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Nerve growth factor-induced fiber outgrowth from isolated rat adrenal chromaffin cells: Impairment by glucocorticoids

(adrenal medullary cells/neurite outgrowth/steroids)

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ABSTRACT **Addition of nerve growth factor to cultures of dissociated rat adrenal medullary cells caused fiber outgrowth from chromaffin cells. These fibers exhibited all the characteristics of neurites, particularly the formation of typical growth cones exhibiting intense catecholamine-specific fluorescence. Because this nerve growth factor-mediated neurite outgrowth could be abolished by physiological concentrations of glucocorticoids, it is concluded that the high glucocorticoid concentrations normally present in the adrenal medulla prevent the fiber outgrowth from medullary chromaffin cells *in vivo*. In dissociated sympathetic neurons the same concentrations of glucocorticoids markedly reduce but do not completely abolish neuronal fiber outgrowth.**

Sympathetic neurons and adrenal medullary cells originate from the neural crest and resemble each other in many respects (1). Both cell types synthesize the enzymes necessary for the formation of the adrenergic transmitter. In addition, the mechanisms of storage and release of the transmitter are similar (cf. refs. 2 and 3). However, there are also distinct differences: sympathetic neurons liberate the transmitter into the immediate surroundings of the effector organs from specialized structures in their nerve terminals. In contrast, the adrenal chromaffin cells lack these specialized processes and release the transmitter into the general circulation,—i.e., they are endocrine cells. An additional difference between adrenal chromaffin cells and adrenergic neurons is the response to nerve growth factor (NGF). Until recently the major part of the available information indicated that adrenal chromaffin cells do not respond to NGF (4, 5). This assumption was based on the observation that concentrations of antiserum to NGF that lead to an extensive destruction of the peripheral sympathetic nervous system do not affect the adrenal medulla and that high doses of NGF, which lead to a marked volume increase of sympathetic ganglia, do not change the volume of the adrenal medulla (5). However, recent experiments have shown that adrenal chromaffin cells and sympathetic neurons respond to NGF with selective induction of tyrosine hydroxylase and dopamine β-hydroxylase (6), although the general growth promoting effect in the adrenal medulla is absent (5). Moreover, autoradiographic studies have shown that intravenously injected ^{125}I-labeled NGF is selectively accumulated in adrenal chromaffin cells as in adrenergic neurons (6). After transplantation experiments of adrenal medullary cells had shown that these cells also possess the ability to form processes (7, 8), we investigated whether the particular topographical location of the adrenal medulla—i.e., its close proximity to the adrenal cortex and concomitant exposure to high glucocorticoid concentrations—is responsible for the lack of formation of neuronal fibers.

We report that NGF produces marked fiber outgrowth from isolated rat chromaffin cells and that NGF-mediated fiber outgrowth can be abolished by concentrations of glucocorticoids that correspond to those in the adrenal venous blood. These concentrations of glucocorticoids also enhance NGF-mediated induction of tyrosine hydroxylase in a manner similar to that in organ cultures of sympathetic ganglia.

MATERIALS AND METHODS

Culture Methods. Adrenal glands were obtained from 7- to 12-day-old rats (Hanover Wistar strain) of either sex. Medullae were carefully freed from capsular and cortical tissue under sterile conditions and incubated with 1% collagenase and elastase (Serva) in Hanks' balanced salt solution supplemented with 20% fetal calf serum (Flow Laboratories) for up to 30 min. Subsequently, medullae were enzymatically dissociated with 0.125% trypsin (ICN Pharmaceuticals) dissolved in glucose-enriched phosphate-buffered saline. After centrifugation and resuspension in culture medium (glucose-enriched medium 199/20% fetal calf serum/100 μg of gentamycin per ml), the cells were plated on collagen-coated coverslips in modified Rose chambers (cf. ref. 8). After 24–36 hr the medium was changed to remove dead cells and cell debris. Medullae from six adrenals were pooled for each chamber. The cultures were maintained for 7 days and divided into the following experimental groups: (*i*) control, (*ii*) medium containing 4 or 12 ng of 2.5S NGF per ml, (*iii*) medium containing the same concentrations of 2.5S NGF plus 10^{-5}–10^{-7} M disodium dexamethasone 21-phosphate (Decadron, supplied by Merck Sharp and Dohme), (*iv*) medium containing 10^{-5}–10^{-7} M dexamethasone.

