# NERVE GROWTH FACTOR-MEDIATED SELECTIVE INDUCTION OF ORNITHINE DECARBOXYLASE IN RAT PHEOCHROMOCYTOMA; A CYCLIC AMP-INDEPENDENT PROCESS

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## 1. Introduction

Nerve growth factor (NGF) is a protein indispensible for the normal development, survival and maintenance of function of peripheral sympathetic neurons throughout life, and of spinal sensory neurons during a brief period of their development [1,2]. Beyond the general promotion of growth and the stimulation of fiber outgrowth from sympathetic neurons [1], the selective induction of tyrosine hydroxylase (TH) and dopamine $\beta$-hydroxylase (DBH) is one of the most characteristic biochemical effects of NGF on adrenergic neurons and adrenal chromaffin cells [3,4]. Recently, a clonal cell line (PC12) was established from a transplantable rat adrenal pheochromocytoma [5]. This cell line shows a high degree of differentiation, involving synthesis, storage, release and uptake of the adrenergic transmitter [5,6], reflecting functional properties of adrenergic neurons. Moreover, as a further characteristic property of adrenergic neurons, PC12 cells respond with fiber outgrowth to NGF [5], although they lack a selective induction of TH, another characteristic response to NGF [7]. So far, neuronal fiber outgrowth is the only common response of all target cells to NGF, whereas a corresponding common biochemical response has not yet been described. Since PC12 cells respond to NGF with fiber outgrowth but not with selective TH induction, they seemed to be suitable for study of the biochemical responses to physiological levels of NGF which are common to all target cells of NGF.

We report that NGF produces a specific increase in the activity of ornithine decarboxylase (EC 4.1.1.17, L-ornithine carboxy-lyase) (ODC), the rate-limiting enzyme in polyamine biosynthesis [8]. This increase of ODC can be abolished by cycloheximide indicating that the rise in ODC activity is protein synthesis-dependent. In addition, we report that NGF-mediated increase in ODC activity is a cyclic AMP independent process.

## 2. Materials and methods

The PC12 clonal line was adapted to culture in 6 cm Falcon plastic tissue culture dishes, and always subcultured to give at a constant cell density ($10^6$ cells per dish) using Dulbecco modified Eagle's Medium containing 10% (v/v) heat inactivated horse serum, 5% (v/v) fetal calf serum, 50 U/ml penicillin and 50 $\mu$g/ml streptomycin, all supplied by Gibco.

2.5 S NGF was prepared by the method of Bocchini and Angeletti [9]. ODC activity was determined by measuring the release of $^{14}CO_2$ from L-[1-$^{14}$C]ornithine according to the method of Russel and Snyder [10]. Cell extracts were prepared by disrupting the PC12 cells by freezing and thawing in 0.5 ml of 50 mM sodium-potassium phosphate buffer, pH 7.2, containing 5 mM dithiothreitol and 0.2 mM pyridoxal 5'-phosphate. Extracts were then centrifuged at 15 000 X $g$ for 20 min and ODC activity was measured in the supernatants. The enzyme reaction was performed in 10 ml centrifuge tubes equipped with a rubber-polyethylene center well containing 0.2 ml of 1 M hyamine hydroxide. 0.1 ml of an

aliquot of the supernatant fluid was incubated for 90 min at 37°C after addition of 10 μl of L-[1-$^{14}$C] ornithine solution (55 nmol, 250 μCi). The enzyme reaction was terminated by addition of 0.5 ml of 40% tricholoroacetic acid. The enzyme activity proved to be linear both with respect to time of incubation and protein concentration. Cyclic AMP concentrations were determined by radioimmunoassay according to the method of Cailla et al. [11] which is based on the high affinity of the 2′-*O*-succinyl derivative of cAMP to the corresponding antibody. Proteins were determined by the method of Lowry et al. [12].

## 3. Results and discussion

As shown in table 1 NGF produced a marked increase in ODC activity in PC12 cells. Maximal induction was elicited with $10^{-8}$ M NGF, although $10^{-10}$ M NGF already induced a 5-fold increase in enzyme activity.

