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An effect of nerve growth factor on parasympathetic neurite outgrowth

(nerve cell culture/ciliary ganglion neurons/conditioned medium/insulin/growth cone)

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ABSTRACT **Addition of nerve growth factor (NGF) to dissociated parasympathetic ciliary ganglion neurons resulted, within 60 min of its addition, in a 2-fold increase in average neurite length and an accompanying enlargement and spreading of neuronal growth cones. These effects occurred over a concentration range of NGF of 0.1–10 ng/ml and were blocked by affinity-purified antibody to NGF. Epidermal growth factor, fibroblast growth factor, and angiotensin did not have these effects, although insulin at high concentrations was able to induce a response similar to that of NGF. Dissociated sympathetic chain neurons also responded to NGF with increased neurite lengths, and, in addition, NGF considerably extended the survival time of these neurons in culture. However, the effect of NGF on ciliary ganglion neurons was limited to neurite outgrowth, and NGF did not promote the survival of these parasympathetic neurons.**

Nerve growth factor (NGF) promotes neurite outgrowth, neuronal survival, and other physiological changes in peripheral sympathetic and sensory neurons and a number of neuron-like clonal cell lines (1–6). However, there has been no evidence of a response of peripheral parasympathetic neurons to NGF. We present evidence for such a response in the present report. This evidence is based on an assay system we have recently developed (7) in which dissociated parasympathetic ciliary ganglion neurons are cultured in the presence of a substratum-conditioning factor from heart cell conditioned medium (8–10). In this system the rate of neurite outgrowth is sensitive to the addition of specific agents present in conditioned medium (7) and rat hippocampal extracts (11), and, as shown here, it is sensitive to NGF.

MATERIALS AND METHODS

Mouse submaxillary gland 2.5S NGF, prepared according to Smith *et al.* (12), was the generous gift of Arne Sutter and Eric Shooter (Stanford University). The results illustrated in Table 1 and Figs. 2 and 4 were also confirmed by using ultrapure 2.5S NGF from The Research Foundation of the State University of New York and from LAREF (Switzerland). Affinity purified rabbit anti-mouse submaxillary NGF antibody was obtained from LAREF. Fibroblast growth factor (FGF) and mouse submaxillary epidermal growth factor (EGF) were obtained from Collaborative Research (Waltham, MA). Crystalline bovine pancreatic insulin and synthetic angiotensin I and II were obtained from Sigma.

Nerve Cells. Ciliary and lumbosacral sympathetic chain ganglia were removed from stage 33 and stage 35 White Leghorn chicken embryos, respectively, and dissociated into single cells by using trypsin as described (8). Before use, dissociated ganglia were placed in plastic tissue culture dishes in culture medium F12FCS10 (see below) for 3 hr in order to remove the nonneuronal cells (almost all of which adhered to the culture dish) from the neurons (which did not adhere). The suspension of single, dissociated neurons was then plated onto a dish containing a "conditioned" substratum (see below) for subsequent observation of neurite elongation.

Culture Conditions. Culture medium (F12FCS10) consisted of Ham's F12 medium (GIBCO) containing 10% fetal calf serum (Irvine Scientific). Conditioned substrata for supporting neurite outgrowth were prepared as follows: Plastic tissue culture dishes (Falcon) were coated with polyornithine, as described (8). The polyornithine-coated dishes were then incubated with heart cell conditioned medium (see below) for 16–18 hr at 4°C in order to coat the dish with a substratum conditioning factor, which induces neurite outgrowth from ciliary ganglion neurons (9).

Heart Cell Conditioned Medium. A confluent monolayer of heart cells, prepared by tryptic dissociation of embryonic hearts from stage 33 chicken embryos, was cultured for 72 hr in F12FCS10 medium. The conditioned medium was removed from the monolayer and centrifuged at 10,000 × *g* for 30 min to remove any cell debris (8). The heart conditioned medium was passed under nitrogen pressure through a filtration membrane (Amicon) with a 100,000-dalton cutoff. The substratum-conditioning factor is retained by the membrane, so that the resultant ultrafiltrate fraction has been depleted of this factor but still contains protein that dramatically increases the rate of neurite elongation (7). The ultrafiltrate fraction was used either undiluted or in serial 1:1 dilutions with F12FCS10 medium.

