Journal of Supramolecular Structure 13:329–337 (1980)
Membrane Transport and Neuroreceptors 159–167

Short-Latency Ionic Effects of Nerve Growth Factor Deprivation and Readministration on Ganglionic Cells

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Nerve growth factor (NGF) is likely to exert its trophic action on dorsal root ganglion (DRG) and on sympathetic ganglion neurons by controlling a crucial function of these cells. This function would in turn regulate other cellular machineries and, ultimately, lead to the traditional NGF consequences, such as survival and neuritic growth. A corollary of this view is that the key to NGF action must lie in short-latency events, occurring within minutes of NGF administration. Chick embryo DRG dissociates have proved to be an effective experimental system to investigate short-latency responses to NGF, in that 1) measurable functional deficits develop over 6 h of NGF deprivation in vitro and 2) delayed presentation of NGF promptly and fully restores the defective function. The first deficit observed in this experimental system, a decline in RNA-labeling capability, led to the recognition that NGF controls the transport of selected exogenous substrates, all of which are Na^+-coupled and depend on an Na^+ gradient across the neuronal membrane. Subsequent work showed that NGF controlled such transport systems by actually regulating the neuronal ability to control intracellular Na^+. Under NGF deprivation, the DRG cells accumulate Na^+ to levels that reflect, and presumably equate, the extracellular Na^+ concentrations. Conversely, on delayed NGF administration, the accumulated Na^+ is actively extruded to an extent and at a speed that depends on the NGF concentration. The Na^+ response is elicited by both Beta and 7S NGF, but not by other proteins tested. All ganglionic systems that display a requirement for exogenous NGF in culture have also displayed the Na^+ response to NGF. The Na^+ response is grossly paralleled by a K^+ response. DRG dissociates, in which intracellular K^+ has been pre-equilibrated with extracellular $^{86}Rb^+$, lose their $^{86}Rb^+$ over 6 h of NGF deprivation and restore it on delayed NGF administration. The regulation by NGF of mechanisms controlling intracellular Na^+ and K^+ levels in their target neurons is likely to occupy an early and fundamental place in the sequence of events underlying the mode of action of this factor.

Key words: nerve growth factor, peripheral neurons, ion fluxes, transport

Received April 2, 1980; accepted July 4, 1980.

0091-7419/80/1303-0329$02.00

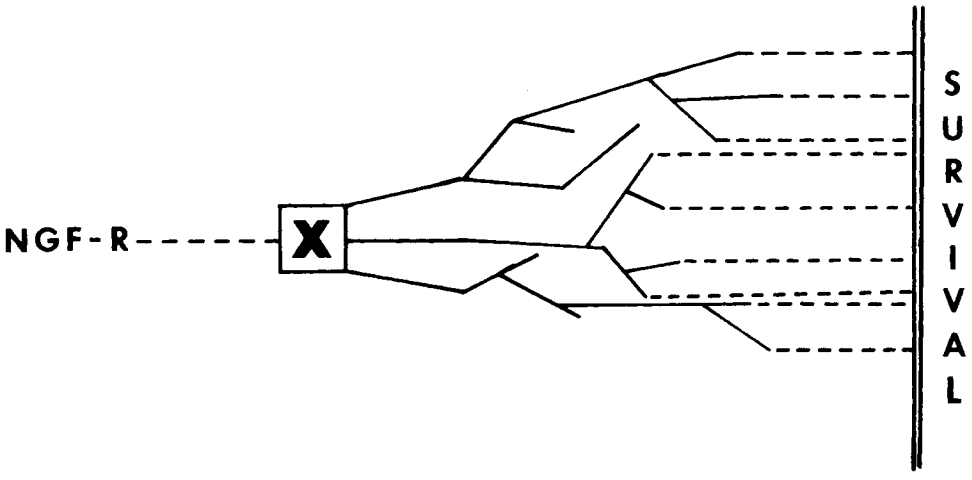

Fig. 1. Diagram of NGF action via critical function "X." The NGF-receptor complex (NGF-R) controls function X, in turn affecting a variety of unspecified cellular machineries leading to the ultimate effect, survival.