Superior cervical ganglia of 1-day-old rats were dissociated mechanically (9, 10) and plated in collagen-coated dishes (20 mm) in the same growth medium as described above. Each dish was supplied with 2.5S NGF at 12 ng/ml. Dexamethasone was added in concentrations of 10^{-5}–10^{-7} M. The culture medium was changed every 2–3 days. Cultures were inspected daily by phase contrast optics, and photographs were taken with Kodak Tri-X pan film.

Preparation and Bioassay of NGF. NGF was prepared from mouse salivary glands as the 2.5S subunit according to the method of Bocchini and Angeletti (11). The biological activity of NGF was determined according to Fenton (12) and amounted in our preparation to 250 biological units per μg of protein.

Catecholamine Histochemistry. After removal of the medium, coverslips were incubated for 3 sec in ice-cold, buffered glyoxylic acid and processed according to De la Torre and Surgeon (13).

Electron Microscopy. Cultures grown on carbon- and collagen-coated coverslips were fixed for electron microscopy in 2.5% phosphate-buffered glutaraldehyde, washed in 0.1 M phosphate buffer, and postfixed with 2% aqueous OsO_4. After staining in an aqueous saturated solution of uranylacetate and

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Abbreviation: NGF, nerve growth factor.

rapid dehydration, cultures were embedded in Araldite (cf. ref. 8).

Tyrosine Hydroxylase Assay. Adrenal medullary cells from one Rose chamber were homogenized in 250 μl of ice-cold 0.005 M Tris·HCl buffer, pH 7.4, containing 0.1% (wt/vol) Triton X-100. After centrifugation at 9000 × *g* for 15 min, 50-μl aliquots from the supernatant were taken for the determination of tyrosine hydroxylase activity according to Coyle (14) with the following modifications. We omitted the dihydropteridine reductase (which in our system did not improve the sensitivity) and used the natural cofactor tetrahydrobiopterin. The assay mixture contained 50 μl of enzyme preparation, 10 μl of 1 M potassium phosphate buffer, 5000 units of catalase in a volume of 10 μl, 5 μl of 5 mM tetrahydrobiopterin prepared in 5 mM HCl containing 20% (vol/vol) 2-mercaptoethanol. The concentration of substrate (L-tyrosine) was 200 μM. After 20 min of incubation at 37° the enzyme reaction was terminated by addition of 0.8 ml of ice-cold 5% trichloroacetic acid.

RESULTS

Growth Characteristics of Isolated Adrenomedullary Cells in Control Medium. In confirmation of previous experiments (8, 15), the dissociated cells could clearly be identified as chromaffin cells, neurons, or Schwann cells. Chromaffin cells were round after plating and became partially polygonal after 2–3 days in culture. The polygonal shape became more pronounced in the presence of dexamethasone (Fig. 1*D*). There was no evidence for fiber outgrowth from chromaffin cells that could clearly be distinguished from neurons. Neurons were present in very small number (about 50 versus 20,000 chromaffin cells) and were characterized by their large nuclei and prominent nucleoli. Chromaffin cells showed strong catecholamine-specific fluorescence, in contrast to neurons, which exhibited a considerably weaker fluorescence.

The ultrastructural features of chromaffin cells kept in culture for 7 days resembled to a great extent their appearance *in vivo* (Fig. 2*A*). However, their specific granular vesicles exclusively exhibited highly electron-dense cores, indicating the presence of a primary amine (16). The cores had a diameter of 35–240 nm, which is less than that described for rat adrenal chromaffin cells at the end of the gestation period (17), but which corresponds to the data given for rat adrenal chromaffin cells grown as explants (8).

Response of Chromaffin Cells to NGF. In contrast to control cultures, the presence of 4 or 12 ng of NGF per ml (corresponding to 1 or 3 units) elicited fiber outgrowth from chromaffin cells within 2 or 3 days (Fig. 1*B*). The pattern of the processes formed was variable, ranging from short, relatively broad processes to large (up to 1000 μm long), thin processes with ends resembling typical growth cones (Fig. 1*B*). The number and length of these processes increased steadily up to the end of the experiment. At this time 50–70% of all chromaffin cells exhibited distinct neuronal processes. An increase in the concentration of NGF to 120 and 1200 ng/ml enhanced the process of fiber formation. Interestingly, in contrast to chicken dorsal root ganglia (12), high concentrations of NGF did not inhibit fiber outgrowth.