Measurement of Neurite Lengths. At times indicated in the text, cultures were fixed in 2% (wt/vol) glutaraldehyde. The total length of all neurites present on each neuron was measured by using a calibrated eyepiece reticle. The data for 50 neurons per culture were used to calculate the mean total length of neurites per neuron for each culture. The calculations were based on data from neurons that had at least one neurite greater than 15 μm in length (i.e., about the diameter of the neuronal soma).

Neuronal Survival. Neuronal survival was assayed by the morphological integrity of the neuronal soma (13). The distinction made is between intact, phase-refractile somas and lysed, phase-dark somas.

RESULTS

Effect of NGF. NGF, when added to dissociated ciliary ganglion neurons, resulted in an approximately 2-fold increase in neurite lengths over untreated, control cultures (Table 1). The increase in neurite lengths was visible within 60 min of

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Abbreviations: NGF, nerve growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor.

Table 1. Maximal effect on neurite lengths of various additions to the culture medium

Neuron	Relative length						
	NGF	NGF + anti-NGF	CM	CM + anti-NGF	EGF	FGF	AII
Ciliary	2.08 ± 0.12†	1.06 ± 0.05	3.42 ± 0.36‡	3.10 ± 0.21‡	1.18 ± 0.10	1.00 ± 0.06	1.07 ± 0.04
Sympathetic	2.47 ± 0.21†	0.94 ± 0.11	1.81 ± 0.09*	1.64 ± 0.27*	1.02 ± 0.05	1.05 ± 0.08	1.02 ± 0.07

Dissociated neurons were exposed to a range of concentrations of the agents indicated above. The largest effect on neurite length observed over the concentration range is reported. NGF, EGF, and FGF were used at 1, 10, and 100 ng/ml. Angiotensin II (AII) was used at 1, 10, 100, and 1,000 ng/ml. Heart cell conditioned medium (CM) was used at dilutions between 1:1 and 1:16 in place of culture medium. Affinity-purified anti-NGF antibody was present at a concentration of 5 μg/ml. Two hours after addition of each agent the cultures were fixed and the mean total neurite length per initiated neuron was determined for each culture and that value was divided by the corresponding value for the untreated control. The resulting ratios proved to be more reproducible in repeated experiments than were the absolute neurite lengths. The effects of each agent were determined on at least two separate occasions and the ratios reported above are the mean and range for the separate determinations. The mean total neurite length per neuron for control cultures was 76 ± 19 μm in four separate determinations. The P values are the statistical significance of the difference between the treated and control cultures: *, $P < 0.01$; †, $P < 0.005$; ‡, $P < 0.001$. The values were calculated by using a t test for unpaired variables from populations of different variance. When no values are given, the difference was not statistically significant (i.e., $P > 0.05$).

the addition of NGF, and by 2–3 hr produced a dramatic difference between treated and control cultures (Fig. 1). In correlation with its ability to increase the rate of neurite elongation, NGF also caused an enlargement and spreading of the ciliary ganglion growth cones (compare Fig. 1 *A* and *B*; this effect is also illustrated at higher magnification in ref. 7).

The increase in neurite lengths resulting from addition of NGF was abolished by affinity-purified anti-NGF antibodies (Table 1), which also abolished the growth cone enlargement induced by NGF. Neither EGF nor FGF, at 1–100 ng/ml, had a significant effect on neurite length (Table 1). Some biological responses previously attributed to NGF have been shown to be a result of contamination by renin, resulting in the production of angiotensin, which induces the responses attributed to NGF (14, 15). However, angiotensin I (data not shown) or angiotensin II (Table I), at 1–1,000 ng/ml, had no significant effect on neurite lengths.

