ca^{2+}]_i$ and survival will also be interesting. Perhaps with more natural forms of stimulation, Ca^{2+} influx through N-type channels can also contribute to survival.

How does Ca^{2+} influx prevent PCD?

Elevated $[K^+]_o$ could conceivably promote survival of NGF-deprived rat SCG in culture by stimulating a Ca^{2+}-dependent release of autocrine or paracrine factors. About 9% of the cells in these cultures were non-neuronal (about 100–200/dish). These cells did not show any particular association with neurons, appearing to be randomly distributed in the dish. Since the cultures were maintained in as much as 2 ml of medium, the large dilution factor indicates that high-$[K^+]_o$ promoted survival cannot be caused by release of paracrine factors by non-neuronal cells. Acheson et al. (1987) reached similar conclusions concerning the effects of high $[K^+]_o$ on survival of embryonic chicken DRG neurons in culture. We have found that the plating density of neurons in culture does not affect the ability of depolarization to enhance survival. Superior cervical ganglion neurons in low-density cultures deprived of NGF and supported by high $[K^+]_o$ had about the same percentage of control survival as those in high-density cultures (10-fold differences in density; data not shown). If a factor were released by the neurons, more should be available in high-density cultures and survival would, presumably, be enhanced via both autocrine and paracrine effects. However, highly localized paracrine or autocrine effects, (for example, synaptic release) in low-density cultures are possible. Recent work suggests that brain-derived neurotrophic factor (BDNF) is, at least partially, responsible for depolarization-enhanced survival of cortical neurons in culture (Ghosh et al., 1994). We have found that BDNF has no effect on the survival of NGF-deprived SCG neurons (unpublished observation) making it unlikely that BDNF release is the mechanism by which depolarization promotes survival of these cells. NGF cannot be a survival factor released by depolarized SCG neurons since the anti-NGF antibody in the culture medium neutralizes its effects and Trk was not activated. Additionally, since the antibody used for Trk immunoprecipitation recognizes all members of the Trk receptor family in rodents (TrkA, TrkB, TrkC; Zhou et al., 1994), none of the other members of the neurotrophin family were released in depolarized neurons. Therefore, depolarization-induced release of neurotrophins is not the mechanism by which depolarization suppresses PCD. Ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) have both survival- and death-promoting effects on these cells, saving some populations of neurons while killing others (Kessler et al., 1994; Kotzbauer et al., 1994). Depolarization prevented death of all cells deprived of NGF, thus probably eliminating CNTF and LIF as autocrine factors. A number of bioactive peptides, which might possibly act as autocrine factors, do not enhance survival of these cells in the absence of NGF (Martin et al., 1992). The effect of depolarization on Trk tyrosine kinase activity was investigated over only a 15 min period in this report. The data clearly indicated that there is no short-term activation of Trk by depolarization. However, the data do not rule out long-term effects such as those reported by Ghosh et al., 1994. We are actively investigating the possibility that other members of the neurotrophin family and, perhaps, their receptors may be upregulated in chronically depolarized neurons and act as autocrine or paracrine factors mediating the effects of depolarization on survival.

One possible explanation for the relationship between $[Ca^{2+}]_i$ and survival illustrated in Figure 10 is that Ca^{2+}-binding protein(s) mediating effects of increased $[Ca^{2+}]_i$ on survival bind Ca^{2+} cooperatively. Such cooperative binding can result in "switch-like" concentration–response relationships. Therefore, rather than a graded concentration–response relationship between survival and $[Ca^{2+}]_i$ such a mechanism would show survival increasing only when a threshold level of $[Ca^{2+}]_i$ is exceeded. The best-characterized protein exhibiting this type of binding is calmodulin, a ubiquitous and abundant Ca^{2+}-binding protein found in all eukaryotic cells (Cohen and Klee, 1988; Rasmussen and Means, 1989; Heinzmann, 1991). In mammals calmodulin has three or four cooperative Ca^{2+}-binding sites. This Ca^{2+}-binding characteristic causes calmodulin to act as a Ca^{2+}-concentration threshold detector, not activating until all sites are bound and then activating in a near all-or-none manner. Gallo et al. (1987) show that high $[K^+]_o$-induced survival of rat cerebellar granule cells in culture is blocked by the calmodulin antagonists, trifluoperazine and calmidazolium, suggesting that calmodulin activation may mediate the effect. In light of our

finding that calmodulin antagonists, including calmidazolium, apparently block survival of chronically depolarized NGF-deprived rat SCG neurons by blocking Ca^{2+} influx, a similar block likely occurs in granule cells and, thus, a role for calmodulin in depolarization-enhanced survival of those cells remains an open question. Calmidazolium did not effect a sustained increase of $[Ca^{2+}]_i$ induced by thapsigargin and elevated $[Ca^{2+}]_o$ in rat SCG neurons. However, calmidazolium was toxic to control cells and cells "saved" from PCD by the thapsigargin/high $[Ca^{2+}]_i$ treatment at similar concentrations. Whether the effects of the calmodulin antagonists on survival of control cells resulted from inhibition of calmodulin or nonspecific toxic effects, we did not determine. Thus, because of the nonspecificity of calmodulin antagonists we were not able to verify or refute a role for calmodulin activation in Ca^{2+}-increased survival of these cells.