Histochemical examination of chromaffin cells by the glyoxylic acid technique revealed an intense yellow-green fluorescence in the cell bodies and in the processes including the growth cone-like structures (Fig. 1*E*).

The histofluorescence-microscopic evidence for the formation of varicosities in the fibers originating from chromaffin cells was confirmed in the electron microscopic pictures (Fig. 2*E*). The size and electron density of dense-core vesicles in the cell body (Fig. 2*B*) did not differ from those in control cultures. The cores of the granular vesicles in the processes were considerably smaller (35–150 nm) than in the perikarya. In addition, processes and, in particular, varicose regions contained small, round or oval translucent vesicles (120–150 nm) as described for fibers growing out from explanted cells (8). A general feature of NGF-treated chromaffin cells was an extensive proliferation of smooth membranes occurring in the form of vesicles and irregular cisternae (Fig. 2*B*) which filled large areas in the cell bodies. A distinct augmentation of rough endoplasmic reticulum and ribosomes was not consistently found.

Effect of Dexamethasone on NGF-Mediated Fiber Outgrowth from Chromaffin Cells and Sympathetic Neurons. When chromaffin cells were grown in medium containing NGF at 12 ng/ml, 10^{-5} M dexamethasone completely abolished fiber outgrowth (Fig. 1*C*). Concentrations of 10^{-6} and 10^{-7} M dexamethasone produced a similar effect, although at 10^{-7} M, sprouting of a few axons was observed. The experiments had to be performed with the synthetic, water soluble glucocorticoid, dexamethasone, because preliminary experiments had shown that the minimal concentration of ethanol necessary for dissolving corticosterone, the main physiological glucocorticoid of the rat, killed the cultured chromaffin cells. For the same reason we could not study the effect of other steroid hormones that would have been of interest with respect to the specificity of the effect of dexamethasone.

Compared with controls, the dexamethasone-treated cells generally flattened out more rapidly and more extensively. Unexpectedly, the granular vesicles of chromaffin cells grown for up to 7 days with 10^{-5} M dexamethasone did not contain cores of low or medium electron density which would be expected if adrenaline were formed to an appreciable extent (Fig. 2*D*).

In order to evaluate whether the effect of glucocorticoids is restricted to chromaffin cells, we studied the effect of dexamethasone on NGF-mediated fiber outgrowth in dissociated sympathetic ganglia (Fig. 3). In contrast to chromaffin cells, dissociated sympathetic ganglia of newborn rats were strictly dependent on NGF—i.e., they degenerated within 24–48 hr after plating when no NGF was added to the culture medium. In the presence of NGF at 12 ng/ml, the sympathetic neurons formed processes up to 1000 μm long within 5 days (Fig. 3*A*). At a concentration of 10^{-5} M, dexamethasone markedly impaired fiber outgrowth (Fig. 3*B*). The fibers appeared much shorter and more blunt, and they split at their ends into numerous, very thin processes. At concentrations of 10^{-6} and 10^{-7} M, dexamethasone had no distinct effect on fiber outgrowth.

Effect of NGF and Dexamethasone on Tyrosine Hydroxylase Levels of Dissociated Adrenal Medullary Cells. In order to obtain information as to whether the abolition of NGF-mediated fiber outgrowth was due to impairment of general cell function, we studied the effect of dexamethasone and NGF on tyrosine hydroxylase levels in isolated chromaffin cells. After these cells had grown for 7 days in the presence of NGF at 12 ng/ml, the tyrosine hydroxylase levels were increased to 145 ± 21% as compared to control cultures [specific activity amounted to 0.46 ± 0.07 (±SEM) nmol of dopa per hr per mg of protein]. Dexamethasone (10^{-5} M) did not produce a statistically significant increase whereas the combined treatment with NGF and dexamethasone resulted in a tyrosine hydroxylase level of 190 ± 11% ($P < 0.01$).

