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NERVE GROWTH FACTOR RESCUES CLONAL PC-12 PHEOCHROMOCYTOMA CELLS FROM "INOSITOL-LESS DEATH"

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Summary: A clonal line of rat pheochromocytoma (PC-12) exhibits an absolute requirement for myo-inositol for survival and multiplication. The myo-inositol requirement for cell viability appears to be related to cell density. Cell death due to myo-inositol deficiency could be completely prevented by the renin-free β-subunit of mouse salivary gland nerve growth factor (β-NGF). Neither insulin nor cytochrome C could rescue the PC-12 cells from "inositol-less death". The myo-inositol dependency of PC-12 cell line is highly specific in that neither scyllo-inositol nor myo-inositol 1,2, cyclic phosphate could substitute for myo-inositol in support of cell survival and growth.

INTRODUCTION

Sympathetic and sensory neurons require nerve growth factor (NGF) for their survival both *in vivo* and *in vitro* (1,2). These neurons also depend upon NGF for their differentiation as well as maintenance of their differentiated functions (3). In contrast, the PC-12 clonal rat pheochromocytoma line, which contains NGF-receptors and responds to NGF similar to sensory and sympathetic neurons (4), does not require NGF for survival in a serum-enriched medium (5). PC-12 cells, however, do exhibit NGF dependency in serum-free media (6). The morphological, physiological and biochemical changes evoked by NGF in PC-12 cells are reversible when the growth medium is replaced by an NGF-free medium (4). Both cell-surface receptors and nuclear NGF-receptors are reported to be present in the responsive cells (For details see 3 and 7). Currently it is believed that the multiple NGF-effects of responsive cells are mediated via NGF binding to cell surface receptors and stimulation of post-receptor mechanisms involving second messenger systems (8). Neither the nature of receptor-molecules nor the biochemical mechanisms that

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Abbreviations: MAP=mean arterial pressure; IDM=myo-inositol deficient medium; ISM=myo-inositol supplemented medium.

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underlie the NGF actions have been well defined. Moreover, it is not clear whether survival and differentiation are independent or integrated receptor mediated NGF-responses.

In the present paper we studied the survival actions of NGF in PC-12 cells. PC-12 cells that had not been previously exposed to NGF (naive) were examined in an attempt to delineate the primary mechanism(s) of the NGF effect on PC-12 cell survival. Myo-inositol deficient, serum-enriched medium proved to be inadequate for survival and viability of these clonal cells, and myo-inositol was found to be an absolute requirement for maintenance and growth. Deprivation of myo-inositol results in death of PC-12 cells, and this "inositol-less death" could be prevented by supplementing medium with appropriate amounts of myo-inositol. Interestingly, PC-12 cells could be rescued from "inositol-less death" by exogenous β-NGF. The experimental system described in the present study may be a fruitful model for elucidating the mechanism(s) of the survival action of NGF as well as other uncharacterized neuronal survival factors.

MATERIALS AND METHODS

Isolation of β-NGF and assessment of its purity: β-NGF was isolated from mouse submaxillary gland by the method of Mobley et al (9). The final preparation of β-NGF was lyophilized and stored at -20°C. The purity of this preparation was checked by SDS-polyacrylamide gel electrophoresis. The NGF preparation also was tested for renin contamination (10) by measuring mean arterial blood pressure in the unanesthetized rabbit. For this study an adult rabbit (2.5 kg) was anesthetized with a mixture of Vetalar (Park-Davis, 35 mg/kg) and Rompun (Haver-Lockart, 5 mg/kg) intramuscularly. The right carotid artery and jugular vein were catheterized with 5 French polyvinyl tubing; this was tunnelled subcutaneously, exteriorized, anchored at the nape of the neck, and suitably capped. The animal was allowed to recover for 24 hours prior to investigation.

