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Evidence that nerve growth factor dependence of sympathetic neurons for survival *in vitro* may be determined by levels of cytoplasmic free Ca^{2+}

(trophic factor/cell death/high K^+/fura-2)

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ABSTRACT **Developing sympathetic neurons established in the presence of nerve growth factor (NGF) die *in vitro* after acute withdrawal of NGF. This *in vitro* model mimics the physiological situation in which neurons die during development or after axotomy when trophic support becomes insufficient. We have previously shown that depolarizing agents including high K^+ and cholinergic agonists prevent neuronal death induced by acute deprivation of NGF *in vitro*. Based on this finding, a Ca^{2+} set-point hypothesis was proposed for the degree of neuronal dependence on tropic factor *in vitro*. Here we have examined the validity of this hypothesis by measuring the level of cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) with fura-2 as a probe for monitoring Ca^{2+}. (*i*) There was a good correlation between cell survival in the absence of NGF and $[Ca^{2+}]_i$ levels of young sympathetic neurons (1 week *in vitro*) chronically exposed to various concentrations of extracellular K^+, which shows that 50% survival occurred at ≈184 nM $[Ca^{2+}]_i$ and complete survival, independent of trophic support, occurred at ≈240 nM $[Ca^{2+}]_i$. (*ii*) The basal level of $[Ca^{2+}]_i$ of sympathetic neurons was relatively low (93.0 ± 10.5 nM) at days 6–8, then increased with incubation time, and finally reached a plateau level of 241 ± 7 nM at around week 3, when the neurons became independent of NGF for survival. (*iii*) Sympathetic neurons maintained in the presence of high or low concentrations of Ca^{2+} displayed altered trophic dependence. Thus, these findings are consistent with this Ca^{2+} set-point hypothesis for the degree of NGF dependence of sympathetic neurons for survival *in vitro*.**

During the development of the nervous system, nerve cells are overproduced and then their final numbers are established by the death of those neurons that fail to have successful contacts with their targets (1–4). Target-derived trophic factors have a decisive role in this mechanism. Developing sympathetic and embryonic sensory neurons established in the presence of nerve growth factor (NGF) die *in vitro* after acute withdrawal of NGF (5, 6). This *in vitro* model mimics the physiological situation in which neurons die during development or after target removal or axotomy. Neuronal death caused by trophic-factor deprivation, both *in vitro* and *in vivo*, is an active process requiring the synthesis of mRNA and protein (5, 7, 8).

There is also growing evidence that non-target-derived influences may be involved in regulating neuronal death. Afferent inputs, in particular, have a critical role in this respect. Thus, removal of afferent inputs increases naturally occurring neuronal death in chicken ciliary ganglion cells (9); the blockade of ganglionic transmission promotes neuronal death in retinal ganglion cells (10) and in chicken sympathetic and ciliary ganglion cells (11). These findings suggest that electrical activity is important for normal development of the nervous system. We found that depolarizing agents, including elevated K^+ (≥33 mM) and cholinergic agonists, prevent death of developing sympathetic neurons after acute withdrawal of NGF *in vitro* (12). Ca^{2+} influx through dihydropyridine-sensitive, L-type Ca^{2+} channels has a major function in the protection by high K^+ (12), whereas the depolarizing agents choline and carbamoylcholine, acting through nicotinic cholinergic receptors, appear to exert their effects through activation of Ca^{2+} release from intracellular stores (12). In either case, prevention of the death of NGF-deprived cells may be accompanied by an increase of cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$). The involvement of L-type Ca^{2+} channels in potassium-mediated survival of chicken ciliary, sympathetic, and dorsal root ganglia has also been demonstrated (13). Based on these findings, a Ca^{2+} set-point hypothesis was proposed in which the $[Ca^{2+}]_i$ effectively determines the degree of neuronal dependence on trophic factor for survival *in vitro* (12). This hypothesis predicts that developing neurons acutely dependent on trophic factor have lower $[Ca^{2+}]_i$ than mature neurons, which are less dependent on trophic factor. Since this age-related decrease in trophic factor dependence occurs *in vitro* (14), we have examined the validity of this hypothesis by measuring $[Ca^{2+}]_i$ as a function of the aging of sympathetic neurons *in vitro*. We have also tested for correlation between neuronal survival and $[Ca^{2+}]_i$ of young sympathetic neurons exposed to various concentrations of extracellular K^+. The data provided here are consistent with the Ca^{2+} set-point hypothesis.