The nerve growth factor (NGF) phenomenon was discovered over 30 years ago [1]. Since then, much has been learned about the physical and chemical properties of the NGF protein(s) and of its receptor on neuronal cell surfaces [2–4]. It is all the more striking, therefore, that so little continues to be known about the nature and the molecular mechanisms of NGF action. There is general agreement by now that one fundamental role – and possibly the only role – of NGF toward its normal neuronal targets is a trophic one, that is, the support of neuronal survival [2]. Cell survival must rely on the appropriate performances of a variety of cell machineries (intake of nutrient, energy metabolism, biosynthetic activities, etc), and control of survival may involve control of any one or several of these essential performances. Conversely, a trophic agent essential for survival of a given cell must control a critical function (X), whose alterations in turn are bound to affect a variety of cellular activities. This "pervasive" concept of the NGF action [2, 5, 6] is represented in its simplest form by the diagram of Figure 1. One task undertaken by this laboratory over the past few years is to begin filling in such a diagram with recognized short-latency consequences of NGF. The emphasis on short-latency events stems from the need to examine cell behaviors that are reversible in a time frame where cell death in the absence of the factor is not yet a prime consideration.

MATERIALS AND METHODS

The experimental system(s) needed for such investigations must meet 2 requirements: 1) in the *absence* of NGF, the temporal development of functional deficits must be revealed by defined changes (decline or rise) of measurable behaviors, and 2) on *delayed* NGF presentation, the same behaviors must display a prompt reversal of the preceding changes, thereby demonstrating that the cells responsible for them are still available and functional. Suspensions of freshly dissected ganglia or of ganglionic cell dissociates have provided such experimental systems.

Most experiments were carried out with cell suspensions obtained from 8-day chick embryo dorsal root ganglia (DRG) by trypsin treatment and subsequent trituration [7–12]. Similar preparations were obtained from 11-day chick embryo sympathetic ganglia and from neonatal mouse DRG [13]. In general, final dispersion of the cells or the undissociated ganglia was in a simple medium (THAM), consisting of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1% bovine serum albumin, in 40 mM Tris-Hepes buffer, pH 7.4 [9]. NGF was prepared from mouse submaxillary glands in either the 7S or the Beta form [14] and added to the medium at the times and in the concentrations desired. Incubation was carried out at 37°C in a shaking water bath [9].

Ganglionic cell aliquots were presented, at selected times and for varying durations, with radioactive exogenous substrates such as ^{3}H-uridine or 2-^{3}H-deoxyglucose (2DG) or with radioactive ions ($^{22}Na^+$ or $^{86}Rb^+$). At the end of the pulse period, cells (or ganglia) were transferred to GF/C glass fiber filters (2.4 cm diameter, Whatman) and washed with ice-cold nonradioactive medium. The filters were dried and their radioactivity counted by liquid scintillation [9, 12].

RESULTS

The first behavior found to reveal a development of functional deficits under NGF deprivation and its reversal on delayed NGF administration was the incorporation of ^{3}H-uridine into RNA [7]. The reason for these changes in RNA-labeling capability was traced back to a decline/restoration of the cellular ability for uridine transport [8]. As illustrated in Figure 2, 1-hour pulses of ^{3}H-uridine result in a constant and maximal accumulation of acid-soluble radioactivity by identical DRG cell aliquots incubated over 6 h in the presence of NGF. In contrast, the same cells incubated *without* exogenous NGF display a 30% decline in their uptake capability by 6 h. If NGF is now presented to such NGF-deprived cells, maximal uptake is restored within 15 min. Thus, the uridine-transport deficit develops slowly under NGF deprivation (approximately 6 h), but is reversed rapidly on NGF administration (approximately 15 min). These temporal patterns were subsequently found to be fairly characteristic of the various ganglionic preparations and the various deficits examined. The reasons for the relatively slow development of the deficit have not yet been investigated.

