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Is Activation of the Na^+K^+ Pump Necessary for NGF-Mediated Neuronal Survival?

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The ability of nerve growth factor to cause rapid activation of the Na^+K^+ pump of its responsive cells was examined by measuring the uptake of $^{86}Rb^+$. A significant increase in $^{86}Rb^+$ uptake in E8 chick dorsal root ganglion sensory neurons after NGF treatment was seen only if the cells had been damaged during the preparation procedure. Such damaged cells could not survive in culture in the presence of NGF, and undamaged cells that did survive in response to NGF exhibited no increased $^{86}Rb^+$ uptake rate. Furthermore, cultured calf adrenal medullary cells did not show an increase in $^{86}Rb^+$ uptake after treatment with NGF, although these cells respond to NGF with an increased synthesis of catecholaminergic enzymes. These results are incompatible with the hypothesis that the mechanism of action of NGF that promotes neuronal survival and enzyme induction results from an initial stimulation of the Na^+K^+ pump.

The physiological role of NGF as a target-derived neurotrophic molecule necessary for the survival and maintenance of peripheral sympathetic and sensory neurons is well established (for review, see Thoenen et al., 1985) although the molecular mechanisms by which NGF evokes these effects after binding to its specific receptors remain unknown. A variety of mitogens and hormones are able to stimulate the Na^+K^+ pump of their target plasma cells' membranes, e.g., epidermal growth factor (EGF) and insulin stimulate the 3T3 cell pump (Rozengurt and Heppel, 1975), and EGF activates the Na^+K^+ pump of quiescent human fibroblasts (Moolenar et al., 1982), neuroblastoma cells (Mummery et al., 1983), and pheochromocytoma cells (Boonstra et al., 1983). Evidence has also been provided showing that administration of the neuronal survival factor NGF to sympathetic and sensory neurons activates their Na^+K^+ membrane pump (Boonstra et al., 1982; Skaper and Varon, 1983b), and the fact that this activation occurs so rapidly has led to the suggestion that the primary mechanism of action of NGF is to stimulate the Na^+K^+-ATPase of neuronal membranes (Skaper and Varon, 1979). The consequences of this activation would then result in secondary effects, such as stimulation of Na^+-dependent glucose and amino acid uptake (McGuire and Greene, 1979; Skaper and Varon, 1979), leading ultimately to the survival of the neurons (Skaper and Varon, 1983a). Some support for the Na^+K^+ pump-stimulation hypothesis was also provided by the observation that a similar stimulation could be evoked by NGF on PC12 pheochromocytoma cells. This stimulation is the indirect consequence of the activation of an electroneutral Na^+/H^+ exchange (Boonstra et al., 1983). PC12 cells do not depend on NGF for their survival and thus can be used in experiments where adequate controls without NGF are used. It should, however, be noted that any of these effects of NGF might be tumor cell-specific, and not necessarily representative of the response of normal cells to NGF. Furthermore, these results have subsequently been contradicted by Chandler and coworkers (1985), who could not show an effect of NGF on Na^+/H^+ exchange.

Although initial experiments designed to demonstrate a direct activation of the Na^+K^+-ATPase of isolated neuronal membranes (Skaper and Varon, 1981a) were negative, we decided to try to establish whether the action of NGF on the neuronal Na^+K^+-ATPase is a direct or indirect effect. The experiments reported here show that a rapid stimulation of the neuronal Na^+K^+-ATPase can be seen, but only under specific experimental conditions, when damage to the neurons is such that they will die even in the presence of NGF. Furthermore, no effect of NGF on the uptake of Rb^+ into bovine chromaffin cells could be detected. We therefore conclude that a direct activation of Na^+K^+-ATPase is not the primary action of NGF leading to neuronal survival and enzyme induction.