MATERIALS AND METHODS

Materials. Mouse NGF (2.5S) was isolated from male mouse submaxillary glands by the method of Mobley *et al.* (15). Antiserum against mouse 2.5S NGF prepared as described (16) was kindly donated by E. M. Johnson and P. Osborne (Washington University School of Medicine, St. Louis). Fura-2 acetoxymethyl ester was purchased from Molecular Probes or Dojin Pharmaceutical (Kumamoto, Japan). Ionomycin from *Streptomyces conglobatus* was obtained from Hoechst. All other materials were of reagent quality.

Cell Culture. Dissociated sympathetic neurons were prepared from superior cervical ganglia of newborn Wistar rats according to the method of Johnson and Argiro (17) modified as described (5).

Evaluation of Neuronal Survival after NGF Deprivation. As a quantitative measure of neuronal death, the amount of the cytoplasmic enzyme adenylate kinase (AK) released into medium was assayed as described (5, 12). Normally, 3–8% of total AK activity was released from cultures of healthy

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Abbreviations: NGF, nerve growth factor; AK, adenylate kinase; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration.

neurons; this value increased to 40–50% upon cell death after the neurons were deprived of NGF by adding antiserum against NGF (0.5%) for 2 days and harvested for the assay. In those cases in which normalization was necessary to compare different sets of data, relative survival (%) was calculated from released AK values (details are given in the legend of Fig. 2). High-K^+ media were prepared as described (12).

Measurements of $[Ca^{2+}]_i$ Levels of Sympathetic Neurons Loaded with Fura-2. Sympathetic neurons were loaded with fura-2 as described (18) and maintained in Hepes-buffered saline (pH 7.2) (18) at 32°C–34°C throughout the measurements. Fura-2 fluorescence was excited by a xenon arc lamp fitted with two grating monochromators and interfaced to a Nikon Diaphot microscope with a CF fluor ×40 objective. Emitted light, passed through appropriate filters, was collected by a low-light CCD camera, Hamamatsu C2400. The video signal was then converted to a 512 × 483 digital image (256 gray levels) and stored in an Argas 100 image-processor system (Hamamatsu, Shizuoka, Japan). The ratio R of fluorescence emitted by fura-2 during excitation at 340 nm to that at 380 nm was calculated from pixel to pixel in which background signals in the absence of light had been subtracted from each fluorescence intensity. Ratio values were then calibrated to the concentration of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) as follows:

$$[Ca^{2+}]_i = K_d \times \{(R - R_{min})/(R_{max} - R)\} \times (F_{380max}/F_{380min}),$$

in which K_d, the dissociation constant for fura-2 Ca^{2+}, is 224 nM (19), R_{min} is the ratio obtained nominally under Ca^{2+}-free conditions in the presence of 10 mM EGTA, R_{max} is the ratio of Ca^{2+}-saturated fura-2 obtained in the presence of 2.5–8 μM ionomycin as a mobile carrier for Ca^{2+}, and F_{380max} and F_{380min} represent the fluorescence values at 380 nm in the presence of EGTA and ionomycin, respectively. In our system, $R_{min} = 0.22$, $R_{max} = 5.6$, and $F_{380max}/F_{380min} = 7.3$. Fluorescence measurements were done on single neurons and small aggregates to collect fluorescence signals from individual neurons. We typically measured ratio values from five to seven small portions of different areas of the cytoplasm of a single neuron at least 5 times per area with an interval of 2–3 sec; we then averaged these values.