DISCUSSION

In recent experiments it was shown that adrenal chromaffin cells accumulate intravenously injected ^{125}I-labeled NGF with high selectivity and that they respond to NGF, as do sympathetic neurons, with selective induction of tyrosine hydroxylase

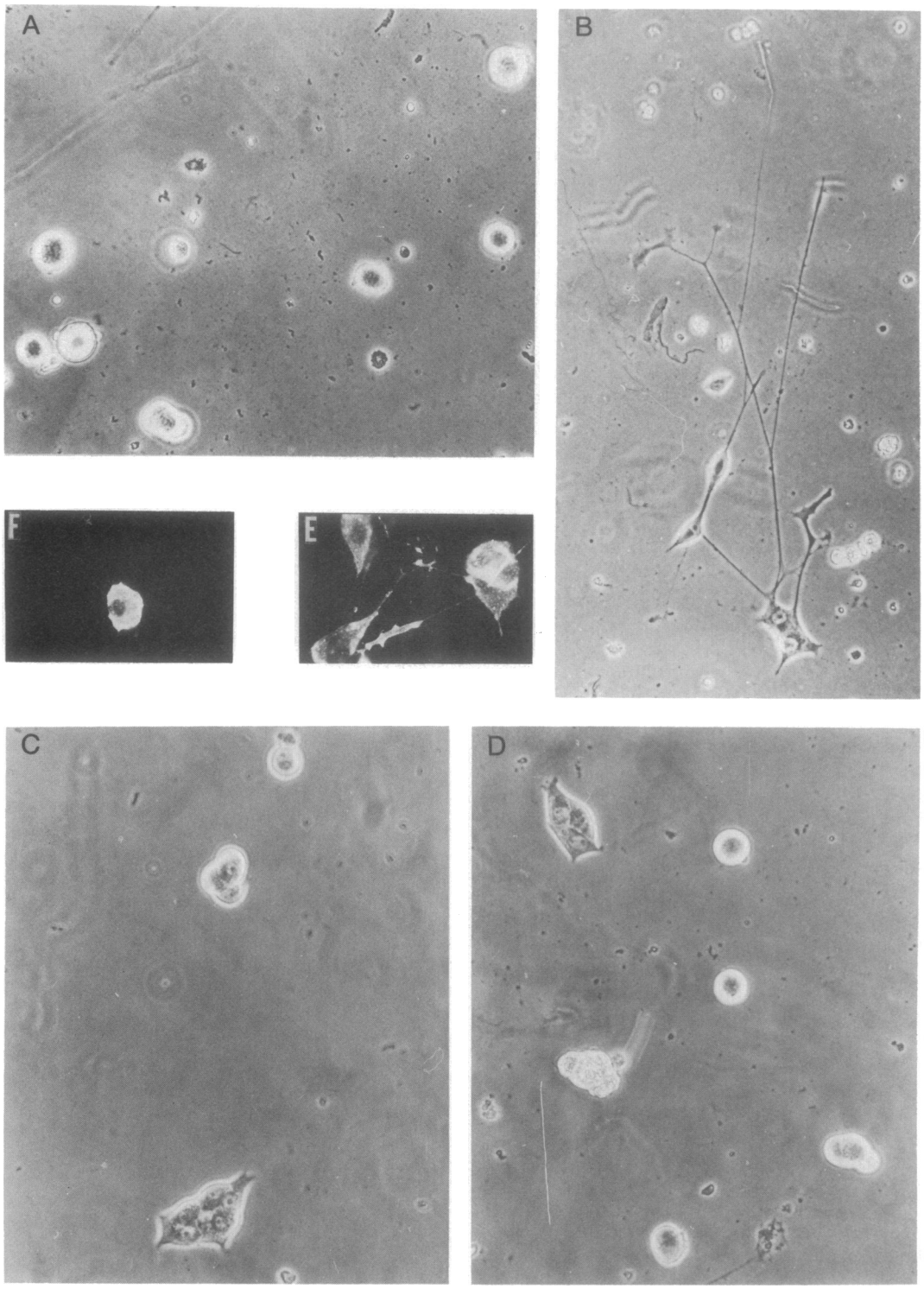

FIG. 1. (*A–D*) Phase contrast micrographs of unfixed rat adrenal chromaffin cells cultured for 7 days as a control (*A*), in the presence of NGF at 12 ng/ml (*B*), with NGF at 12 ng/ml plus 10^{-5} M dexamethasone (*C*), and in the presence of 10^{-5} M dexamethasone (*D*). (*E* and *F*) Fluorescence histochemical demonstration of catecholamines in chromaffin cells cultured for 7 days in the presence of NGF at 12 ng/ml plus 10^{-5} M dexamethasone (*F*) and NGF alone (*E*). (*A–D*, ×320; *E* and *F*, ×500.)

and dopamine B-hydroxylase (6). The present experiments have extended the response of adrenal chromaffin cells to NGF by a further characteristic effect, namely, neuronal fiber outgrowth.

