Regulation of the Multiple Forms of Dopamine \(\beta\)-Hydroxylase by Nerve Growth Factor, Dexamethasone, and Dibutyryl Cyclic AMP in the PC12 Pheochromocytoma Cell Line*

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Treatment with nerve growth factor was found to influence the subunit forms of dopamine \(\beta\)-hydroxylase in PC12 pheochromocytoma cells. In untreated cells, near equal amounts of two subunit forms were observed (apparent \(M_r = 77,000\) and 73,000) by labeling with \([\text{35}S]\)methionine. Upon treatment of PC12 cells with nerve growth factor for several days, the \(M_r = 73,000\) subunit form of dopamine \(\beta\)-hydroxylase was almost exclusively observed. The shift in subunit forms became apparent only after a day of treatment and was maximal with 3 days or more of exposure to nerve growth factor. The dose-response curve was similar to other nerve growth factor-induced responses in PC12 cells. Neurite outgrowth, however, was not essential for the shift in predominance of the \(M_r = 73,000\) subunit form. This effect of nerve growth factor also occurred in suspension cultures or in the presence of low concentrations of inhibitors of transcription sufficient to prevent neurite outgrowth. Pulse-chase experiments with nerve growth factor-treated cells indicated that the \(M_r = 77,000\) form is initially synthesized (5 min) and is then converted to the \(M_r = 73,000\) form by 30–60 min.

Insulin (100 ng/ml) and epidermal growth factor (1 ng/ml) had no effect on the subunit forms of dopamine \(\beta\)-hydroxylase. However, treatment of PC12 cells for several days with dexamethasone (10–4 M) or dibutyryl cyclic AMP (1 mM) leads to predominance of the \(M_r = 73,000\) form of the enzyme.

These experiments suggest that the proportions of the subunit forms of dopamine \(\beta\)-hydroxylase can be regulated in cells by external signals and this may reflect alterations in post-translational processing enzymes and may serve as a potential mechanism to regulate catecholamine metabolism.

Dopamine \(\beta\)-hydroxylase (E.C.1.14.17.1) is the terminal enzyme in norepinephrine biosynthesis. It is present in adrenergic chromaffin cells in immunologically indistinguishable soluble and membrane-bound forms which can be separated by crossed immunoelectrophoresis (Bjerrum et al., 1979). We have shown that, in cultured PC12 pheochromocytoma cells, the subunits of the soluble (apparent \(M_r = 73,000\)) and membrane forms (apparent \(M_r = 77,000\)) of dopamine \(\beta\)-hydroxylase can be separated by SDS-polyacrylamide gel electrophoresis. These appear to be biosynthetically related in that the 73,000-\(M_r\) form is derived from the 77,000-\(M_r\) form (Sabban et al., 1983 (accompanying paper)). These findings raised the possibility that extracellularly supplied signals could alter the distribution of the forms of dopamine \(\beta\)-hydroxylase, and thereby, could play a role in the regulation of this enzyme.

We have examined the effect of various additives on the relative amounts of the multiple subunit forms of dopamine \(\beta\)-hydroxylase in the PC12 pheochromocytoma cell line. The PC12 cell line is established from a transplantable rat pheochromocytoma (Greene and Tischler, 1976). When these cells are exposed to NGF, a protein required in vivo and in vitro for the survival and differentiation of sympathetic and sensory neurons (Levi-Montalcini, 1976; Greene and Shooter, 1980; Thoenen and Barde, 1980), they cease proliferation and display many of the differentiated properties of sympathetic neurons. These properties include outgrowth of neuritic processes, increased electrical excitability, enhanced responsiveness to acetylcholine and the presence of synaptic-like vesicles (Greene and Tischler, 1976; Dichter et al., 1977; Tischler and Greene, 1976; Greene and Tischler, 1982). At the biochemical level, this treatment is accompanied by an increase in the specific activities of choline acetyltransferase (Greene and Rein, 1977; Schubert et al., 1977; Edgar and Thoenen, 1978) and acetylcholinesterase (Rieger et al., 1980), as well as by increased levels of several other proteins (McGuire et al., 1978; Guroff et al., 1981). However, the specific activities of tyrosine hydroxylase and dopamine \(\beta\)-hydroxylase decrease in NGF-treated PC12 cells (Greene and Tischler, 1976; Edgar and Thoenen, 1980; Greene and Tischler, 1982). In contrast, treatment of these cells with the synthetic glucocorticoid dexamethasone leads to induction of tyrosine hydroxylase (Edgar and Thoenen, 1978) via increased levels of mRNA for this enzyme (Baetge et al. 1981). In this paper, we present evidence that the distribution of the subunit forms of dopamine \(\beta\)-hydroxylase is specifically influenced by treatment for several days with NGF, dexamethasone, or dibutyryl cyclic AMP. This treatment leads to a shift from the presence of both forms in about equal amounts to a predominance of one form (apparent \(M_r = 73,000\)) of the enzyme.