Blood pressures were recorded continuously from the carotid artery on a Beckman dynograph (Model R611) six-channel recorder via a Statham P23 Ia transducer. Baseline mean arterial pressure (MAP) was recorded for one minute prior to injection of test solutions and for one minute immediately following the injections. After determining base line MAP graded doses of angiotensin II amide (CIBA), dissolved in 5% dextrose/water (in 2 ml volume), were injected through the catheterized jugular vein and the MAP was recorded. After standardizing the MAP response to angiotensin, two injections of 2ml 5% dextrose/water were repeated at intervals of 20 min in order to rinse the catheter of angiotensin II. Purified NGF dissolved in 5% dextrose (in 2 ml) was then injected. MAP was recorded at 1, 10, 20, 30 and 40 minutes after the injection. After this time, administration of the angiotensin test solution was repeated to confirm the normal response.

Cell Cultivation: PC-12 cells were obtained from Dr. H.R. Herschman. Cells were cultured in Dulbecco s modified Eagle s medium (DMEM) supplemented with 10% heat inactivated horse serum, 5% newborn fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml fungizone (referred to as basal growth medium). Culture media components were purchased from KC Biological Inc., Lenexa, Kansas, USA. Incubation was carried out in a humidified atmosphere of 5% CO_2 in air. The medium was replaced every three days. The cells were subcultured by shake dispersion upon reaching 80% confluency.

Cell culture studies with respect to myo-inositol requirement: Twenty-four hours prior to myo-inositol requirement studies the cells were washed and subcultured in the basal growth medium to maintain the cells in a logarithmic phase of growth. The following day the cells were harvested and washed three times with DMEM containing no myo-inositol. These cells were resuspended (10^4-10^5 cells/ml) in myo-inositol deficient growth medium (IDM). This medium was prepared by using myo-inositol lacking DMEM supplemented with 10% dialyzed heat inactivated horse serum and 5% dialyzed fetal calf serum. Both antibiotics and fungizone were maintained at a concentration similar to that in the basal growth medium. In culture studies involving longer periods of time, the cells were pelleted at the end of a 72 hour incubation period, the excess cells were removed, and the required number of cells were resuspended in fresh IDM. The cells were maintained at the initial cell density. In constructing the cell growth curve, appropriate corrections were made for this dilution.

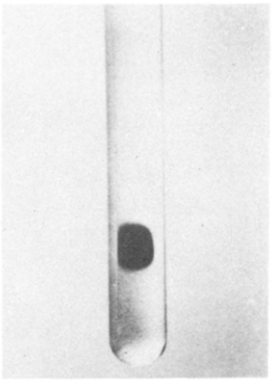
In all experiments the cells were cultured in plastic 60mm tissue culture dishes. Horse serum and fetal calf serum used in the present study were obtained from Biocell Laboratories. Both sera were dialyzed against 0.68% solution of NaCl at 4°C. The cell counts were made using a hemocytometer and cell viability was confirmed by nigrosine dye exclusion test. Dialysis was performed using tubing with molecular weight cut off of 8000 dalton. Scyllo-inositol was purchased from Calbiochem-Behring Corp, CA. Myo-inositol 1, 2, cyclic phosphate was generously provided by Dr. H. Diringer. The absolute purity of these two cyclitols was not tested and possible myo-inositol contamination cannot be ruled out. Bovine insulin and cytochrome C were purchased from Sigma.

RESULTS

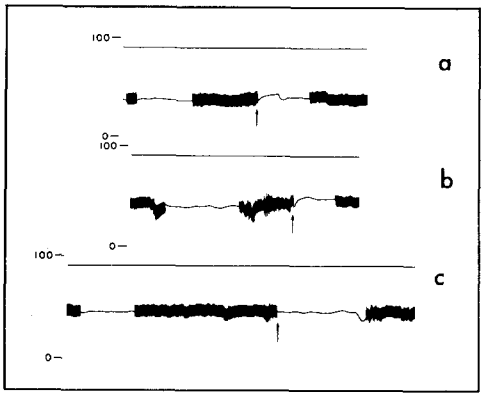
Fig. 1-A shows a 7.5% polyacrylamide SDS-urea gel electrophoresis analysis of purified β-NGF; the gel is stained with Coomassie Blue R-250. Fig. 1-B shows the results of one rabbit renin bioassay of this β-NGF preparation. The rabbit showed an immediate rise in blood pressure in response to infusion of 50 or 100 ngs of angiotensin II. Under the same conditions, however, no response was observed at any time when 3 mg of the purified β-NGF preparation was infused through the jugular vein. These results confirm a lack of significant renin-angiotensin contamination of the β-NGF preparation. The growth response of PC-12 cells was studied in IDM in the absence and presence of known concentrations of β-NGF and/or myo-inositol.