Figure 1 shows the time course of the increase in ODC activity after addition of $10^{-8}$ M NGF to the culture medium. A first significant ($P < 0.01$) increase was consistently observed at 2 h. A peak of activity, 40- to 50-fold that of control, was observed at 4 h. In spite of the presence of NGF, peak ODC activity was very short-lasting, and declined during the next 3 h. However, basal enzyme levels were not reached again. The enzyme activity remained elevated (about 10-fold) up to 25 h (fig.1) until NGF was removed.

Induction of ODC in PC12 cells by NGF was found

Table 1
Effect of NGF and/or cyclic nucleotides on ODC activity in cultured PC 12 cells

| Additions to media | Concentration (M) | ODC activity (nmol/h/mg protein) |
|---|---|---|
| None | | 0.127 ± 0.032 |
| NGF | $10^{-10}$ | 0.678 ± 0.064 |
| NGF | $10^{-9}$ | 2.46 ± 0.14 |
| NGF | $10^{-8}$ | 4.04 ± 0.12 |
| NGF | $10^{-7}$ | 3.64 ± 0.25 |
| $Bu_2$cAMP | $10^{-5}$ | 0.677 ± 0.003 |
| $Bu_2$cAMP | $10^{-4}$ | 3.41 ± 0.28 |
| $Bu_2$cAMP | $10^{-3}$ | 8.36 ± 0.82 |
| $Bu_2$cAMP | $10^{-2}$ | 9.07 ± 0.24 |
| $Bu_2$GMP | $10^{-3}$ | 0.079 ± 0.008 |
| $Bu_2$cAMP | $10^{-3}$ | |
| + NGF | $10^{-8}$ | 29.54 ± 0.62 |
| $Bu_2$cGMP | $10^{-3}$ | |
| + NGF | $10^{-8}$ | 4.77 ± 0.37 |

PC12 cells were plated at a density of $10^6$ cells per 6 cm culture dish. Various compounds at the concentration indicated were added to the culture medium. ODC activity was determined after 4 h. Values represent the means of 3 determinations ± SEM

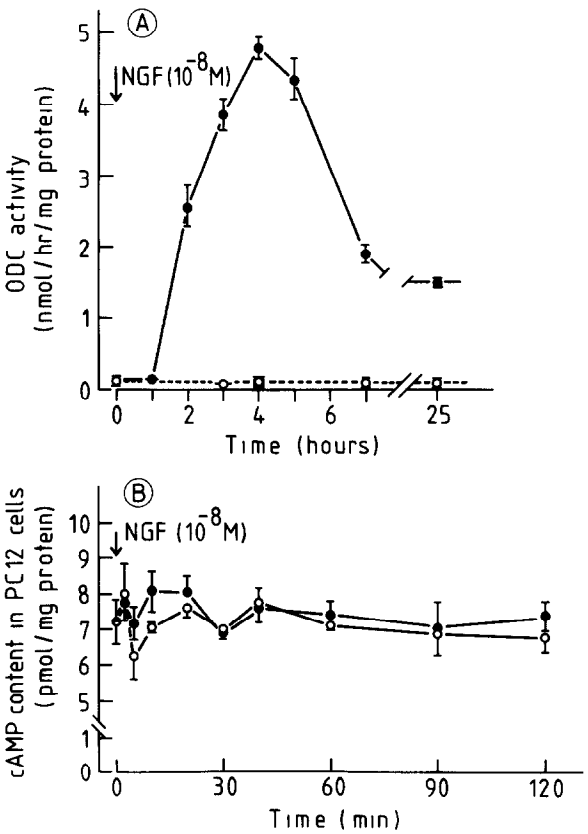


Fig.1. Effect of NGF on ODC activity and cAMP levels in PC12 cells. Cells were subcultured into 6 cm tissue culture dishes containing Dulbecco modified Eagle's Medium supplemented with 10% horse serum and 5% fetal calf serum. (A) (○– – – –○), control, no addition; (●———●) $10^{-8}$ M NGF; (□), 6 μg/ml cycloheximide + $10^{-8}$ M NGF. The cells were collected after indicated times and ODC activity was determined as described under section 2. (B) (○———○), control, supplemented with cytochrome *c* (0.3 μg/ml); (●———●), $10^{-8}$ M NGF. At indicated times cells were collected and cAMP content was determined according to [11]. Values given are the means of 6 determinations ± SEM.