Significant activation of the Trk tyrosine kinase occurred only with NGF treatment; depolarization had little effect. Thus, activation of Trk by increased $[Ca^{2+}]_i$ probably does not occur and is not the mechanism by which depolarization promotes survival. The increased tyrosine phosphorylation of a protein with a molecular weight of about 44 kDa by both NGF and depolarization is intriguing since this is the approximate molecular weight of ERKs (Boulton et al., 1991; Cobb et al., 1991; Davis, 1993). Increased tyrosine phosphorylation of these kinases is caused not only by growth factor treatment but many other stimuli as well, including KCl in PC12 cells (Tsao et al., 1990) and in the brain by electroconvulsive shock (Stratton et al., 1991). ERKs are activated by phosphorylation on tyrosine and threonine/serine residues. When activated, ERKs phosphorylate a large number of substrates including other kinases, microtubule-associated proteins, and transcription factors. Many of the effects of growth factors are thought to be mediated by these phosphorylations. ERKs can be activated by several other kinases involved in growth-factor signaling including Raf-1 kinase. Raf-1 can be activated either by protein kinase C (PKC; Kolch et al., 1993) or by a PKC-independent receptor tyrosine kinase pathway. Protein kinase C has Ca^{2+}-dependent forms and is also activated by diacyl glycerol (DAG) (Huang, 1989). PKC may be involved in depolarization-enhanced survival of chicken sympathetic neurons in culture (Wakade et al., 1988). In these cells chronic depolarization increases PKC activity, and phorbol esters, which mimic the action of DAG on PKC, will substitute for depolarization in supporting survival. The PKC activator phorbol 12-myristate 13-acetate (PMA) has no effect on survival of NGF-deprived rat SCG neurons at concentrations that promote survival of chicken neurons (Martin et al., 1992). However, this type of experiment is difficult to interpret since PMA causes profound down regulation of PKC in rat SCG neurons (Matthies et al., 1987). If Ca^{2+} prevents PCD through activation of a PKC-dependent pathway, phorbol ester treatment of depolarized, NGF-deprived neurons might downregulate PKC and block survival. However, we have found that the ability of 50K to promote survival of rat SCG neurons deprived of NGF is not affected by PMA treatment (unpublished observation). Thus, PMA has no effect on survival of rat SCG neurons under any condition. It appears, therefore, that PKC is not involved in depolarization-enhanced survival of rat SCG neurons. Further investigation of potential roles for ERKs, calmodulin, and additional Ca^{2+}-binding proteins in depolarization-induced survival of these cells is beyond the scope of this report and will be pursued in future work.

Separation of growth and survival

One of the most interesting results reported here is that NGF promotes both growth and survival of SCG neurons while chronic depolarization supports survival but little growth. This finding suggests that growth and survival are mediated via separate signal transduction pathways. Neurites of NGF-deprived neurons were stabilized by elevated $[K^+]_o$ with only a little growth and retraction occurring. This finding is similar to results reported recently by Teng and Greene (1993) in PC12 cells. When PC12 cells are exposed to NGF, they extend neurites and differentiate into a phenotype resembling that of sympathetic neurons. After differentiation, removal of NGF causes neurites to degenerate and the cells revert to a phenotype similar to the one they had before exposure to NGF. When Teng and Greene depolarized differentiated PC12 cells with elevated $[K^+]_o$, NGF removal did not cause neurite degeneration. Instead, neurites were stabilized; little extension or retraction occurred. This stabilization is blocked by DHP Ca^{2+} channel antagonists suggesting that the effect of chronic depolarization is mediated by Ca^{2+} influx through L-type channels. The stabilizing effect of high $[K^+]_o$ on PC12 cell neurites is suggested to be a result of stabilization of the cytoskeleton caused, perhaps, by Ca^{2+}-activated phosphorylation of cytoskeletal proteins. This hypothesis is supported by the work of Riederer et al. (1992) showing that sustained depolarization of fetal rat telencephalic neurons in culture enhances development and maturation of their cytoskeleton. It is likely that the stabilization of neurites in NGF-deprived SCG neurons by elevated $[K^+]_o$ is the same phenomenon observed in PC12 cells exposed to high $[K^+]_o$. If the cytoskeletal stabilization hypothesis is correct it is possible that such stabilization may also be, at least partially, responsible for depolarization-enhanced survival.

In summary, the data presented here indicate that, while chronic depolarization prevented PCD of NGF-deprived rat sympathetic neurons in culture by causing a DHP-sensitive Ca^{2+} influx, the relationship between increased $[Ca^{2+}]_i$ and survival was not clear-cut. This finding suggests that the connection between $[Ca^{2+}]_i$ and survival may be more complex than previously assumed, perhaps involving localized Ca^{2+} pools or $[Ca^{2+}]_i$ thresholds. Little growth was supported by chronic depolarization in the absence of NGF. The NGF receptor, Trk, was probably not involved in mediating the effect of depolarization on survival and the non-specificity of calmodulin antagonists did not allow investigation of a role for calmodulin. A $\approx$44 kDa protein that may be an ERK became phosphorylated on tyrosine residues in response to both NGF and depolarization indicating that this protein is a point of convergence for depolarization and NGF signaling pathways. This protein and its potential role in transduction of growth factor and Ca^{2+}-promoted survival should be most interesting to characterize. Synergistic effects of growth factors and depolarization on survival have been recently demonstrated in embryonic chicken ciliary ganglion neurons in culture (Schmidt and Kater, 1993). Such synergism might be caused by activation of the same signaling pathways. This is an appealing idea since convergence of depolarization and neurotrophic signals on the same pathways is a parsimonious and relatively simple means by which afferent input to a neuron (e.g., electrical activity/release of factors from presynaptic cells) and target-derived influences (growth factors) could combine to determine which neurons are spared from PCD.

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