**EXPERIMENTAL PROCEDURES**

Most of the experimental procedures have been previously described (Sabban et al., 1983 (accompanying paper)). NGF was prepared by the method of Mobley et al. (1976) and had a specific activity

*The abbreviations used are: SDS, sodium dodecyl sulfate; NGF, nerve growth factor.

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of 0.25 ng/unit in the PC12 bioassay (Greene, 1977). Epidermal growth factor was prepared by the method of Savage and Cohen (1972) and was the kind gift of Dr. Frederick Maxfield, Department of Pharmacology, New York University Medical Center. Dexamethasone (Sigma), dibutyryl cyclic AMP (Boehringer-Mannheim), insulin (Eli Lilly), and actinomycin D (Calbiochem) were obtained from commercial sources. Camptothecin was supplied by the Drug Development Branch, National Cancer Institute.

PC12 cells were grown in monolayer culture as previously described. For suspension culture, the cells were plated in uncoated plastic Petri dishes. The cells do not attach to these dishes in the presence or absence of NGF, but rather remain in suspension in small clumps.

All labeling with [35S]methionine was carried out in the presence of the same additives to which the cells had been previously exposed.

RESULTS

Effect of Nerve Growth Factor on the Subunit Forms of Dopamine β-Hydroxylase—We have shown that PC12 cells synthesize two molecular forms of dopamine β-hydroxylase subunits (apparent Mr = 77,000 and 73,000, Fig. 1). In un-treated PC12 cells, these are present in approximately equal amounts (Sabban et al., 1983 (accompanying paper)).

PC12 cells were treated with NGF (50 ng/ml, ~2 nM) for 5 or more days, conditions which lead to outgrowth of long neuronal processes. The cells were then labeled with [35S]methionine in the presence of NGF, and their solubilized homogenates were subjected to immunoprecipitation with antiserum to dopamine β-hydroxylase. SDS-polyacrylamide gel electrophoresis and fluorographic analysis of the immunoprecipitates revealed that the 73,000-Mr subunit form of dopamine β-hydroxylase predominated while the 77,000-Mr form was barely detectable (Fig. 1). This effect was very reproducible (consistent in at least 20 experiments with NGF treatment). In order to rule out the possibility that NGF-treated cells simply do not contain more of a protease which is degrading the dopamine β-hydroxylase during the immunoprecipitation, varying amounts of the protein from untreated cells were mixed with those from NGF-treated cells. The 77,000-Mr subunit form of dopamine β-hydroxylase persisted in these mixing experiments (Fig. 1). It also should be noted that it is unlikely that the presence of the 73,000-Mr subunit form is due to proteolytic degradation during the immunoprecipitation since the cells were initially lysed in phospho-buffered saline containing 2% SDS and boiled. The immunoprecipitates were carried out in the presence of detergents (0.4% SDS; 2% Triton X-100) and of a protease inhibitor (100 units/ml of Trasylol). Thus, it is likely that the immunoprecipitation accurately represents the distribution of the multiple dopamine β-hydroxylase forms in the cell.