Measurements of Cell Diameter of Sympathetic Neurons. Cell diameter of sympathetic neurons was measured by use of a Videoplan imaging analysis system (Kontron, Zurich).

RESULTS

Correlation Between Cell Survival and Levels of $[Ca^{2+}]_i$ of Sympathetic Neurons Chronically Exposed to Various Concentrations of Extracellular K^+. Sympathetic neurons, grown for 7 days in the presence of NGF, were phase-bright cells with an average diameter of 22.6 ± 3.9 μm (mean ± SD; n = 52). When these neurons were loaded with fura-2, the fluorescence intensity was homogeneously distributed; no clear distinction between cytoplasmic and nuclear levels of free Ca^{2+} was observed in 80–90% of these young neurons. Thus, the basal level of $[Ca^{2+}]_i$ of sympathetic neurons (6–8 days *in vitro*) loaded with fura-2 was 93.0 ± 10.5 nM (average of five independent experiments ± SEM, each consisting of measurements on 6–13 neurons; n = 48), which compared well with previous determinations (18, 20, 21). Chronic exposure to a high-K^+ medium (35 mM) for these neurons induced an essentially constant and homogeneously sustained level of $[Ca^{2+}]_i$ that could be maintained as long as the neurons were exposed to the depolarizing stimulus (up to 3 days); for example, the sustained level of $[Ca^{2+}]_i$ of neurons depolarized for 1 day was 247 ± 14 nM (average of three independent experiments, each consisting of measurements on 10 neurons; one of which is shown in Fig. 1*A*). The increase in $[Ca^{2+}]_i$ under depolarizing conditions was abolished after replacement of the high K^+ by normal medium (Fig. 1*A*). This finding clearly suggests that long-term exposure to high K^+ did not totally inactivate the Ca^{2+} influx through Ca^{2+} channels and shows that there was a sustained, elevated level of $[Ca^{2+}]_i$ under these chronically depolarizing conditions. Next, we determined the basal $[Ca^{2+}]_i$ of young sympathetic neurons as a function of the concentration of extracellular K^+; the effect was dose dependent, and the half-maximal concentration of K^+ was 20 mM (at 170 nM $[Ca^{2+}]_i$) (Fig. 1*B*). This agrees well with the EC_{50} (21 mM K^+) for the effect of K^+ on neuronal survival (12). By combining these results, we

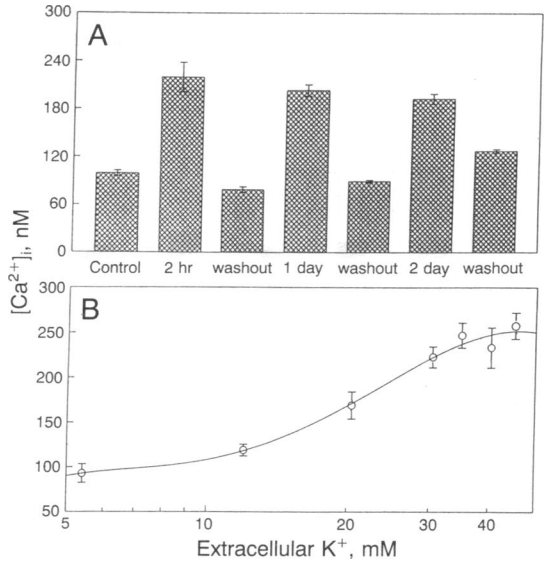

FIG. 1. Effects of extracellular K^+ concentrations on $[Ca^{2+}]_i$ levels of young sympathetic neurons. (*A*) Sympathetic neurons (1 week *in vitro*) were exposed to high-K^+ medium (35 mM) for various periods of time (2 hr, 1 day, 2 days) in the absence of NGF, and their $[Ca^{2+}]_i$ levels were determined. We have done three independent experiments, each consisting of measurements of 10 neurons in which each neuron was calibrated individually with ionomycin, one of which is shown here. When the high-K^+ buffer was replaced by normal buffer (washout), the sustained level of $[Ca^{2+}]_i$ was abolished (after ≈5 min) (mean ± SEM; n = 10). (*B*) Sympathetic neurons were exposed to various concentrations of extracellular K^+ for 13 hr in the absence of NGF, and the $[Ca^{2+}]_i$ levels were determined individually with ionomycin (mean ± SEM; n = 8).