The NGF-stimulated increase in neurite lengths of ciliary ganglion neurons was detectable at 0.1 ng of NGF per ml and reached a maximum at 10 ng/ml (Figs. 2 and 3). NGF also increased the average length of neurites in cultures of dissociated sympathetic chain neurons under identical assay conditions (Table 1). The maximal increase for sympathetic neurons was typically larger than for ciliary ganglion neurons (Fig. 2 and Table 1) and occurred at a lower concentration of NGF, 1 ng/ml (Fig. 2).

Comparison to Conditioned Medium. We have recently reported that protein contained in a fraction of heart cell conditioned medium will also increase the average neurite length in cultures of dissociated parasympathetic ciliary ganglion neurons under conditions identical to those used above (7). The effect of conditioned medium on neurite lengths was due to an increase in the rate of neurite elongation (7). Conditioned medium also caused an enlargement and spreading of ciliary ganglion growth cones similar to that observed with NGF (7).

The effects of conditioned medium and NGF are similar, but the evidence does not support the conclusion that the action of conditioned medium is due to NGF. The effect of conditioned medium is not blocked by the anti-NGF antibody (Table 1). Also, the magnitude of the conditioned medium effect on ciliary ganglion neurite lengths is significantly greater than that of NGF, whereas the reverse is true for sympathetic neurons (Table 1).

Effect of Insulin. Because NGF is structurally similar to insulin (16, 17), we determined the effects of the latter on parasympathetic ciliary ganglion neurite lengths. As shown in Fig. 3, insulin also caused an increase in neurite lengths com-

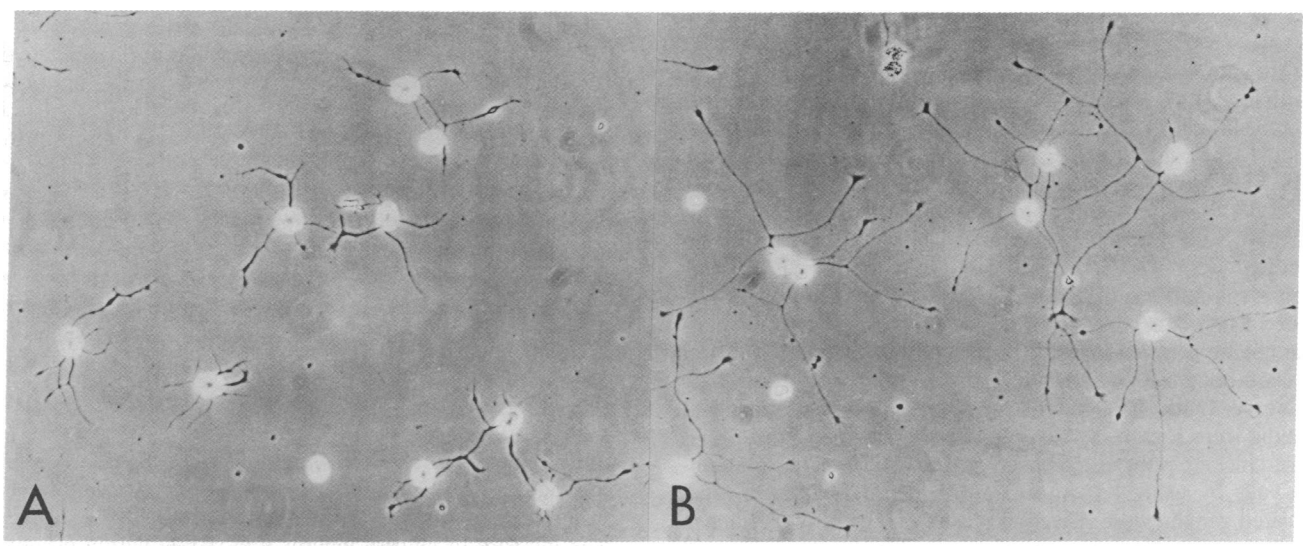

FIG. 1. Effect of NGF on ciliary ganglion neurons. NGF (10 ng/ml) was added to dissociated ciliary ganglion neurons 45 min after plating (*B*), at which time neurite outgrowth had already commenced. A duplicate culture without added NGF served as a control (*A*). At 3 hr after the addition of NGF these phase-contrast micrographs were taken. (×170.)