Time Course of Effect of Nerve Growth Factor—The kinetics of the NGF-induced alterations were studied. PC12 cells were treated with NGF (50 ng/ml) for varying lengths of time and then labeled with [35S]methionine for 4 h. The distributions of the labeled 73,000-Mr and 77,000-Mr subunit forms were then examined by immunoprecipitation (Fig. 2). Only by two days of treatment was a clear shift to predominance of the 73,000-Mr form apparent. This change reached a maximum by about three days of NGF exposure. No alteration in distribution was noted in response to incubation with NGF for 12 h (data not shown). The reduction in the amount of dopamine β-hydroxylase synthesized relative to total protein synthesis in NGF-treated cells (Fig. 2) was consistently observed. This is compatible with the decrease in specific activity of dopamine β-hydroxylase which occurs in NGF-treated cells (Greene and Tischler, 1976).

Dose-Response of Nerve Growth Factor Effect—The concentration of NGF necessary for the shift in synthesis to predominantly the 73,000-Mr subunit form was determined. PC12 cells were grown in the presence of 0–100 ng/ml of NGF for 7 days, then labeled with [35S]methionine for several hours and used for immunoprecipitation with anti-dopamine β-hydroxylase antiserum. Fig. 3 shows that the change in the forms was maximal by 40 ng/ml (~1.5 nM). This is similar to the concentration necessary for maximal levels of neurite outgrowth, and for other maximal responses in PC12 cultures (cf. Greene and Tischler, 1982).

Neurite Outgrowth Is Not Necessary for the Effect of Nerve Growth Factor on Dopamine β-Hydroxylase—The time course of NGF treatment suggested that, while accompanying the early events of NGF treatment, the regulation of the subunit forms of dopamine β-hydroxylase may precede, and therefore proceed, independently of neurite outgrowth. To test this possibility, PC12 cells were treated with NGF (50 ng/ml) in suspension for 10 days and then labeled with [35S]methionine for 4 h and compared to sister cultures treated similarly, but in the absence of NGF. Under these conditions, while NGF-
shows both forms in near equal amounts as in monolayer culture. Experiments described below with RNA synthesis inhibitors and NGF, and with dexamethasone and dibutyryl cyclic AMP also confirm that neurite outgrowth is not required for regulation of the dopamine β-hydroxylase subunit forms.

Role of RNA Synthesis—The relatively slow time course of the dopamine β-hydroxylase response to treatment with NGF suggests that alterations in mRNA levels could be necessary for the enhanced levels of the 73,000-Mr subunit form in the presence of NGF. While PC12 cells cannot survive for several days in the presence of high concentrations of transcription inhibitors, low concentrations (0.2 μg/ml of camptothecin and 0.01 μg/ml of actinomycin) allow many of the cells to remain viable. This is especially true of cells grown in the presence of NGF. In the presence of these low concentrations of transcription inhibitors, no neurites are formed in the presence of NGF (Burstein & Greene, 1978) and the cells fail to undergo NGF-promoted induction of several specific proteins (cf. Greene & Tischler, 1982, for review). Thus, the PC12 cells were treated for 7 days with low concentrations of actinomycin and camptothecin in the absence and presence of NGF and then labeled for 4 h with [35S]methionine. Although many of the cells do not survive, the labeling pattern of the total proteins synthesized was remarkably unchanged. The only detectable difference was the disappearance of one extremely minor band in the region of apparent Mr = 77,000 (Fig. 4). Immunoprecipitation with anti-dopamine β-hydroxylase antisera showed that the effect of NGF on the forms of dopamine β-hydroxylase persisted in the presence of low concentrations of actinomycin and camptothecin (Fig. 4). The inhibitors themselves did not have an effect on the relative proportions of the two forms of dopamine β-hydroxylase in NGF-untreated cultures.