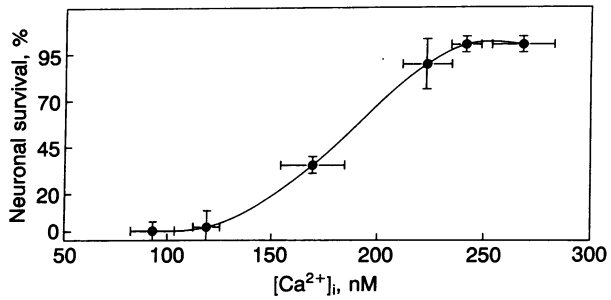

FIG. 2. Correlation between neuronal survival and $[Ca^{2+}]_i$ levels of sympathetic neurons in culture. The data on AK release as a function of extracellular K^+ were taken from figure 4*A* in an earlier paper (12). Percentage relative survival (%) was calculated from these data assuming that the cell survival of young neurons, maintained either in the presence of NGF or in its absence at normal K^+ (5.4 mM) for 2 days, is 100% or 0%, respectively. This normalization procedure was necessary for comparing different sets of data. The relative survival (%) was plotted against the $[Ca^{2+}]_i$ of young neurons exposed to a corresponding concentration of K^+ for 13 hr (Fig. 1*B*).

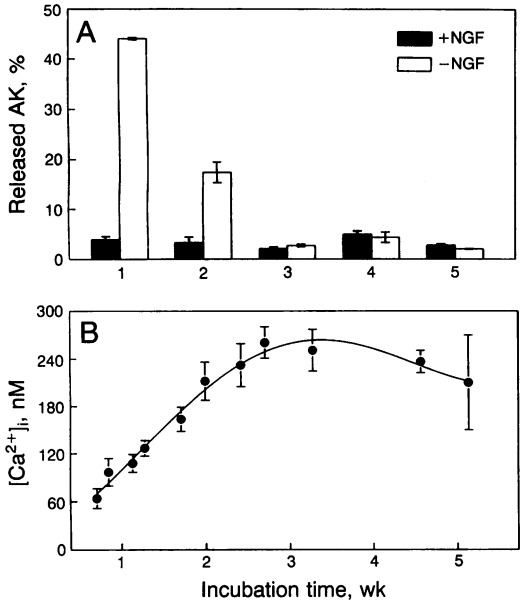

FIG. 3. NGF-independent survival and $[Ca^{2+}]_i$ levels of sympathetic neurons as a function of advancing age *in vitro*. (*A*) Sympathetic neurons maintained *in vitro* for various periods of time (1–5 weeks) were incubated in assay medium either in the presence (solid bar) of NGF or in its absence by adding antiserum against NGF (open bar) for 2 days and were assayed for AK release. Each bar represents mean ± SEM of triplicate values. (*B*) The level of $[Ca^{2+}]_i$ of sympathetic neurons maintained for the indicated periods of time in the presence of NGF was determined as described (mean ± SEM; n = 5–18). A plateau level of $[Ca^{2+}]_i$ (241 ± 7 nM) was calculated from the data at day 18 (232 ± 27 nM; n = 5), day 19 (260 ± 19 nM; n = 14), day 24 (251 ± 10 nM; n = 13), and day 32 (237 ± 14 nM; n = 7).