In constrast to neuronal cells originating from sympathetic ganglia, the adrenal chromaffin cells do not require exogenous NGF to survive a culture period of 7–12 days. Under comparable experimental conditions sympathetic neurons degenerate within 24–48 hr (18), suggesting that either adrenal chromaffin cells do not need NGF at all for survival or that dissociated adrenomedullary cells can produce NGF or a NGF-like substance in amounts sufficient to keep the chromaffin cells alive, but not sufficient to initiate fiber outgrowth. That adrenal chromaffin cells are not as absolutely dependent on NGF as are sympathetic neurons in the early postnatal phase can also be deduced from the fact that large doses of anti-NGF antibodies, which lead to extensive destruction of the peripheral sympathetic nervous system, do not affect the adrenal medulla (5, 19).

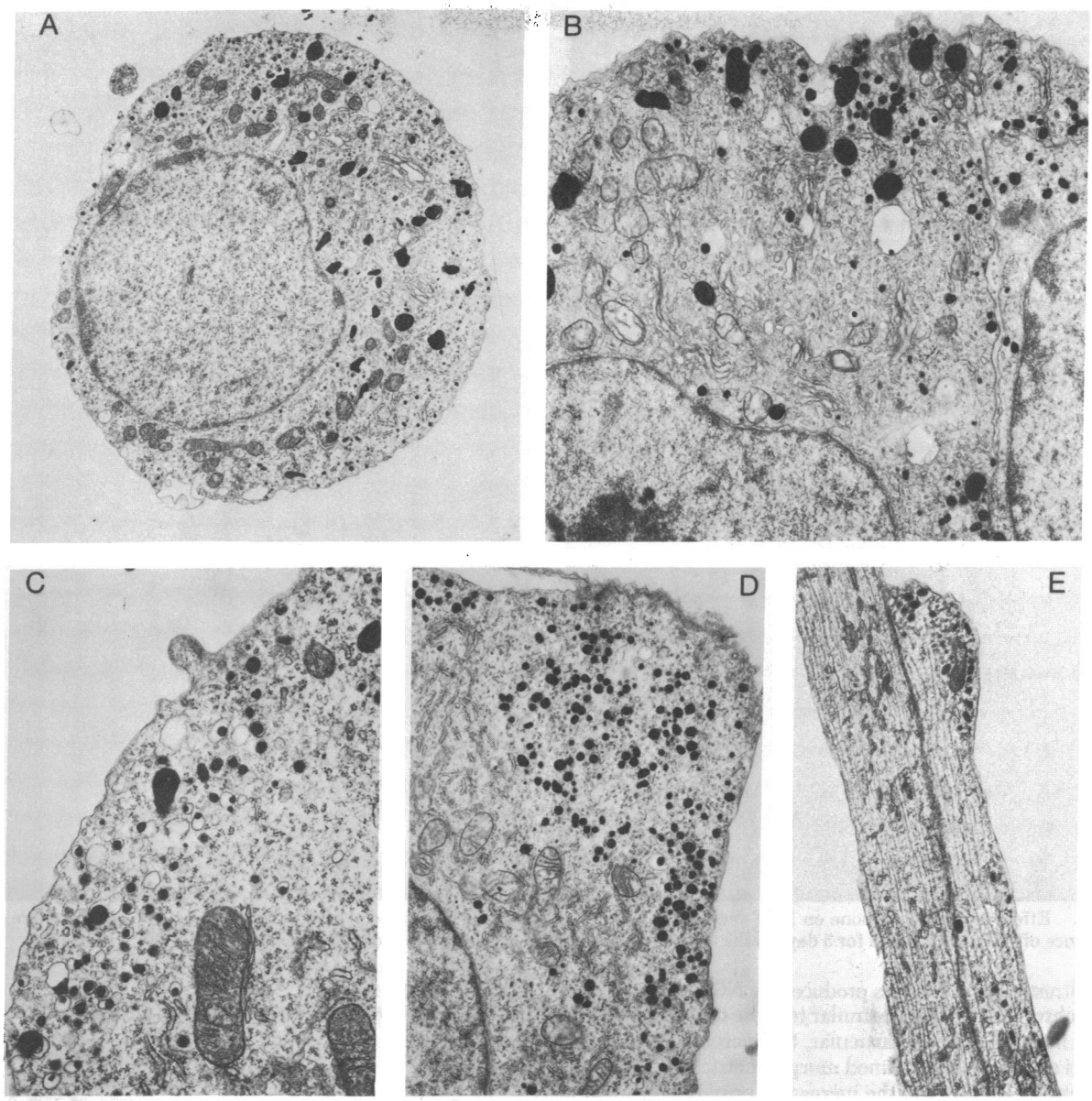

FIG. 2. Electron micrographs of rat adrenal chromaffin cells cultured for 7 days as a control (*A* and *C*), in the presence of 10^{-5} M dexamethasone (*D*), and NGF at 12 ng/ml (*B* and *E*). Note that the cores of the vesicles are exclusively of the noradrenaline type. (*B*) Extensive development of smooth membranes; (*E*) two processes, one of which shows a varicosity with dense core vesicles. (*A*, ×6200; *B*, ×14,500; *C*, ×20,000; *D*, ×9500; *E*, ×15,200.)

On the other hand NGF or NGF-like substances are formed by adrenal medullae in organ culture (20). It is not yet known whether this NGF activity results from *de novo* synthesis by one or several cell types in the adrenal medulla or whether it reflects the activation of a precursor supplied by the culture medium. That adrenal medullary cells are able to form NGF or a NGF-like substance is also indicated by recent observations of explants of rat adrenal medulla that exhibited neuronal fiber outgrowth without addition of NGF to the culture medium (8). This fiber outgrowth could be blocked by monospecific antibodies to NGF (unpublished observations).