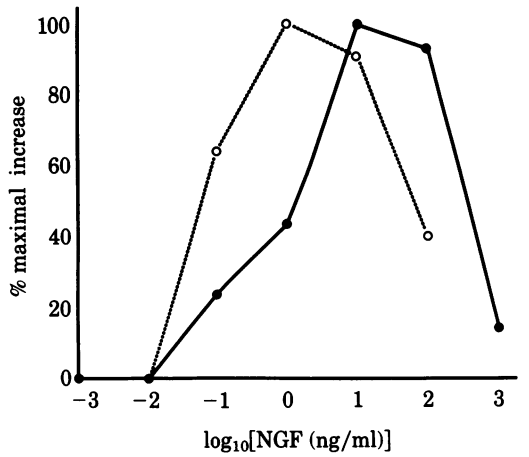

FIG. 2. Effect of NGF on mean neurite length in sympathetic and ciliary ganglion neurons. NGF, at various concentrations, was added to dissociated neurons 45 min after plating. Two hours later the mean total neurite length per initiated neuron was determined for each NGF concentration and the largest increase over the control without NGF was set equal to 100%. For sympathetic neurons (○) the maximal increase in neurite length was 2.4-fold over the control; for ciliary ganglion neurons (●) the maximal increase was 1.85-fold.

parable to that seen with NGF, but only at relatively high concentrations (50% maximum at 1 μg/ml). At these concentrations insulin also caused a change in growth cone morphology similar to that produced by NGF or conditioned medium.

Neuronal Survival. When cultured in unconditioned medium, parasympathetic ciliary ganglion neurons did not survive intact past 18–24 hr, and sympathetic chain neurons did not survive past 48 hr. NGF at 1–10 ng/ml prolonged the survival of the sympathetic neurons for at least 8 days but had no effect on the survival of ciliary ganglion neurons, which disintegrated as usual at 18–24 hr in concentrations of NGF between 1 and 500 ng/ml (Fig. 4). This result is not simply a reflection of the inability of ciliary ganglion neurons to survive in culture, because in conditioned medium or appropriate tissue extracts these neurons are able to survive for at least several weeks in the present culture conditions (9, 13, 18).

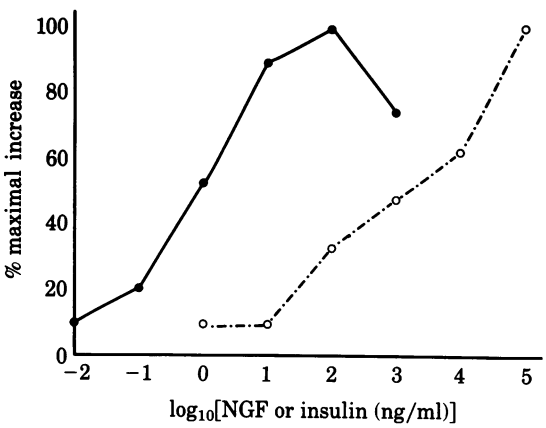

FIG. 3. Effect of NGF and insulin on ciliary ganglion neurite lengths. NGF or insulin, at various concentrations, was added to dissociated ciliary ganglion neurons 45 min after plating. Two hours later the mean total neurite length per initiated neuron was determined for each NGF and insulin concentration and the largest increase over the control without NGF for each agent was set equal to 100%. For NGF (●) the maximal increase was 1.9-fold over the control; for insulin (○) the maximum was 2.2-fold.

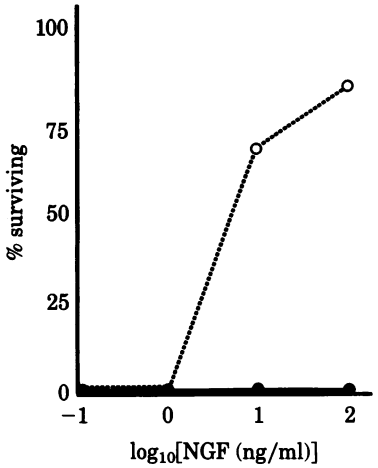

FIG. 4. Effect of NGF on neuronal survival. Dissociated ciliary ganglion (●) or sympathetic chain (○) neurons were cultured in medium supplemented with NGF at various concentrations. The percentage of neurons surviving at 49 hr after plating was determined with respect to the number originally present at 6 hr after plating.