Fig. 1-C depicts the results of growth response studies of PC-12 cells cultured over a period of 120 hrs in IDM, IDM supplemented with NGF (50 ng/ml) [IDM + NGF], myo-inositol supplemented medium (7.5 μg myoinisitol/ml) [ISM]; or myo-inositol supplemented medium containing β-NGF (50 ng/ml) [ISM + NGF]. The cells continued to grow under each of these four conditions for at least the first 24 hrs of the culture period. The cell counts indicated relative growth promoting efficiency as follows ISM >ISM + NGF >IDM + NGF >IDM. None of these media supported a logarithmic multiplication phase suggesting a limitation of some low molecular component in the serum. The cells continued to multiply under ISM and ISM + NGF conditions and the growth responses were comparatively better than in IDM or IDM + NGF conditions. Multiplication

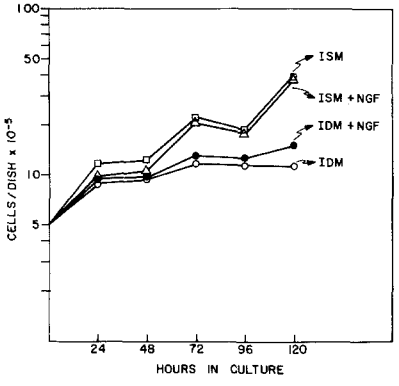
1A. STAINED GEL



1B. MEASUREMENT OF MEAN ARTERIAL BLOOD PRESSURE



1C. GROWTH RESPONSE OF PC-12 CELLS TO myo-INOSITOL ± NGF



1D. VIABILITY OF PC-12 CELLS

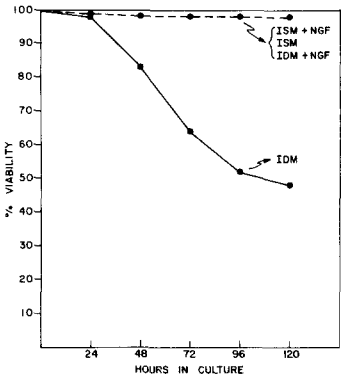


Figure 1A: SDS-Urea-polyacrylamide gel electrophoresis of β-NGF (170 μg) stained with Coomassie Blue R-250.

Figure 1B: Measurement of mean arterial blood pressure in an unanaesthetized rabbit in response to Angiotensin II 50 ng (a) and 100 ng (b) or β-NGF 3 mg (c).

Figure 1C: Growth response of PC-12 cells in the absence or presence of myoinositol (7.5 μg/ml) with or without β-NGF (50 ng/ml). PC-12 cells (1 x 10^5 cells/ml; 5 ml/dish) were cultured in IDM, IDM + NGF, ISM and ISM + NGF for 120 hrs as described under material and methods. Cell counts were made every 24 hr. At 72 hr the cells were pelleted, washed and resuspended in fresh media. Cultures were maintained in respective media with initial cell density after removing the excess cells. The values given in the graph have been corrected for this dilution. Cell counts are average of determinations on triplicate cultures.

Figure 1D: Viability of PC-12 cells under four different conditions as in 1C.

of the cells appears to have ceased under IDM conditions as indicated by the plateau in cell number after a 72 hr culture period.