Effect of Insulin, Epidermal Growth Factor, Dexamethasone, and Dibutyryl Cyclic AMP on Dopamine β-Hydroxylase—The specificity of the effect of NGF was tested by the additions of other compounds to which PC12 cells are known to respond (cf. Greene and Tischler, 1982, for review) and which have specific activity of the effect of NGF on the forms of dopamine β-hydroxylase.
been found to modulate the catecholamine synthesizing enzymes in some systems. These substances were epidermal growth factor, insulin, dexamethasone, and dibutyryl cyclic AMP. Treatment for 8 days with insulin (100 ng/ml) or epidermal growth factor (1 ng/ml) did not alter the distribution of the dopamine β-hydroxylase subunit forms (Fig. 5). However, dexamethasone (10^{-6} M) and dibutyryl cyclic AMP (1 mM) reproduced the effect observed with NGF (Fig. 5). Neurites were not formed under these conditions, although dibutyryl cyclic AMP led to flattening of the cells and formation of some cytoplasmic extensions. As in the case of NGF, it was also necessary to treat the cells for more than one day with dexamethasone or dibutyryl cyclic AMP for the effect on dopamine β-hydroxylase to occur.

The effect of dexamethasone raised the possibility that hormones present in the complete culture medium may be necessary for the NGF effect on dopamine β-hydroxylase. Thus, PC12 cells were treated with NGF in serum-free medium. The control, without NGF, is not possible since PC12 cells are not viable in the absence of both serum and NGF (Greene, 1977). In serum-free medium, NGF still led to the predominance of the 73,000-Mr subunit form of dopamine β-hydroxylase.

**Relationship between the Dopamine β-Hydroxylase Subunit Forms**—Since NGF-treated cells contain predominantly the 73,000-Mr subunit form of dopamine β-hydroxylase, they seem appropriate for testing the biosynthetic relationship between the two subunit forms (Subban et al., 1985). Thus, pulse-chase experiments were carried out to study whether a precursor of the 73,000-Mr, subunit could be detected in NGF-treated PC12 cells. When the cells were labeled for 5 min with [35S]methionine and the newly synthesized dopamine β-hydroxylase was immunoprecipitated, the 77,000-Mr, subunit form predominated (Table I). We did not detect any higher molecular weight immunoreactive forms. After a 10-min chase period in the presence of excess unlabeled methionine, both the 73,000-Mr and 77,000-Mr, forms were present. By 30–60 min, the latter form was almost completely gone, while the 73,000-Mr, subunit form predominated (Table I). Thus, as in untreated cells, there appears to be a precursor-product relationship between the 77,000- and 73,000-Mr, forms.

**DISCUSSION**

The PC12 cell line appears to be an excellent model system in which to study the regulation of the multiple forms of dopamine β-hydroxylase by NGF and other compounds. These cells provide the opportunity to directly compare cells before and after treatment. We have shown that treatment with NGF, glucocorticoids, and dibutyryl AMP leads to a shift from an approximately equal distribution of the 77,000- and 73,000-Mr, subunit forms of dopamine β-hydroxylase to a state in which the 73,000-Mr, subunit form greatly predominates.

We established by three different types of experiments that the alteration in the ratio of the subunit forms of dopamine β-hydroxylase can occur in the absence of neuronal-like outgrowth. First, the shift occurs upon treatment with NGF in suspension. Similarly, treatment with dexamethasone or dibutyryl cyclic AMP does not lead to neurite outgrowth, yet alters the ratio of the dopamine β-hydroxylase subunit forms. Thirdly, treatment with low concentrations of actinomycin D or camptothecin prevents neurite outgrowth in the presence of NGF (Burstein and Greene, 1978), but does not prevent the effect of NGF on dopamine β-hydroxylase.