Materials and Methods

Materials. Calf adrenal medullae were obtained from the local slaughterhouse. Fertilized white Leghorn hen eggs were incubated in a forced-draft incubator at 38°C and staged according to the criteria of Hamburger and Hamilton (1951). $^{86}RbCl$ (sp act, 1–8 mCi/mg Rb) was obtained from Amersham (Braunschweig), ouabain, HEPES (tissue cultue grade), and bovine serum albumin (fraction 5) from Sigma, Tris from Boehringer (Mannheim). NGF (2.5 S) was isolated from submaxillary glands of adult male mice, as described by Suda et al. (1978). Trypsin was from Worthington. Tissue culture medium (F14) was made from F12 powdered medium (Gibco) and supplemented as described by Vogel et al. (1972). Horse serum was obtained from Gibco.

Measurement of $^{86}Rb^+$ uptake by E8 chick sensory neurons in suspension. Sensory neurons were isolated from dorsal root ganglia (DRGs) of E8 chick embryos and prepared as described by Barde et al. (1982). Briefly, about 150 sensory ganglia were collected in Ca^{2+}, Mg^{2+}-free PBS, incubated in 0.1% trypsin for 30 min at 37°C, and washed twice with 10 ml F14 medium containing 10% (vol/vol) heat-inactivated horse serum. The ganglia were then dissociated in 2 ml medium by gentle trituration with a siliconized Pasteur pipette (aperture diameter, 1 mm), the tip being held at a distance of about 10 mm from the bottom of a 15 ml Falcon tube.

For some experiments a different dissociation technique was used. The Pasteur pipette was constricted to a tip diameter of less than 0.5 mm, and the tip was held near the bottom of the Falcon tube to dissociate the ganglia by 15–20 passages. After preplating (150 min) to remove

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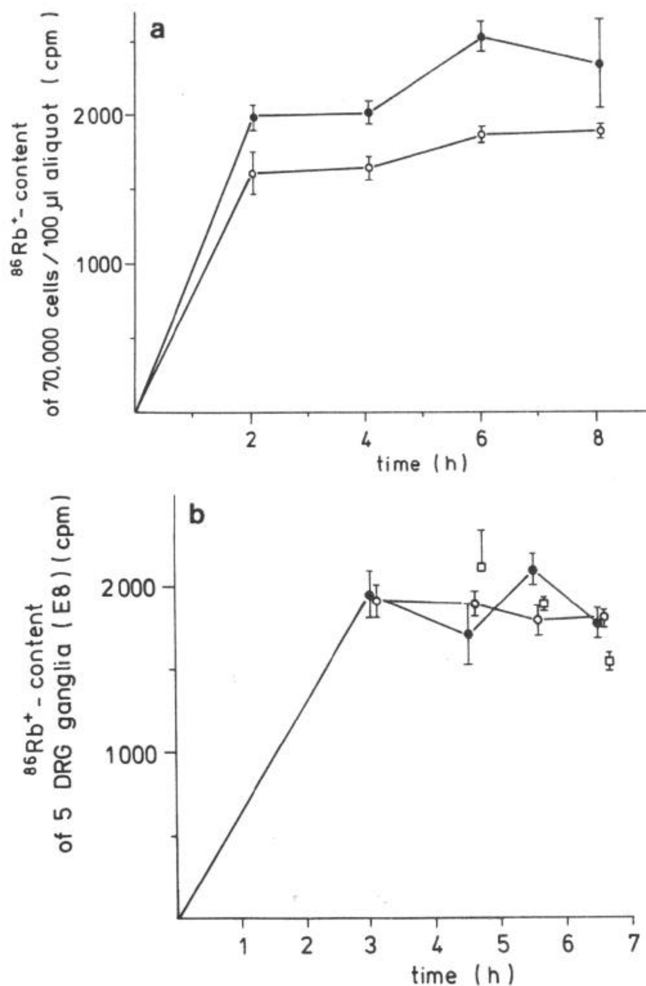

Figure 1. *a,* ^{86}Rb$^+$ accumulation by E8 DRG cells incubated in suspension. 70,000 cells/100 µl aliquot were incubated in THAM together with ^{86}Rb$^+$Cl (0.5 µCi/ml) with (●) and without (○) NGF (100 ng/100 µl). *b,* ^{86}Rb$^+$ accumulation by E8 DRG ganglia in suspension. Five ganglia were incubated in 100 µl THAM with (●) or without (○) NGF (100 ng/100 µl). To some aliquots (□), NGF (100 ng/100 µl) was given 30 min prior to measurement of uptake. At the time points indicated, the ganglia were washed 3 times with 10 ml of cold THAM, lysed in Aqualuma, and counted for radioactivity.