The transport deficit caused by NGF deprivation in DRG cells was not restricted to uridine. Beside other pyrimidine and purine nucleosides [8], amino acids represented by α-amino isobutyric acid (but not, for example, leucine) are also affected [8, 10], and so is the transport of hexose as represented by 2DG [8, 9, 10]. Figure 3 shows the decline in 2DG uptake capability displayed by DRG cells incubated in THAM medium in the absence of NGF. Maximal differential was again achieved in about 6 h. Delayed administration of NGF restores within minutes maximal capability for 2DG uptake (as it did for uridine uptake). In addition, both the magnitude and the speed of the restoration could be altered by varying the concentration of NGF [9]. Further investigation of the transport systems responsive to NGF [10] led to the finding that all of them were also Na^+-coupled transports, ie, that they depended on an Na^+ gradient for their drive. Leucine transport, not affected by NGF, was also not dependent on the occurrence of transmembrane Na^+ gradients.

The dual dependence of selected transports on both NGF and the Na^+ gradient raised the question of whether NGF might not control those transports by actually controlling the Na^+ gradient across its target-neuron membranes. The investigation of putative

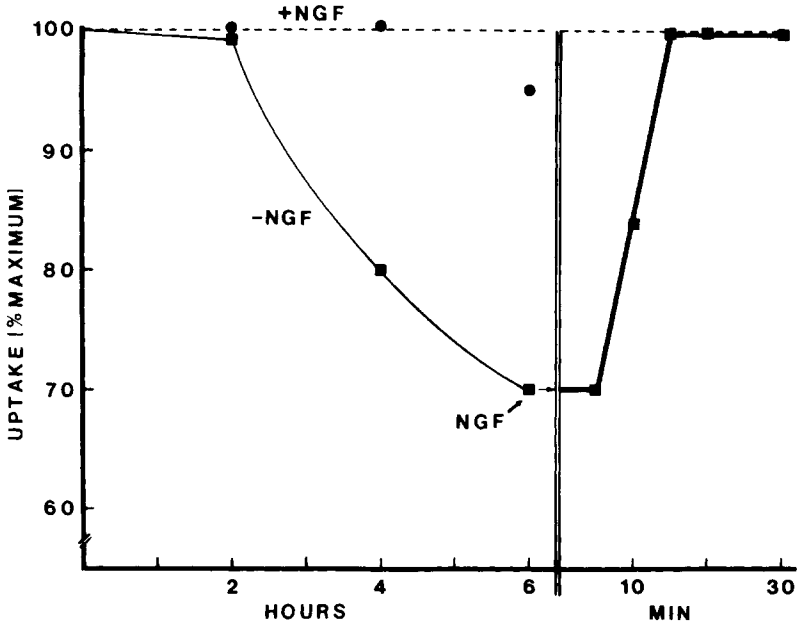

Fig. 2. Accumulation of acid-soluble radioactivity from ^{3}H-uridine following incubation of chick DRG cells in the presence and absence of NGF. A DRG dissociate was incubated with (•) 10 Biological Units (BU)/ml of 7S NGF or no (▪) NGF over a 6 h period. One-hour pulses of ^{3}H-uridine were applied at different times. After 6 h, NGF-deprived cells received NGF and aliquots were collected at the times indicated, pulsed for 5 min with ^{3}H-uridine, and analyzed for acid-soluble radioactivity as described under Methods.

short-latency effects of NGF on *ionic behaviors* was first directed to the accumulation of Na^+ in NGF-deprived cells and its extrusion in NGF-treated ones [11, 12], using $^{22}Na^+$ as a tracer. Figures 4 and 5 summarize various aspects of the Na^+ behavior in NGF-deprived and NGF-treated cells:

1. NGF deprivation causes progressive accumulation of Na^+, reaching a maximum at about 6 h (Fig. 4, heavy line).

2. Plateau levels of Na^+ accumulation are about 6-fold higher in the absence than in the presence of NGF (Fig. 4, broken line), a far greater NGF-related differential than any previously observed in transport systems.