Examination of cell viability during every 24 hr culture period also revealed significant changes (Fig. 1D). Cell viability exceeded 98% at the end of the first 24 hr culture period under all four culture conditions. Cell viability progressively declined in IDM but was maintained at a steady state in the IDM + NGF, ISM and ISM + NGF media. These results indicate that myo-inositol is an absolute requirement for growth and

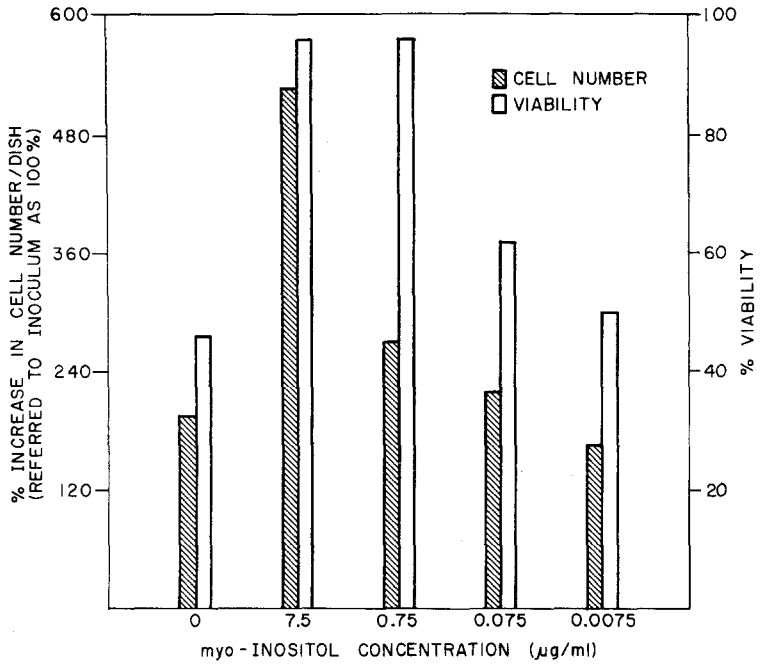


Figure 2: Growth and viability response of PC-12 cells to different concentrations of exogenous myo-inositol in the medium. PC-cells (1 x 10^5 cells/ml, 5 ml/dish) were maintained for a period of 72 hr in the absence or presence of varying amounts of myo-inositol in the medium. Each bar represents the average of determinations on triplicate cultures.

viability of this PC-12 cell line. NGF could substitute for myo-inositol and maintain viability in IDM.

The growth and viability responses of PC-12 cells as a function of the concentration of myo-inositol in the medium were then studied separately. The results obtained with varying concentrations of myo-inositol for a period of 72 hrs using an initial cell density of 1 x 10^5 cell per ml, are shown in Fig. 2. The maximal effective concentration of myo-inositol for cell viability was approximately 0.75 μg/ml. The cell number increased about 530% at 7.5 μg myoinositol/ml while a significantly reduced (270%) increment in cell number was observed at 0.75 μg myo-inositol per ml. Concentrations of myo-inositol lower than 0.75 μg/ml did not sustain cell viability above the basal level.

The effect of myo-inositol deficiency on growth and viability was also tested as a function of cell density and the results are given in Table 1. The absolute requirement of myoinositol for growth and survival appears to change with size of population. The differential responses observed at high and low-cell densities either in the absence or presence of myoinositol indicate that cell-cell contact is a physiologically important

TABLE 1. DETERMINATION OF PC12 CELL VIABILITY AS A FUNCTION OF CELL DENSITY

INITIAL CELL DENSITY	LOW DENSITY: 5×10^3/dish		HIGH DENSITY: 5×10^7/dish	
TREATMENT	cell number	viability	cell number	viability
Inositol deficient medium	5×10^4	22%	6.8×10^7	62%
Inositol supplemented medium	1.45×10^5	97%	8.45×10^7	78%

PC12 cells were cultured at initial cell density ranging with very sparse (1×10^3 cells/ml; 5ml/dish to very crowded (1×10^7 cells/ml; 5 ml/dish) in the absence or presence of myo-inositol (0.75 μg/ml) in myo-inositol deficient medium. Cell number and viability were determined at the end of the 74hr period. Each value represents the averages of determinations of triplicate cultures.

regulatory phenomena involved in myo-inositol dependent cell survival and growth responses.