It should not be concluded on the basis of the present experiments that transcription is not involved in the alteration in the subunit forms of dopamine β-hydroxylase in response to NGF. The low concentrations of inhibitors employed do not completely block transcription in the PC12 system (Burstein and Greene, 1978). Indeed, the time course of the effect showed that the cells had to be treated for at least two days with NGF or other agents in order for the distribution of the dopamine β-hydroxylase subunit forms to be maximally altered. This suggests that the regulation is a relatively long term effect and that transcription, therefore, may be involved. We were unable to directly test the involvement of RNA synthesis as the cells cannot survive for such long periods in the absence of transcription. It is of interest that the levels of transcription inhibitors employed, while not affecting the action of NGF on dopamine β-hydroxylase, effectively block a number of responses to NGF such as neuritic outgrowth (Burstein and Greene, 1978), induction of NGF-inducible large external glycoprotein (McGuire and Greene, 1980) and elevated acetylcholinesterase activity (Greene and Rukenstein, 1981). Hence, if the action of NGF on dopamine β-hydroxylase has a transcriptional component,

### Table I

<table>
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<tr>
<th>Pulse</th>
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<th>77,000-Mr form</th>
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this action is separable from that involved in other long term NGF responses.

It cannot be concluded that the shift to predominance of the 73,000-Mr subunit form in NGF-treated cells is a consequence of decreased synthesis of dopamine \(\beta\)-hydroxylase. While treatment with dibutyryl cyclic AMP or dexamethasone also leads to predominance of the 73,000-Mr subunit form, these agents do not appreciably decrease the synthesis of dopamine \(\beta\)-hydroxylase.

The initial mechanisms by which NGF, dexamethasone, and dibutyryl cyclic AMP affect the multiple forms of dopamine \(\beta\)-hydroxylase are presently unclear. Most actions of corticosteroids appear to be mediated via a cytoplasmic acceptor and nuclear binding sites (cf. Higgins and Gehring, 1978). Dibutyryl cyclic AMP appears to promote its actions by leading to activation of cAMP-regulated protein kinase (Corbin et al., 1981), and the mechanism of action of NGF is not resolved. Also, despite their shared ability to regulate the multiple forms of dopamine \(\beta\)-hydroxylase, the above three agents have rather dissimilar actions on PC12 cells. For instance, dibutyryl cyclic AMP and dexamethasone do not mimic NGF's ability to promote neurite outgrowth or to resolve. Also, despite their shared ability to regulate the multiple forms of dopamine \(\beta\)-hydroxylase, the above three agents do not appreciably decrease the synthesis of dopamine \(\beta\)-hydroxylase.

While treatment with dibutyryl cyclic AMP or dexamethasone causes a long term increase in the levels of tyrosine hydroxylase (Edgar and Thoenen, 1978), the initial mechanisms by which NGF, dibutyryl cyclic AMP, and dexamethasone affect the ratio of the dopamine \(\beta\)-hydroxylase forms is also presently unclear. One possibility is that these agents may be inducing a protease responsible for the processing of the 77,000- to the 73,000-Mr subunit form. This is consistent with the pulse-chase experiments which indicate that in NGF-treated cells, the 77,000-Mr form is initially synthesized and then rapidly converted to the 73,000-Mr form. In the NGF-treated cells, there is an increase in the rate of formation of the 73,000-Mr form since here, by a 10-min chase (following a 5-min pulse), the 77,000- and 73,000-Mr forms are present in near equal amounts, while in untreated cells, they are 60-90 min to reach an equal ratio of the subunit forms (Sabban et al., 1983).

Alternatively, the treated cells may possess a larger proportion of dopamine \(\beta\)-hydroxylase-containing storage vesicles containing a processing enzyme. It has been shown that NGF induces the appearance of small 40–60 nm synaptic-like vesicles in PC12 cells (Greene and Tischler, 1976; Tischler and Greene, 1978; Luckenbill-Edds et al., 1979).

We have presented evidence in the accompanying paper (Sabban et al., 1983) that the different forms of dopamine \(\beta\)-hydroxylase may have different specific activities. Hence, the various treatments tested here (NGF, corticosteroids, cAMP analogue), by virtue of their actions on the multiple forms of dopamine \(\beta\)-hydroxylase, have the potential to bring about long term regulation of the activity of this enzyme. It will be of interest to ascertain whether such regulation of forms and of activity occurs in normal dopamine \(\beta\)-hydroxylase-containing cells.

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