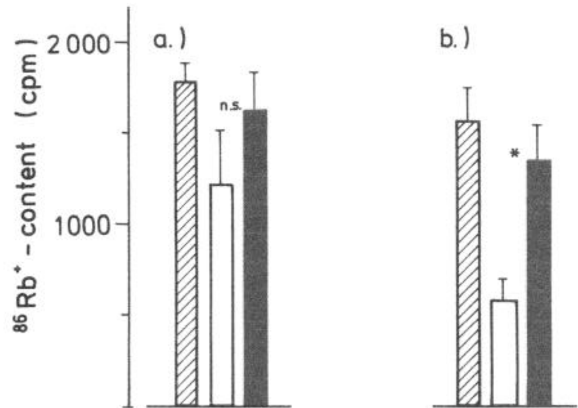

Figure 2. Effect of NGF readdition on Rb$^+$ uptake E8 DRG cells in suspension. ▨, NGF (100 ng/100 µl) present throughout incubation (7.5 hr); □, without NGF; ■, NGF (100 ng/100 µl) added 30 min prior to measurement of uptake (at 7.0 hr). *a,* ^{86}Rb$^+$ content of cells dissociated to maximize their survival (see Materials and Methods). ^{86}Rb$^+$ content of cells dissociated by harsh treatment (see Materials and Methods). The experiment was done 3 times; values shown are from one experiment representative of the other 2. Each point is the mean ± SD of ^{86}Rb$^+$ uptake measured in 3 aliquots taken from the suspension. Aliquots consisted of 70,000 cells in 100 µl THAM ± NGF.

non-neuronal cells (Barde et al., 1982), the neurons were collected by centrifugation and resuspended in Tris-HEPES–albumin-medium (THAM) [140 mM NaCl, 5 mM KCl, 1 mM $MgCl$, 0.1 mM $CaCl_2$, 40 mM Tris-HEPES (pH 7.4), 1% (wt/vol) bovine serum albumin], as described by Skaper and Varon (1981a). The suspension was then divided into aliquots of 100 µl containing 70,000 neurons, to which ^{86}RbCl was added to a final concentration of 1.2 µM (0.5 µCi/ml). Where shown (Figs. 1, 2), the suspensions were supplemented with NGF to a final concentration of 1 µg/ml. The aliquoted suspensions were shaken in a water bath at 37°C, and the incubations stopped (Figs. 1, 2) by adding 1 ml of ice-cold THAM. The diluted cell suspensions were immediately transferred to 2.4 cm Whatman GF/A filters that had been preincubated in 100 mM nonradioactive RbCl solution for 2 hr. Filters were washed twice with 1 ml ice-cold THAM, air-dried, and the radioactivity counted.

Some aliquots of preplated neurons (15,000 cells) were plated in F14 medium with 10% horse serum on 35 mm Falcon culture dishes that had been precoated with polyornithine (0.5 mg/ml; Sigma) and 4 µg/ml laminin (Edgar et al., 1984). NGF and ^{86}RbCl were added to the cultures for the same period employed for the cell suspensions. In addition, neuronal survival in the cultures was determined after 24 hr (Barde et al., 1982).

Sympathetic neuronal cultures. Embryonic chick sympathetic neurons (E12) were grown in dissociated cell culture as described by Edgar et al. (1984) on polyornithine- and laminin-coated 16 mm 24-multiwell dishes (Costar) at a cell density of 10,000 ± 2000 neurons/well. Cultures were pregrown with F14 containing 10% horse serum supplemented with 20 ng/ml NGF. After 18 hr, the cells were washed 3 times with 1 ml prewarmed F14 medium with 10% horse serum, NGF was added to some cultures at a concentration of 100 ng/ml, and at the time points shown (Fig. 3) the cells were pulsed for 10 min with ^{86}RbCl at a concentration of 10 µM (4 µCi/ml). Thereafter the cells were washed 3 times with 1 ml ice-cold THAM, lysed in 1 ml Aqualuma, and the radioactivity was counted.