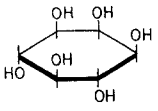
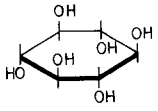
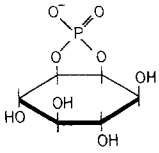
The specificity of the myo-inositol dependency was studied by substituting with scyllo-inositol or myo-inositol 1, 2, cyclic phosphate. Scyllo-inositol, a stereoisomer of inositol, is a component of nervous tissue (11). Myo-inositol 1,2, cyclic phosphate is a product formed during enzymatic hydrolysis of phosphatidylinositol (12). In these studies myo-inositol in a concentration of 0.75 μg/ml sustained a 2.5 fold increase in cell number (Table 2). Neither scyllo-inositol nor myoinositol 1, 2, cyclic phosphate had a demonstrable growth promoting effect when tested as a substitute for myo-inositol. Though these two cyclitols did not support cell viability as efficiently as myo-inositol, cell viability was maintained at a higher level in the presence of myo-inositol 1,2, cyclic phosphate than scyllo-inositol.

The specificity of NGF in sustaining cell viability under myo-inositol deficient conditions was tested by comparing the effects of insulin and cytochrome C. Neither insulin which has some structural similarity to NGF (13), nor cytochrome C which is similar in size and isoelectric point could support viability (data not shown). These results suggest that the action of NGF in preventing the cells from "inositol-less" death is mediated via NGF-receptor linked function(s).

DISCUSSION

The present study establishes that myo-inositol is an essential growth factor for the survival and multiplication of PC-12 cells. The need for exogenous myo-inositol becomes

TABLE 2: EFFECTS OF MYO-INOSITOL, SCYLLO-INOSITOL AND MYO-INOSITOL 1,2 CYCLIC PHOSPHATE ON PC12 CELL SURVIVAL

CYCLITOL TESTED CONCENTRATION	NONE --	MYO-INOSITOL 0.75 μg/ml	SCYLLO-INOSITOL 0.75 μg/ml	MYO-INOSTIOL 1,2 CYCLIC PHOSPHATE 0.75 μg/ml
STRUCTURE				
INITIAL CELL NUMBER per dish (5 ml)	5×10^5	5×10^5	5×10^5	5×10^5
CELL NUMBER AFTER 72 HRS CULTURE	7.8×10^5	1.25×10^6	7.46×10^5	6.95×10^5
VIABILITY	51%	98%	72%	81%

PC12 cells were grown (1×10^5 cells/ml; 5 ml/dish) in myo-inositol deficient medium supplemented with myo-inositol, scyllo-inositol or myo-inositol 1,2 cyclic phosphate. After 72 hrs, the cell counts and viability were determined on triplicate dishes.

apparent only when the serum used in the medium has been efficiently dialyzed. At the cellular level myo-inositol is believed to function as an essential "vitamin" as well as a requisite "metabolite" for the synthesis of inositol phosphatides. It will require further study to determine whether the loss of cell viability is due to impairment of either the "vitamin" or "metabolite" functions of myo-inositol.

PC-12 cells appear to survive and multiply during the first 24 hours of culture in the absence of myo-inositol; thereafter both cell growth and viability appear to be greatly reduced. The increase in cell number observed in the presence of NGF under myo-inositol deficient conditions suggests that NGF not only rescues PC-12 cells from "inositol-less death" but also promotes cell multiplication. The present study also reveals that both myo-inositol and NGF have important role(s) in the survival mechanism(s) of PC-12 cells.

Cell viability and survival often are attributed to the functional activities of "primary genes" (also referred to as "house-keeping genes") (14,15). These genes are believed to remain largely unregulated. However, the loss of cell viability in the absence of myo-inositol or NGF indicates that these two growth factors may function as primary regulators of "house-keeping genes" in PC-12 cells. Whether such regulatory

control is exerted at the level of the cell surface or via intracellular sites remains to be determined. The precise mechanism(s) by which NGF rescues PC-12 cells from "inositol-less death" is a fascinating question currently under investigation.

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