3. Plateau levels of Na^+ accumulation in NGF-deprived cells are about the same as those induced by treatment with ouabain or dinitrophenol (Fig. 4), suggesting that complete equilibration has occurred in all cases between intracellular and extracellular Na^+. The interpretation is further supported by the strict proportionality to be found between plateau levels achieved in the absence of NGF and varied outside Na^+ concentrations (Fig. 4, side numbers).

4. When NGF is provided to cells that had been preloaded with Na^+ through 6 h of NGF deprivation (Fig. 5), intracellular Na^+ is released within minutes. As already shown by 2DG-transport studies, NGF concentrations determine the speed as well as the magnitude of the Na^+ release, suggesting that the features of a binding reaction (between NGF and its surface receptors) are directly reflected in the features of consequent cellular events such as Na^+ exclusion and their derivative effects on Na^+ gradient and Na^+-gradient-dependent transports.

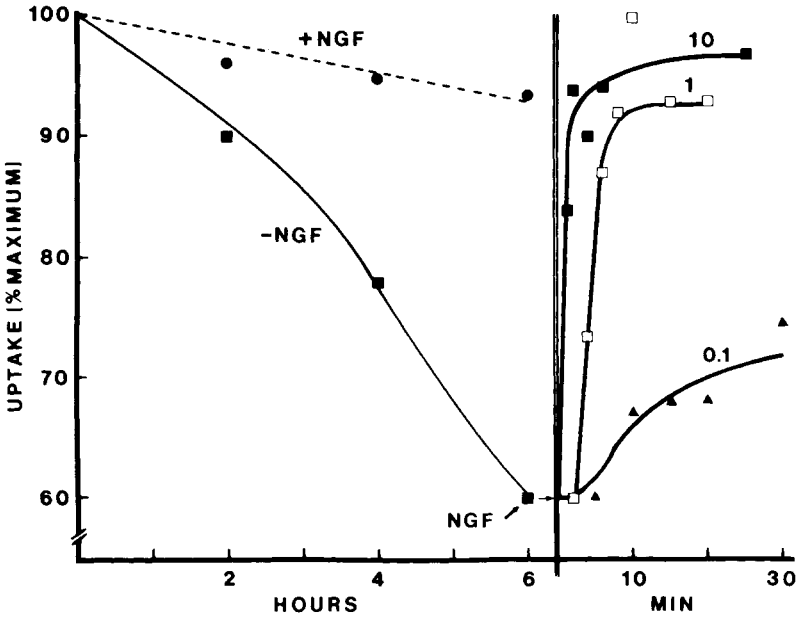

Fig. 3. Effect of NGF on the uptake of ^{3}H-deoxyglucose by chick DRG cells. Samples of DRG dissociates were incubated for 6 h with (•) 10 BU/ml 7S NGF or without (▪) NGF. After 6 h, NGF was added to NGF-deprived cells at (▲) 0.1, (□) 1, or (▪) 10 BU/ml. Cell aliquots were collected at different times throughout, pulsed with ^{3}H-deoxyglucose for 6 min, and processed for radioactivity measurements as described under Methods.