The major difference between adrenomedullary transplants, tissue culture explants, and dissociated adrenomedullary cells on the one hand and the *in vivo* situation on the other hand is the proximity of the adrenal cortex *in vivo*. As a consequence, transplants, explants, and dissociated cells are not exposed to the high concentrations of glucocorticoids that reach the adrenal medulla *in vivo* via a capillary plexus from the adrenal cortex (21). The glucocorticoid concentrations achieved in the adrenal medulla are two orders of magnitude larger than in the general circulation (22). The fact that physiological glucocorticoid concentrations prevent the NGF-mediated fiber outgrowth *in vitro* suggests that there is a causal relationship between the glucocorticoid concentration in the adrenal medulla *in vivo* and the absence of fiber outgrowth.

In contrast, the sympathetic neurons located in pre- or paravertebral ganglia are never exposed to such high concentrations of glucocorticoids under physiological conditions. Moreover, the impairment of NGF-mediated fiber outgrowth by glucocorticoids in sympathetic neurons is even smaller than that in adrenal chromaffin cells.

Another characteristic response of NGF observed *in vivo*—i.e., the increase in tyrosine hydroxylase activity (6)—could also be observed in dissociated adrenomedullary cells. As in sympathetic ganglia the effect of NGF was potentiated by appropriate concentrations of glucocorticoids. However, the small quantities of available cells did not allow us to decide whether increased enzyme activity resulted from enhanced synthesis or from delayed degradation.