generated a correlation curve between neuronal survival and levels of $[Ca^{2+}]_i$ of young neurons, which shows that 50% survival occurred at ≈184 nM $[Ca^{2+}]_i$ and complete survival, independent of trophic support, occurred at ≈240 nM $[Ca^{2+}]_i$ (Fig. 2). With consideration of this estimate, we predicted that sympathetic neurons exposed to high K^+ for 2 days and whose basal $[Ca^{2+}]_i$ reached 140 nM after the change from high-K^+ medium will die after deprivation of NGF at normal K^+ (the survival was expected to be 15%). Indeed, these neurons did not survive after being deprived of NGF at normal K^+ (data not shown; relative survival was estimated to be 13% from the data on released AK). This strongly suggests that continued exposure of neurons to high-K^+ medium is necessary for the saving effect of depolarization to manifest itself. These data are consistent with previous results showing that upon removal of elevated K^+, neurons revert back to a trophic factor-dependent state (13, 22).

The Basal Level of $[Ca^{2+}]_i$ of Sympathetic Neurons as a Function of Advancing Age *in Vitro*. It is known that older sympathetic neurons are much less acutely dependent on NGF for survival both *in vivo* and *in vitro* (14, 23); trophic deprivation for a few days does not cause death of these neurons. Under our culture conditions, neurons maintained for 3 weeks *in vitro* became well hypertrophied (cell diameter, 33.5 ± 3.4 μm; n = 50) and were able to survive independently of trophic support (Figs. 3*A* and 4). Moreover, this acquisition of NGF-independent survival was associated with an increase of the basal $[Ca^{2+}]_i$; that of young neurons was relatively low (93.0 ± 10.5 nM; n = 48) at days 6–8, increased with incubation time, and finally reached a plateau level of 241 ± 7 nM (mean ± SEM of the data at days 18, 19, 24, and 32) at around week 3 (Fig. 3*B*). It should be mentioned that distribution of fura-2 fluorescence in 3-week-old neurons was heterogeneous; cytoplasmic levels of free Ca^{2+} were 20% ± 4% higher than nuclear levels of free Ca^{2+}, and heterogeneity of free Ca^{2+} distribution within the cytoplasmic region was also developed. Nonetheless, the average $[Ca^{2+}]_i$ of 3-week *in vitro* neurons was significantly higher than that of young neurons as described. It is interesting that this plateau level of $[Ca^{2+}]_i$ of mature neurons was very similar to the sustained level (247 ± 14 nM) of young neurons (1 week *in vitro*) chronically exposed to high K^+ (35 mM). Thus, there appears to be a general correlation between elevated $[Ca^{2+}]_i$ and NGF-independent survival. Fig. 4 shows the morphological changes of 3-week *in vitro* neurons in response to deprivation of NGF for 6 days. These neurons survived up to 2 weeks, although they became slowly atrophied. The process of this neuronal atrophy was reversible after the addition of NGF (data not shown). From these findings, we estimate that the optimal $[Ca^{2+}]_i$ level of sympathetic neurons for trophic factor-independent survival occurs at ≥240 nM.

Neuronal Dependence on Trophic Factor Is Regulated by the Concentration of Extracellular Ca^{2+}. Dissociated sympathetic neurons initially maintained in a medium containing NGF and 3 mM Ca^{2+} and grown in this medium for 1 week became partially resistant to NGF deprivation (Fig. 5*A*). Conversely, sympathetic neurons maintained for 3 weeks in the presence of NGF, during which time they were exposed to normal