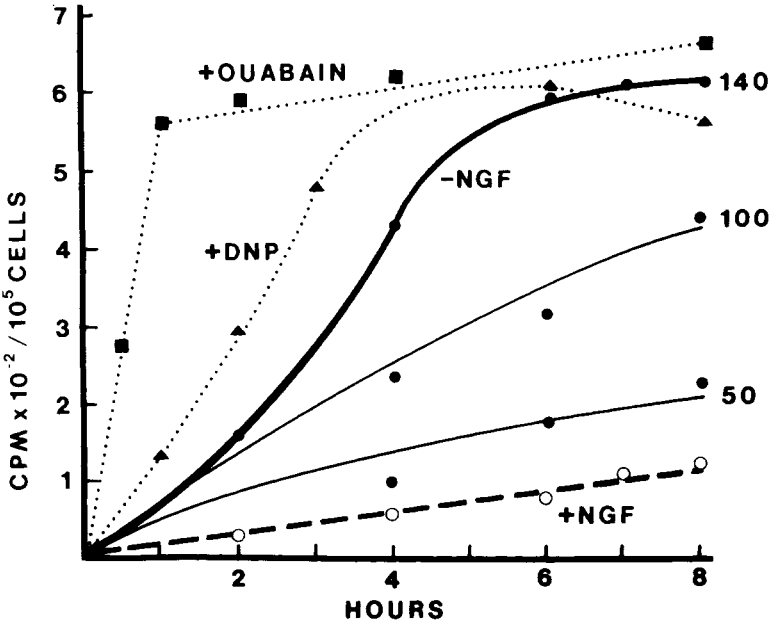

Fig. 4. Effect of NGF, ouabain, dinitrophenol, and extracellular sodium concentration on accumulation of ^{22}Na by chick DRG cells. A DRG cell dissociate was incubated with ^{22}NaCl with the following additions: (▪) 1 mM ouabain; (▲) 1 mM dinitrophenol (DNP); (○) 1 BU/ml 7S NGF; (•) no NGF with 50, 100, or 140 mM NaCl (NaCl concentrations are indicated to the left of the figure). ^{22}NaCl was adjusted to maintain constant specific activity. At different times over 8 h, aliquots of cell suspension were taken for determination of radioactivity as described under Methods.

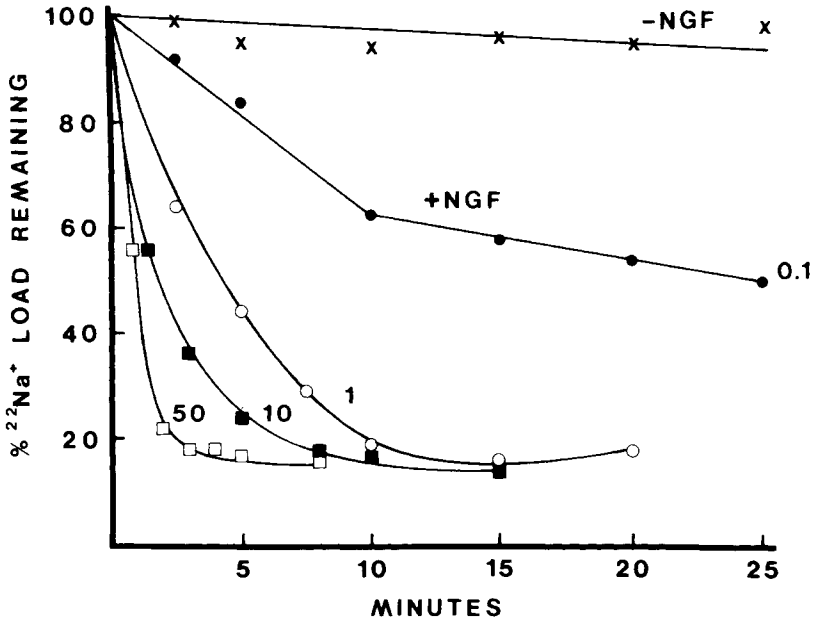

Fig. 5. Effect of NGF concentration on the time course of the Na^+ extrusion response. A DRG cell dissociate was incubated with $^{22}NaCl$ for 6 h in the absence of NGF. At this time, 7S NGF was added at the following concentrations: (X) none; (•) 0.1 BU/ml; (○) 1 BU/ml; (■) 10 BU/ml; (□) 50 BU/ml. At the times indicated, aliquots of cell suspension were taken for determination of radioactivity as described under Methods. Values are expressed as a percentage of the $^{22}Na^+$ load just prior to NGF addition.

5. On delayed NGF presentation, intracellular Na^+ concentrations decrease progressively against a constantly high extracellular concentration of Na^+ (and isotope). Therefore, release of intracellular Na^+ requires an active extrusion mechanism. Other data [12] suggest that it is to such an extrusion mechanism that the action of NGF may be itself directed.

The Na^+ response to NGF has the following additional features (data not shown):

1. It is specific to NGF [12]. Both Beta and 7S NGF are effective in a similar manner. In contrast, serum proteins and hormones and several other selected agents (eg, insulin, progesterone) neither prevent Na^+ accumulation in the absence of NGF nor are required for its reversal by delayed NGF presentation.