Preparation of calf adrenal medullary cells. Calf adrenal medullary cells were grown in dissociated cell culture for 3 d as described by Naujoks et al. (1982). Cell density was 200,000 cells/16 mm Costar well. ^{86}RbCl (25 µM, 10 µCi/ml), diluted in THAM ± NGF (100 ng/ml), was added for the time periods shown in Table 4, after which the medium was removed and the cells washed 3 times with 1 ml ice-cold THAM, lysed, and counted for radioactivity.

Results

Effect of NGF on ^{86}Rb$^+$ uptake into E8 chick sensory neurons in suspension

The experiments illustrated in Figure 1 are adapted from the experimental system described by Skaper and Varon (1981b). After cell dissociation and enrichment of neuronal cells by preplating, the cell suspension was divided into aliquots, which were supplemented with ^{86}Rb$^+$. At time points between 2 and 8 hr, the ^{86}Rb$^+$ content of the cells was measured. The ^{86}Rb$^+$ content of the cells rose to a plateau within the first 2 hr of incubation, after which the steady-state levels remained constant both in the presence and absence of NGF (Fig. 1*a*). Although the ^{86}Rb$^+$ content of the cells was slightly lower when incubated in the absence of NGF, no significant decrease in steady-state levels was noted throughout the 8 hr observation period, irrespective of the presence or absence of NGF (Fig. 1). Similar results were obtained when whole ganglia were incubated in the same buffer with and without NGF (Fig. 1*b*).

In order to determine the effect of restoring NGF to NGF-deprived cells, sensory neurons were maintained in suspension in the presence and absence of NGF for 7.5 hr, at which time

Table 1. Effect of different dissociation procedures on the survival of E8 DRG neurons in culture

Dissociation procedure	Neuronal survival after 24 hr culture (%)
A. Normal mild procedure, i.e., trituration maximally 10× with a Pasteur pipette, tip diameter ≥1 mm	53.2 ± 3.2
b. Rigorous procedure, i.e., trituration >10× through a Pasteur pipette, tip diameter ≤0.5 mm, with repeated trituration after centrifugation to resuspend the cells	8.7 ± 5.2

A total of 15,000 neurons were plated on each 35 mm polyornithine/laminin-precoated culture dish. Surviving neurons were counted after 24 hr culture by scanning strips equivalent to 10% of the total surface area of each dish. Amounts are percentages of survival in 3 dishes ± SEM.

point 1 μg/ml NGF was added to cells that had previously been without NGF. The suspensions were incubated for a further 30 min before collection. Figure 2*a* shows that NGF increased the $^{86}Rb^+$ content of deprived neurons to a level approaching that found in cells that had been continuously exposed to NGF from the start of the incubation. The changes in response to NGF were, however, not statistically significant. Similarly, no increase of $^{86}Rb^+$ content could be observed after readdition of NGF to whole ganglia suspensions after 6 hr (Fig. 1*b*).

In contrast to these results, however, ganglia dissociated with a fire-polished Pasteur pipette of tip diameter reduced to less than 0.5 mm, using more than 10 strokes, with the cells resuspended after centrifugation using a similarly constricted pipette, showed a significant decrease of $^{86}Rb^+$ content in cells deprived of NGF for 8 hr (Fig. 2*b*). Furthermore, these cells reacted to the restoration of NGF by more than doubling their $^{86}Rb^+$ content. This significant increase brought the $^{86}Rb^+$ levels up to those seen in cells that had been continuously exposed to NGF. It was found, however, that this harsher dissociation procedure not only affected the response of the $^{86}Rb^+$ uptake into the cells, but also markedly influenced cells' ability to survive in culture: Table 1 shows that after mild dissociation conditions, some 53 ± 3.2% of the neurons survived for at least 24 hr, in agreement with previous results (Barde et al., 1982). In contrast, the harsh dissociation conditions necessary for a significant effect of NGF restoration on $^{86}Rb^+$ levels adversely affected the cells, so that only 8.7 ± 5.2% were able to survive for 24 hr in culture.