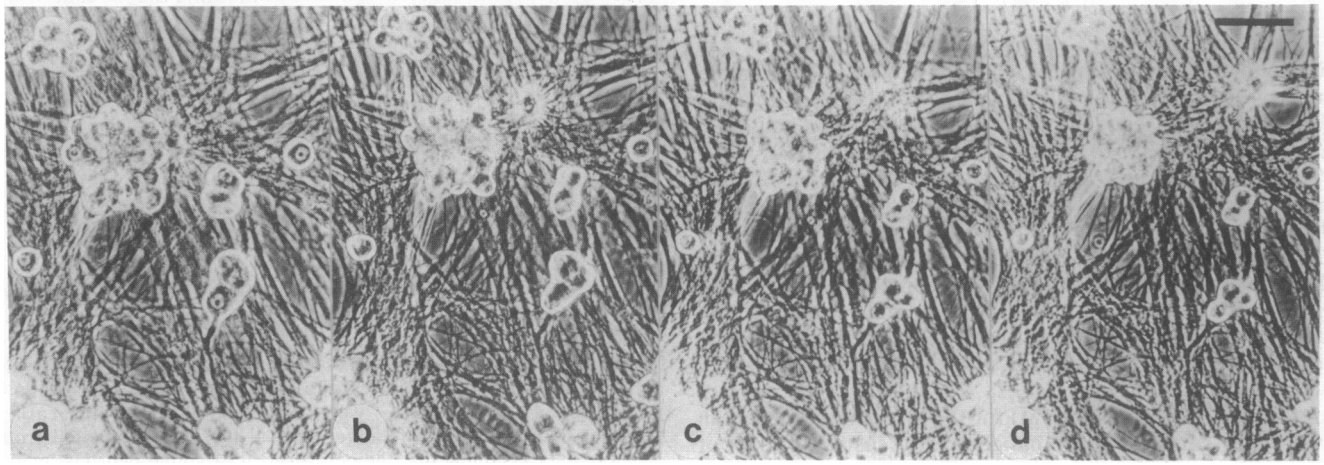

FIG. 4. Phase-contrast micrographs of 3-week *in vitro* neurons after NGF deprivation. Sympathetic neurons maintained for 3 weeks in the presence of NGF *in vitro* were deprived of NGF by adding antiserum against NGF, and photographed at day 0 (*a*), day 2 (*b*), day 4 (*c*), and day 6 (*d*). All the individual neurons seen in *a* survived after deprivation of NGF for 6 days (*d*), although they became atrophied. The cell diameters of the neurons shown in *a* and *d* were 33.5 ± 3.4 μm (n = 50) and 26.4 ± 2.5 μm (n = 48), respectively. (Bar = 100 μm.)

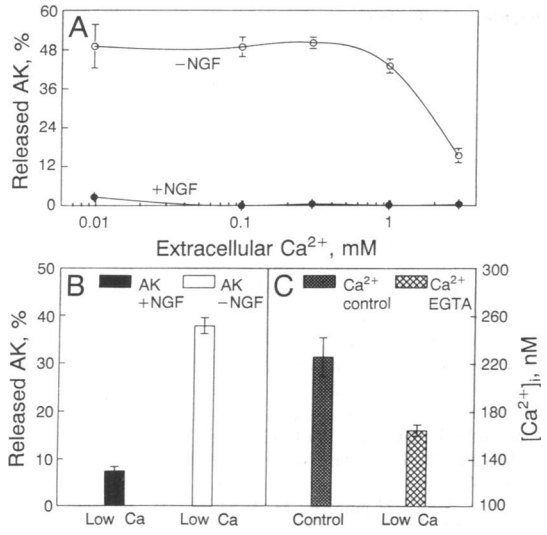

FIG. 5. Extracellular Ca^{2+} alters trophic factor dependence of sympathetic neurons for survival *in vitro*. (*A*) Dissociated sympathetic neurons were maintained for 1 week in medium containing various concentrations of Ca^{2+}. The neurons were then assayed for AK release at normal Ca^{2+} (1.8 mM) either in the presence of NGF (solid circle) or in its absence (open circle). Mean of three determinations ± SEM. (*B*) Sympathetic neurons, maintained for 3 weeks in the presence of NGF during which they were exposed to a medium containing EGTA (final Ca^{2+} concentration, 0.3 mM) for the last week, were incubated for 2 days in the presence of NGF (left column) or in its absence (right column) and were assayed for AK release under normal conditions. Mean of three determinations ± SEM. For comparison, young neurons were deprived of NGF for 2 days and assayed for AK release (50% ± 4%; $n = 3$). Relative survival (%) calculated from this value and the data shown in this figure was 25%. (*C*) Cytoplasmic Ca^{2+} levels of the neurons maintained for 3 weeks at normal Ca^{2+} (left column; mean ± SEM; $n = 14$) and under conditions described in *B* (right column; mean ± SEM; $n = 5$).