2. It is restricted to NGF-responsive ganglionic systems [13]. Dissociates from both DRG and sympathetic ganglia, but not from ciliary ganglia, display the Na^+ behavior just described (accumulation without NGF, extrusion with it). The same behavior is exhibited by intact rather than dissociated ganglia. Newborn mouse DRG neurons are known to require NGF in dissociated cultures but to display neurite outgrowth even without NGF in explant cultures. Correspondingly, mouse DRG dissociates display the Na^+ response, while intact ganglia do not.

Changes in intracellular Na^+ are likely to be accompanied by changes in other ions. In particular, Na^+ extrusion is usually coupled to K^+ intake via the ouabain-sensitive Na^+/K^+, ATPase pump. Studies currently underway reveal that DRG cells do display a concurrent

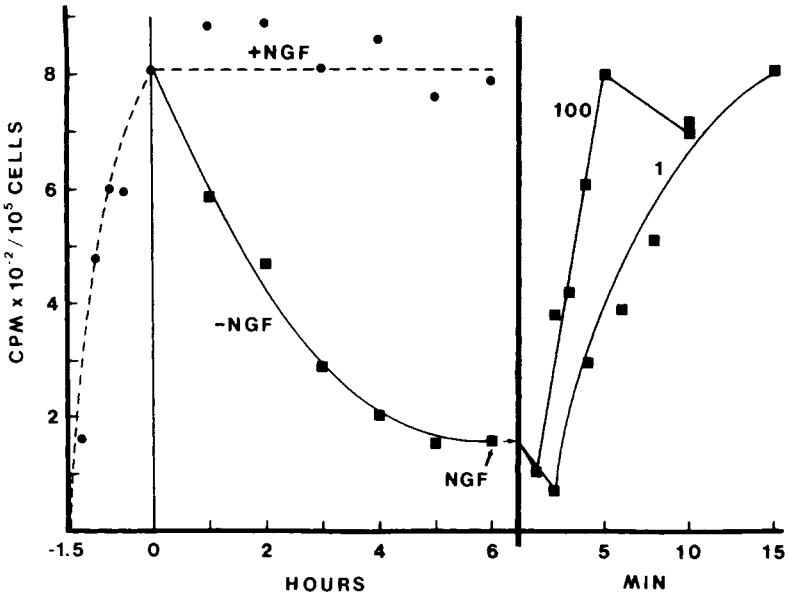

Fig. 6. Effect of NGF on potassium retention and re-uptake by chick DRG cells. Left half: A DRG dissociate was incubated with $^{86}Rb^+$ (a potassium analog) for 90 min with 7S NGF (10 BU/ml). Incubation was continued for an additional 6 h with $^{86}Rb^+$ in the continued presence (•) of NGF, or following removal (▪) of the NGF. Right half: NGF-deprived cells incubated with $^{86}Rb^+$ were then presented with NGF (▪) at 1 or 100 BU/ml and sampled at different times for radioactivity.

K^+ response, as illustrated in Figure 6. Freshly prepared cells are pre-incubated for 90 min in THAM containing $^{86}Rb^+$ (a K^+ analog), as well as NGF. This results in maximal exchange between intracellular and extracellular K^+ + $^{86}Rb^+$ mixtures. The cells are then centrifuged out, resuspended in $^{86}Rb^+$-THAM with or without NGF, and incubated for another 6 h. At that time, NGF-deprived cells receive NGF once again. The 6 h deprivation of NGF causes an unequivocal loss of intracellular K^+ (as traced by the $^{86}Rb^+$), contrasting with the full K^+ retention ensured by the continuous presence of NGF. The K^+ loss in NGF-deprived cells is promptly reversed by delayed NGF administration, with the expected dose-dependent relationships. These preliminary findings encourage the speculation that, in some manner yet to be explored, NGF controls the effective performance of the Na^+/K^+ pump in its target neurons.