Effect of NGF on $^{86}Rb^+$ uptake by cultured sympathetic neurons

In order to determine whether the Na^+K^+ pump of viable cells in culture could be stimulated at all by NGF, sympathetic neurons were cultured for 18 hr either in the presence of NGF (20 ng/ml) or with elevated K^+ concentrations (35 mM) for 24–48 hr. Under both these conditions, essentially all the sympathetic neurons can be maintained for at least 6 d (Wakade et al., 1983). Figure 3 shows the subsequent uptake of $^{86}Rb^+$ in cells that were washed after 18 hr and subsequently cultured with and without NGF. It can be seen that the rates of $^{86}Rb^+$ uptake were the same, at least for the first 10 hr of incubation, after which cells without NGF lost their ability to take up $^{86}Rb^+$. After 16 hr in

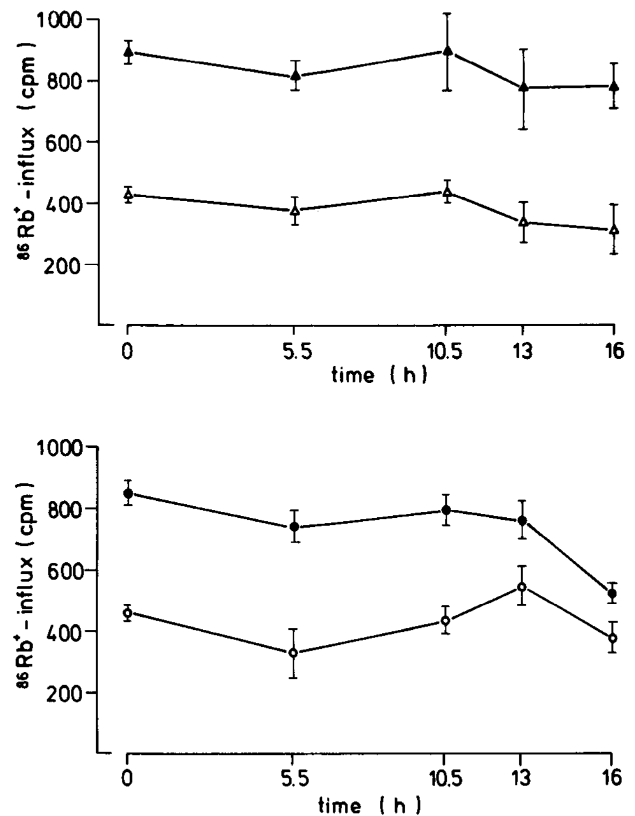

Figure 3. $^{86}Rb^+$ uptake rates in cultured sympathetic neurons with and without NGF. Embryonal chick sympathetic neurons (E12) were grown in dissociated cell culture. Cell density was 10,000 cells/well. Cultures were grown 18 hr in medium containing NGF (20 ng/ml), and at time 0 washed 3 times with 1 ml of prewarmed (37°C) F14 medium with 10% horse serum, and then switched to medium containing no NGF (●), or to medium containing 100 ng/ml NGF (▲). Each point shows the mean ± SEM. $^{86}Rb^+$ uptake (cpm) of triplicate measurement from one experiment. At the time, points shown, $^{86}Rb^+$ was added for 10 min, at a final concentration of 4 μCi/ml; thereafter the cells were washed 3 times with cold THAM, lysed, and radioactivity counted. Controls to each point of measurement were done with 5 mM ouabain added 5 min before the 10 min pulses with $^{86}Rb^+Cl$. △, Cells with NGF (100 ng/ml) and ouabain; ○, cells with ouabain and without NGF.

the absence of NGF the cells started to detach from the substrate so that no further experiments could be done. Unspecific uptake of $^{86}Rb^+$ was measured by adding 5 mM ouabain 5 min before the cells were pulsed. Interestingly, the unspecific uptake of $^{86}Rb^+$ into cells deprived of NGF increased after 10 hr, although the first dead cells could be observed only after 16 hr.