medium containing 1.5 mM EGTA for the last week, thus reducing the concentration of free Ca^{2+} to 0.3 mM, became susceptible to NGF deprivation; they released AK activities into medium in response to acute withdrawal of NGF (Fig. 5*B*). Consistent with the neurons' retained NGF dependence, their $[Ca^{2+}]_i$ was decreased (Fig. 5*C*). The relative survival calculated from the observed $[Ca^{2+}]_i$ (164.3 ± 3.7 nM; $n = 5$) by using the Ca^{2+}-survival relationship (Fig. 2) was 32%, predicting that two-thirds of the neurons should die under these conditions. Indeed, most of them died as shown in Fig. 5*B*; the relative survival calculated from released AK values was 25%. In a separate experiment, we treated neurons, maintained for 3 weeks, with normal medium containing 5 mM EGTA during the last 2 days of incubation (the concentration of free Ca^{2+} was 20 μM); some neuronal death was observed upon subsequent deprivation of NGF [the released AK value was 19.2% ± 1.4% ($n = 3$) in the absence of NGF and 4.1% ± 0.9% ($n = 3$) in the presence of NGF], whereas no cell death was seen for these neurons cultured continuously in the presence of normal Ca^{2+} (Fig. 3*A*). These results clearly suggest that trophic factor dependence of sympathetic neurons for survival *in vitro* is regulated by the $[Ca^{2+}]_i$.

DISCUSSION

These data provide evidence for our Ca^{2+} set-point hypothesis (12) for NGF dependence of sympathetic neurons *in vitro*. This hypothesis conjectures that neurons at optimal levels of $[Ca^{2+}]_i$ will survive autonomously independent of trophic factor support and predicts that developing neurons, which are acutely dependent on trophic factors, have lower levels of $[Ca^{2+}]_i$ than mature neurons, which are less dependent on trophic factor. Indeed, the age-related decrease in trophic factor dependence was associated with an increase of the basal $[Ca^{2+}]_i$. The level of $[Ca^{2+}]_i$, ≈240 nM, reached agrees very well with the sustained level of $[Ca^{2+}]_i$ found in young neurons chronically exposed to high-K^+ medium that similarly allowed them to survive autonomously, independent of trophic support. Thus, we have established a correlation between cell survival and levels of cytoplasmic Ca^{2+} of sympathetic neurons *in vitro*: 50% survival under these assay conditions is provided at ≈184 nM and complete survival is provided at ≈240 nM $[Ca^{2+}]_i$. On the other hand, we speculate that excessive influx of Ca^{2+} beyond optimal, neuronal $[Ca^{2+}]_i$ levels, as after exposure to excitatory amino acids, may raise $[Ca^{2+}]_i$ inordinately, resulting in toxic or fatal effects (24). Indeed, Gallo *et al.* (22) reported pharmacological data suggesting that elevated $[Ca^{2+}]_i$ promotes the survival of immature cerebellar neurons in culture but has an adverse effect on mature neurons in which excitation-mediated increases of $[Ca^{2+}]_i$ cause them to die (25). We suggest that depolarization can raise the $[Ca^{2+}]_i$ level of immature neurons toward an optimal level, whereas further elevation of $[Ca^{2+}]_i$ in mature neurons would be toxic. Thus, we envision three important Ca^{2+} states: a low level (<184 nM) that places the neurons (particularly developing neurons) at risk because of an increased dependence on trophic factor; an intermediate, optimal level (≥240 nM), in which neuronal survival is independent of trophic factor (for mature neurons); and an elevated level (not yet defined) that is toxic. The precise levels of these states, which depend on the method of calibration used, may also vary from cell type to cell type.