Table 2
Specificity of NGF for induction of ODC activity in PC12 cells

| Addition to media | ODC activity (pmol/h/mg protein) |
|---|---|
| None | 105 ± 10 |
| NGF (0.3 μg/ml) | 5833 ± 457 |
| Insulin (0.3 μg/ml) | 84 ± 44 |
| Epidermal growth factor (0.3 μg/ml) | 199 ± 15 |
| Cytochrome *c* (0.3 μg/ml) | 84 ± 14 |

Exponentially growing PC12 cells ($10^6$ cells/dish) were incubated in the presence of various proteins at the concentrations indicated. After a total incubation period of 4 h cells were harvested and assayed for specific ODC activity. Results are the means of 2 experiments

to be abolished by cycloheximide (6 μg/ml) (fig.1) indicating that the NGF-mediated enzyme increase requires intact protein synthesis.

In order to determine whether NGF-mediated increase in ODC activity is specific, we examined the effects of a number of proteins on ODC induction in PC12 cells (table 2). Addition of insulin (insulin shares structural similarities with NGF [13]) or cytochrome *c* (a protein with very similar physicochemical properties to NGF [14]) or epidermal growth factor (a protein which, as NGF, is synthesized in the tubular cells of male mouse submaxillary gland [15]), caused no or only a small (epidermal growth factor +90%) increase in enzyme activity in PC12 cells. Only NGF elicited a marked increase in ODC activity. The specificity of the NGF-mediated induction is further supported by the finding that addition of monospecific antibodies to NGF (molar ratio 33 to 1) to the medium abolished subsequent ODC induction by NGF.

The mechanism of ODC induction in PC12 cells by NGF is not clear. Under various experimental conditions rapid and transient increases in cAMP levels have been reported to precede the induction of ODC in several organs in vivo [16] and cell lines in vitro [17,18] suggesting that cAMP may initiate ODC induction. The fact that $N^6$,$O^{2'}$-dibutyryl cyclic AMP ($Bu_2$ cAMP) but not $N^2$,$O^{2'}$-dibutyryl cylic GMP caused a marked ODC increase (table 1) could be taken as evidence for such an assumption. However, addition of $10^{-8}$ M NGF to the culture medium did not produce significant cAMP changes up to 2 h after addition of NGF (fig.1B) when ODC levels were already significantly elevated (fig.1A). In order to exclude the possibility that rapid small cAMP changes evoked by NGF escaped detection due to rapid phosphodiesterase digestion, the effect of NGF on cAMP levels was studied in the presence of the phosphodiesterase inhibitor theophylline. Even with theophylline present, NGF did not produce a significant increase in cAMP. The independence of NGF mediated ODC induction from cAMP is further supported by the observation that NGF and $Bu_2$ cAMP mediated ODC induction are more than additive. Maximal induction is elicited by NGF at a concentration of $10^{-8}$ M (table 1) and by $Bu_2$ cAMP at $10^{-3}$ M. At these concentrations NGF produced a 32-fold, $Bu_2$ cAMP a 66-fold increase in ODC. The combination of $10^{-8}$ M NGF and $10^{-3}$ M $Bu_2$ cAMP resulted in a 233-fold increase in ODC activity.

The present results show that a specific and early effect of NGF on PC12 cells is a marked induction of ODC. The increase in ODC is dependent upon protein synthesis, and is not mediated by initial cAMP changes. That ODC induction might represent a general characteristic response of other target cells to NGF is supported by the observations that NGF-induced increase of ODC activity have been observed in dorsal root and rat sympathetic ganglia [19] and in rat brain [20].

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