DISCUSSION

The findings reviewed in the preceding section provide us with a number of details with which the initial diagram for NGF action (Fig. 1) may be spelled out to a greater extent, as illustrated now in Figure 7. The formation of NGF-receptor complexes on the cell surface triggers a series of still unknown events leading to alterations of a function X, also still unknown. A major consequence of these alterations is the control of mechanisms regulating Na^+ and K^+ movement across the neuronal membrane. This results in changes of both intracellular ion concentrations and transmembrane ion gradients. Na^+ gradients, in turn, may regulate the intake of important nutrients (as they do in chick DRG neurons). K^+ gradients are presumed to control membrane potentials, hence a variety of cell behaviors. Finally, intracellular levels of Na^+ and K^+ may be critical for several other cell

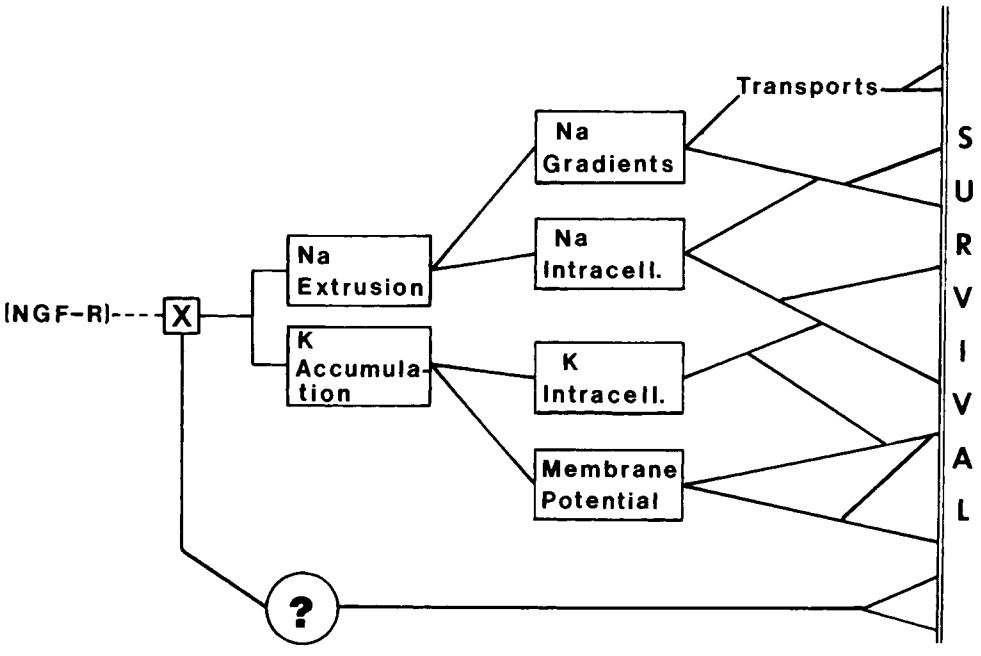

Fig. 7. Proposed schematic diagram for the action of NGF based upon the findings presented here.

activities, such as selective enzyme activation or inhibition or organization of subcellular structures. It would be tempting, therefore, to speculate whether ionic control is itself the function X postulated in the diagrams of Figures 1 and 7. Certainly, the potential consequences of ionic regulation are pervasive and important enough to explain control of cell survival by NGF, although explicit evidence in that direction still remains to be sought. However, there are other short-latency effects of NGF that have not yet been accurately located within the scheme of Figure 7. Such are the transient elevations of cyclic AMP reported in intact chick embryo DRG [15, 16] and neonatal superior cervical ganglia [17].

Further investigation of the NGF mode of action along the lines suggested in the above diagrams are planned in 3 main directions: 1) events linking the Na^+ and K^+ responses to the binding reaction between NGF and cell surface receptors (which must be the initial event), 2) the consequences of the Na^+ and K^+ responses, which may link the latter to survival (or death) of the NGF-dependent neurons, and 3) other events triggered by NGF, which are correlated with, but independent of, the Na^+ and K^+ responses. Results in these areas may provide additional leads to the identification of the putative function X.

ACKNOWLEDGMENT

These studies were supported by USPHS grant NS-07606 from the National Institute of Neurological and Communicative Disorders and Stroke.

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