Effect of readdition of NGF on $^{86}Rb^+$ content in cultures of sympathetic neurons after 12–16 hr

If NGF was added again to the cells when Na^+K^+ pump-specific $^{86}Rb^+$ uptake had started to decrease, no significant uptake could be seen (Fig. 4). Similarly, after 16 hr the NGF-deprived cells did not increase their Na^+K^+ pump activity in the 90 min subsequent to NGF restoration. After the 90 min period, no further experiments could be done, as cells had begun to detach from the culture dishes.

Effect of NGF on $^{86}Rb^+$ uptake by calf adrenal medullary cells

Calf adrenal medullary cells were cultured for 3 d in the absence of NGF as described by Naujoks et al. (1982). The addition of

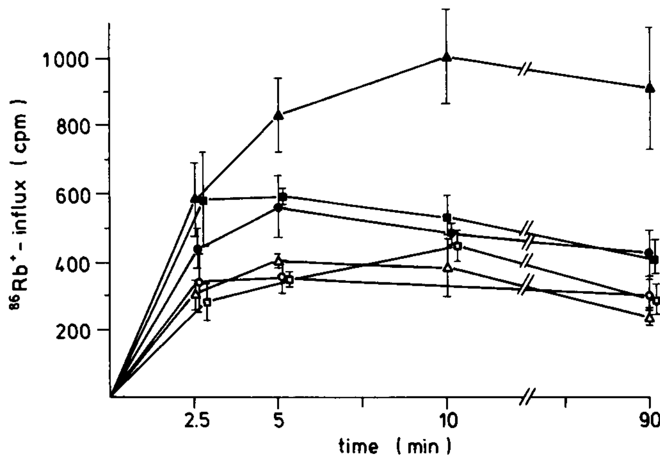

Figure 4. Effect of NGF readdition on $^{86}Rb^+$ uptake in E12 sympathetic neurons deprived from NGF for 16 hr. Neuronal cultures were grown for 18 hr in the presence of NGF (20 ng/ml), then washed 3 times with F14 medium with 10% horse serum and deprived of NGF for 16 hr. At 16 hr (time point 0), they were incubated for the times shown in THAM containing the following additions: ●, $^{86}Rb^+$ (4 μCi/ml), no NGF; ○, $^{86}Rb^+$ (4 μCi/ml), ouabain (5 mM); ■, $^{86}Rb^+$ (4 μCi/ml), NGF (100 ng/ml); □, $^{86}Rb^+$ (4 μCi/ml), ouabain (5 mM), NGF (100 ng/ml); ▲, control cultures with NGF (100 ng/ml) present throughout the 16 hr after washing, $^{86}Rb^+$ (4 μCi/ml) at time point 0; △, control cultures with NGF (100 ng/ml) present throughout the 16 hr after washing, with 5 mM ouabain 5 min before adding $^{86}Rb^+$ (4 μCi/ml) at time point 0. After incubation for the indicated time periods, the cells were washed 3 times with ice-cold THAM, lysed, and the radioactivity was counted. Values at 2.5 min show the mean $^{86}Rb^+$ uptake ± SEM of 8 dishes; at all other time points 3 dishes were used.

NGF to these cells did not affect their rate of accumulation of $^{86}Rb^+$ (Table 2).

Discussion

The results presented here argue strongly against the hypothesis that NGF acts primarily to stimulate the Na^+K^+ membrane pump of its responsive cells. The provision of NGF to embryonic chick ganglionic neurons that require NGF for survival did not result in a significant increase in the ability of the cells to take up $^{86}Rb^+$. The same was true for the effect of NGF on calf adrenal medullary cells, which do not need NGF for survival but in which NGF produces a selective enzyme induction.