Sympathetic neurons from superior cervical ganglia of the rat are mature at birth in terms of synaptogenesis and electrophysiological properties (26, 27). However, further biochemical and morphological maturation occurs in the first few postnatal weeks: enzyme activities for transmitter synthesis are increased during this time both *in vivo* and *in vitro* (28, 29), which is associated with the development of cellular hypertrophy. This study shows that this period also coincides with that of observed elevation of $[Ca^{2+}]_i$ in sympathetic neurons. Moreover, Vidal *et al.* (30) provide evidence for the involvement of L-type Ca^{2+} channels in the high K^+-mediated increase of tyrosine hydroxylase activity and decrease of choline acetyltransferase activity in cultured sympathetic neurons. These findings suggest that an increased basal level of cytoplasmic Ca^{2+} may have a central role not only in neuronal survival but also in the development of biochemical and morphological maturation of sympathetic neurons *in vitro*.

In this regard, it should be pointed out that we are concerned here with overall, averaged levels of $[Ca^{2+}]_i$ of single neurons. This overall increase in $[Ca^{2+}]_i$ is also associated with the development of heterogeneity of free Ca^{2+} distribution. This heterogeneity may be important for activation and regulation of cellular functions of the matured neurons. It may thus be possible that maturation of these neurons accompanies the development of messenger systems, such as inositol phospholipid mobilization, which interacts with the calcium system in a spatial- and/or temporal-specific manner. This notion remains to be tested by using high-resolution fluorescence imaging analysis of $[Ca^{2+}]_i$ distribution of matured neurons.

Our previous results (12) indicate that neither Ca^{2+} channel blockers nor a chelator for systolic Ca^{2+} abolished the survival-promoting effect of NGF, suggesting that NGF does not act by raising $[Ca^{2+}]_i$ of sympathetic neurons. This is consistent with the fact that NGF does not directly stimulate the uptake of extracellular Ca^{2+} in PC12 cells (31). Indeed, NGF elicited only a small increase of $[Ca^{2+}]_i$ in PC12 cells that is not long lasting (unpublished observation). Tolkovsky

et al. (32) reported that NGF does not elevate $[Ca^{2+}]_i$ of sympathetic neurons. Streit and Lux (33) reported that there is no evidence for a short-latency effect of NGF on calcium currents in PC12 cells. It is thus likely that elevated levels of $[Ca^{2+}]_i$ are a result of a general trophic effect. There is a suggestion that trophic factors may alter Ca^{2+} homeostasis by regulating activities of pumps or Ca^{2+} channels in translational- and transcriptional-dependent ways (34). How would the elevated basal levels of $[Ca^{2+}]_i$ lead to neuronal survival? One possibility is that mature neurons with an increased basal level release an autocrine factor, distinct from NGF, that promotes neuronal survival. This hypothesis implies that elevated $[Ca^{2+}]_i$ levels of mature neurons may facilitate the synthesis and release of this autocrine factor. Alternatively, increased levels of $[Ca^{2+}]_i$ may be accompanied by the activation of various second messenger systems through the binding of Ca^{2+} to Ca^{2+}-binding proteins. Ca^{2+}-binding protein(s) involved in the Ca^{2+} homeostasis of sympathetic neurons remains to be identified.

Recently, Johnson and his coworkers have proposed a model for neuronal death caused by tropic factor deprivation in which the survival-promoting effects of NGF are a result of its ability to suppress an endogenous, active death program (5, 35). According to this hypothesis, increased levels of $[Ca^{2+}]_i$ may inhibit the expression of genes or gene products responsible for death-inducing activity. The mechanism of action of Ca^{2+} ions in suppression of the death program remains unclear.

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