DISCUSSION

We have observed that mouse submaxillary NGF from three different purified preparations caused a rapid increase in the rate of elongation of parasympathetic ciliary ganglion neurites and an accompanying enlargement and spreading of their growth cones. These effects of NGF occurred over the typical concentration range reported for its other biological activities, 0.1–10 ng/ml (17, 19), and were abolished by affinity-purified anti-NGF antibodies. The effects were not due to angiotensin, nor were they manifested by EGF or FGF.

In contrast to our results, others have reported no effect of NGF on parasympathetic neurite outgrowth *in vitro* (18, 20, 21). The reason for this discrepancy is probably the nature of the assay system used in each case to test for the effects of NGF. In our assay system, the effects of added supplements, such as NGF, are tested on dissociated neurons under conditions in which the culture substratum is fully adequate (i.e., preconditioned) for neurite outgrowth and there is no requirement for extended neuronal survival in order for the rapid effects on elongation rate and growth cone morphology to be manifested. The previous studies that reported negative results tested the ability of NGF to induce neurite outgrowth rather than augment its rate. For NGF to have induced outgrowth under the conditions of those assays it would have had to either (*i*) precondition the polyornithine substratum itself (18) or (*ii*) prolong neuronal survival for the 1–3 days required to observe outgrowth around whole ganglia (20, 21). As shown in our study, NGF has neither of these effects on ciliary ganglion neurons.

There is another report in the literature indicating a relationship between NGF and parasympathetic neurons. Max *et al.* (22) observed the uptake and retrograde axonal transport of NGF by chicken and rat ciliary ganglion neurons *in vivo*. High-affinity receptors for NGF are considered a prerequisite for such uptake and retrograde transport (23, 24).

Thus, an agent with demonstrated specificity for sympathetic and sensory neurons, at least in the peripheral nervous system, has now been shown to be taken up and transported by peripheral parasympathetic neurons *in vivo* and to increase the rate of neurite elongation of these neurons *in vitro*. It seems to us that there are two general ways of interpreting these results. The first is that NGF may be involved in the

normal development of these neurons, possibly in a more limited way than it is involved in the development of sympathetic neurons. The second possibility, which we currently favor, is suggested by the following considerations. In our assay system, sympathetic neurons, the more usual target for the actions of NGF, required 1/10 as much NGF as did ciliary ganglion neurons for a similar increase in neurite lengths. Heart cell conditioned medium also caused an increase in neurite lengths similar to that observed with NGF, but this effect was insensitive to anti-NGF antibodies. Also, the neuronal specificity of the conditioned medium effect was just the reverse of that of NGF, because a given concentration of conditioned medium had a much greater effect on the parasympathetic than on the sympathetic neurons. It may be, therefore, that in the present assay system NGF is demonstrating crossreacting biological activity with a more specifically parasympathetic trophic factor found in heart cell conditioned medium (13, 25, 26). In view of the effectiveness of insulin at high concentrations in this same assay system, it may be that there are a number of insulin-like neurotrophic factors, of which NGF is one (17), that are each specific for a particular class of neurons but are similar enough in structure to be crossreactive under certain circumstances.

The present results suggest that parasympathetic ciliary ganglion neurons, in addition to sympathetic and sensory neurons and certain neuron-like tumor cells, may be a useful system in which to study the biological action of NGF. One interesting feature of the ciliary ganglion system is that NGF has an effect on neurite outgrowth but not on neuronal survival. This dissociation of two effects normally associated in the response of sympathetic neurons (1, 2, 27) suggests the possibility that NGF may produce these effects by separate mechanisms, one of which can be studied in isolation by using ciliary ganglion neurons.

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