The methods we routinely employ to dissociate neurons from embryonic ganglia were designed to maximize their survival (Edgar et al., 1981; Barde et al., 1982). Neurons prepared in this way were able to maintain their steady-state levels of $^{86}Rb^+$ for at least 8 hr after start of culture (Fig. 1). When finally, after 10 hr, the $^{86}Rb^+$ uptake levels began to decline in the absence of NGF, again the readdition of NGF had no effect on $^{86}Rb^+$ uptake (Fig. 4), and we tentatively conclude that after this time, neuronal viability had begun to decline irreversibly. In addition, the provision of NGF to dissociated bovine chromaffin cells did not result in an increased accumulation of $^{86}Rb^+$. These cells were used because, although they respond to NGF with an increased synthesis of catecholaminergic enzymes, they do not require NGF for survival in culture. Thus, adequate controls employing cells that were viable could be performed (Naujoks et al., 1982; Acheson et al., 1984).

At first sight these results and conclusions might seem to contradict those published previously (Skaper and Varon, 1981b), but this is not necessarily the case: in order to see any significant effect of NGF on the activity of the Na^+K^+ pump, it was necessary to use the rigorous dissociation procedure developed by Skaper and Varon (1979; see also Montz et al., 1985). After dissociation by this method, the steady-state levels of $^{86}Rb^+$ of the neurons declined rapidly in the absence of NGF and could be restored by providing the cells with NGF, as has previously been described (Skaper and Varon, 1981a). However, because the dissociation procedure necessary for this effect by NGF results in neurons that are largely incapable of survival in culture, we conclude that the reported effects of NGF on the embryonic chick neuronal Na^+K^+ pump are artifactual: the effects are only seen to occur after the cells have been damaged. Indeed, even with our gentler dissociation procedure, inevitably some damage must have been inflicted on the neurons, and this is reflected in the slightly lower steady-state $^{86}Rb^+$ levels seen when the cells were incubated without NGF (Fig. 1).

It should be noted that McCarthy and Partlow (1976) have investigated systematically the effect of different Pasteur pipette tip diameters on sympathetic neurons. They came to the conclusion that the sheer forces caused by flame-constricted Pasteur pipettes (diameters 0.5 mm or lower) decrease the viability of the cells.

Why should NGF exert a rapid effect on the Na^+K^+ pump of damaged cells? Recently it has been reported that the mechanical damage inflicted on neurons during dissociation results in the partial inhibition of Na^+K^+-ATPase activity, possibly as a consequence of the increased peroxidation and hydrolysis of membrane lipids also seen after damage (Demediuk et al., 1985). It is therefore tempting to speculate that the recently reported neuronotrophic effect of catalase *in vitro* (Walicke et al., 1986) results from its ability to hinder the peroxidation of membrane lipids after neuronal membrane damage during dissociation.

Although NGF acts as a panacea for injured neurons in restoring their steady-state $^{86}Rb^+$ uptake levels, this effect can clearly be seen to be unrelated to the survival-promoting and enzyme-inducing actions of NGF, occurring only when the cells are incapable of surviving. Nevertheless, this injury artifact might tell us something about the ability of NGF to rapidly restore the properties of damaged membranes—perhaps by preventing the catabolism or degradation of membrane components—and so make the membranes less leaky.

Table 2. Effect of NGF on $^{86}Rb^+$ accumulation by cultured calf chromaffin cells

Time (min)	$^{86}Rb^+$ accumulated (cpm): Control	NGF added (100 ng/ml)
5	2411 ± 243	2173 ± 506
10	4502 ± 617	4198 ± 187
30	11,500 ± 782	11,114 ± 992

Cells were grown in 15 mm Costar wells at a density of 200,000/well. At time 0 the culture medium was removed, the cultures were washed 3 times with THAM, and $^{86}Rb^+$ (10 μCi/ml) diluted in THAM ± NGF was added. At 5, 10, and 30 min the cells were again washed 3 times, lysed, and radioactivity was counted. The data show mean values (cpm) of triplicate measuremets ± SEM. No significant difference between treated and control cultures for each time period was seen ($p \geq 0.35$).

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