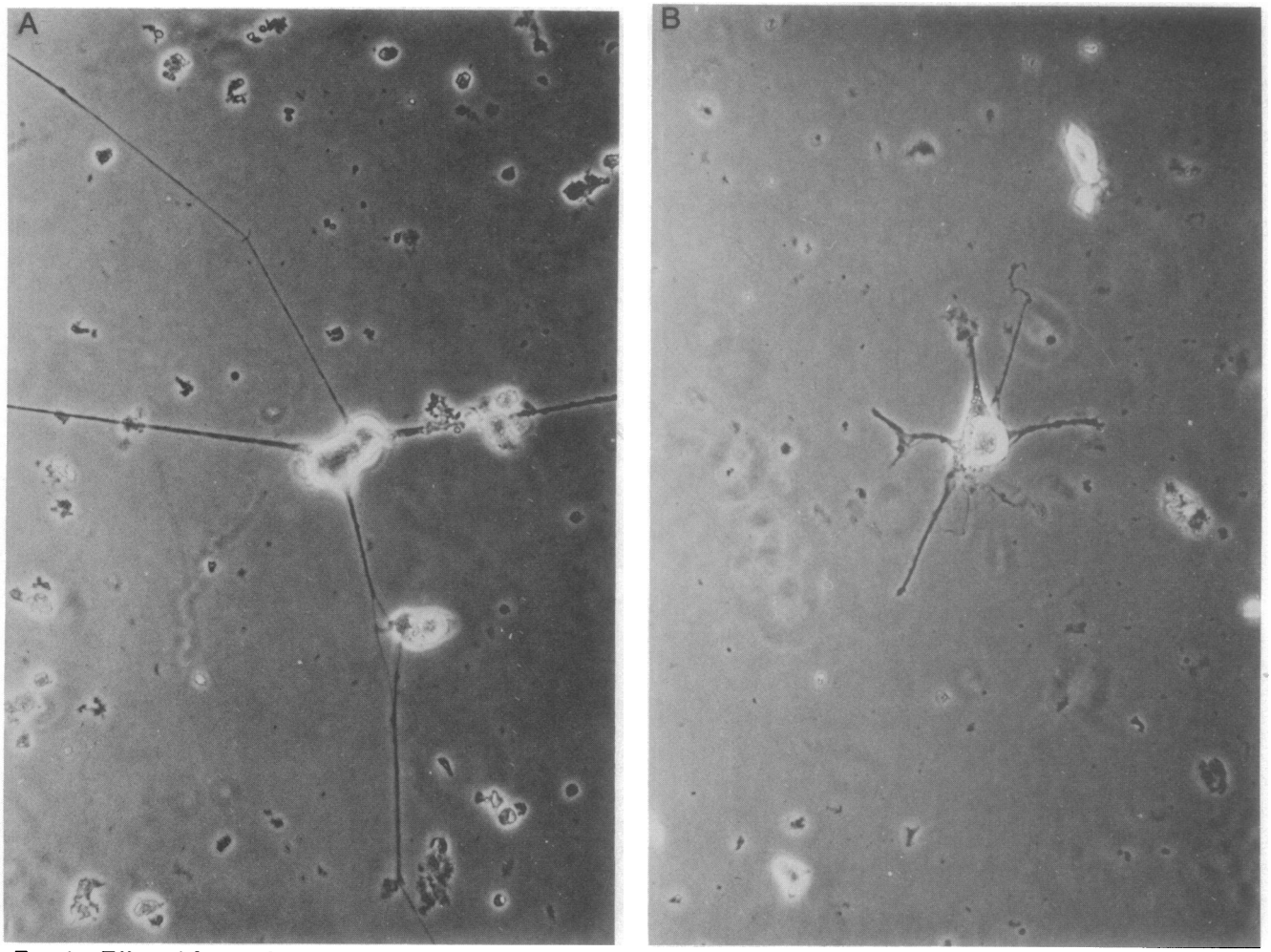

FIG. 3. Effect of dexamethasone on NGF-mediated fiber outgrowth from dissociated sympathetic neurons. The neurons were grown in the presence of NGF at 12 ng/ml for 5 days in the absence (*A*) or presence (*B*) of 10^{-5} M dexamethasone.

The ultrastructural changes produced by NGF in dissociated adrenal chromaffin cells are similar to those observed in sympathetic neurons (23). In particular, the increased volume of the Golgi apparatus determined morphometrically in sympathetic ganglia is similar to the increased formation of smooth membranes in NGF-treated chromaffin cells (Fig. 2*B*). No efforts have been made to quantify these changes morphometrically in the present experiments.

In conclusion, adrenal chromaffin cells kept in tissue culture respond to NGF by fiber outgrowth. The outgrowing fibers exhibit all the characteristics of neurites including the formation of typical growth cones. Because fiber outgrowth can be abolished by concentrations of glucocorticoids that are easily achieved in the adrenal medulla under physiological conditions (22), it is suggested that this represents the explanation for the absence of fiber outgrowth from chromaffin cells *in situ* in contrast to the fiber outgrowth observed in adrenomedullary transplants and explants.

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