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# NERVE GROWTH FACTOR ENHANCES THE LEVEL OF THE PROTEIN KINASE C SUBSTRATE B-50 IN PHEOCHROMOCYTOMA PC12 CELLS[1]

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Exposure of PC12 cells to nerve growth factor results in arrest of cell growth and induction of differentiation to sympathetic neuron-like cells, bearing neurites. In this study we identify a 48 kDa PC12 phosphoprotein as the neuron-specific protein kinase C substrate B-50 ($M_r$ 48 kDa; IEP 4.5) on basis of comigration with purified B-50, immunoreactivity and phosphopeptide mapping. B-50 is present in both undifferentiated and differentiated PC12 cells. Exposure of PC12 cells to nerve growth factor for two days results in a 2.5-fold increase in the amount of B-50 as measured by RIA. Indirect immunofluorescence microscopy reveals that B-50 is mainly localized at the cell membrane and in growth cones. Our data are in line with the hypothesis that B-50 plays a role in neurite outgrowth and indicate that PC12 cells provide a suitable model to study this hypothesis. © 1986 Academic Press, Inc.

One of the major endogenous substrates of protein kinase C in synaptosomal plasma membranes (SPM) is the protein B-50 ($M_r$ 48 kDa, IEP 4.5) (1-3). In adult rat brain this neuron-specific phosphoprotein is predominantly localized in presynaptic terminals (4,5). Neo-natal rat brain membranes are rich in endogenous B-50 phosphorylation (6,7). Moreover, B-50 is present in nerve growth cones isolated from fetal rat brain (8,9), in outgrowing hippocampal neurites (10) and in axonal sprouts of the regenerating sciatic nerve (11). These observations indicate that B-50 might be involved in neurite outgrowth, a suggestion that is supported by recent evidence from several laboratories

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Abbreviations: NGF, Nerve growth factor; RIA, Radio immuno assay; SAP, Staphylococcus Aureus protease V8; SDS-PAGE, Sodium dodecyl sulphate poly acrylamide gel electrophoresis.

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indicating that B-50 is very similar to a growth-associated protein called GAP 43 (12-14), GAP 48 (15), pp46 (16) or F1 (17,18).

One of the best studied models for examining the mechanism of neuronal differentiation is the rat pheochromocytoma cell line PC12 (19). Upon addition of nerve growth factor (NGF), PC12 cells acquire a phenotype resembling sympathetic neurons, including neurite outgrowth and cessation of growth (20). In this paper we demonstrate the presence of the protein kinase C substrate B-50 in PC12 cells and present for the first time evidence that NGF-induced neurite outgrowth is paralleled by increased levels of B-50.

## MATERIALS AND METHODS

Cell culture: Rat pheochromocytoma PC12 cells were grown at 37°C in Dulbecco's modified Eagle's medium, containing 10% fetal calf serum (Flow Laboratories, Inc., Mc Lean, VA, USA) and 5% heat inactivated horse serum (Flow Laboratories) as described by Tischler and Greene (21). Cells were plated at a density of 60.000 cells/$cm^2$ and grown for 48 hours in the presence (NGF+) or absence (NGF-) of 100 ng/ml NGF (7 S; Collaborative Research Corp., Waltham, MA, USA). After removal of the culture medium, the cells were rinsed twice with phosphate buffered saline (PBS), dislodged by resuspension and collected by centrifugation. Cell pellets were homogenized in 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM Tris/HCl pH 7.4 (buffer A) supplemented with 10% glycerol at 4°C (about 500.000 cells/ml).

Protein phosphorylation: Phosphorylation assays were performed in a reaction mixture in buffer A, containing 15 ug PC12 cell homogenate protein, 7.5 uM ATP, 2 uCi [$\gamma$-$^{32}$P]ATP (S.A. 3000 Ci/mmol, Amersham, UK) with or without 1 ug purified protein kinase C in a final incubation volume of 25 ul. The reaction was started after a 5 minute preincubation at 30°C by addition of ATP and stopped after 15 seconds with 12.5 ul denaturing solution (final concentration: Tris/HCl, 62.5 mM, pH 6.8; SDS, 2%; glycerol, 10%; bromophenol blue, 0.001%; 2-mercaptoethanol, 5%). Purified [$^{32}$P]-labeled B-50 tracer was prepared according to Oestreicher et al. (22), SPM from adult rat brain as described by Kristjansson et al. (23) and protein kinase C isolated by the method of Aloyo et al. (1). Protein was determined according to Lowry et al. (24), using bovine serum albumin (BSA) as a standard.

Immunoprecipitation: The phosphorylated samples were diluted with 263 ul buffer B (200 mM NaCl, 10 mM EDTA, 10 mM $NaH_2PO_4$ and 0.5% NP-40) and 100 ul anti B-50 antiserum (8502, diluted 1:50 in buffer B), resulting in a final SDS concentration of 0.2% and a total volume of 400 ul. The antiserum was allowed to bind overnight at 4°C. Formaldehyde inactivated Staphylococcus Aureus cell membranes (SAC; kindly provided by Dr. W.H. Jansen, RIVM, Bilthoven, The Netherlands) were washed with buffer B. 50 ul of a 4% SAC-suspension was added to bind the immunoglobulins and incubated for 30 minutes at room temperature. After 10 minutes centrifugation at 4800 x in a Sorvall HS4 rotor, the SAC-pellet was resuspended in 30 ul diluted denaturing solution (1:2, v/v) and the samples were heated for 10 minutes at 100°C prior to 11% polyacrylamide electrophoresis (SDS-PAGE) according to Zwiers et al. (2).

Western blotting and immunostaining: 11% SDS-PAGE separated proteins were transferred to nitrocellulose sheets (25). Blots were rinsed with Tris buffered saline pH 7.4 containing 1% Tween-20 (TBS-T) and incubated for two hours at room temperature with affinity purified polyclonal rabbit anti-B-50 IgGs (antiserum 8103, dilution 1:2000) (26) or pre-immune IgGs in TBS-T containing 0.5% gelatin. Subsequently, blots were rinsed in TBS-T and incubated for one hour with horse radish peroxidase conjugated to swine-anti-

rabbit antibodies (Dako, Denmark), diluted 1:500 in TBS-T containing 0.5% gelatin. IgG binding was visualized using the staining method of Buckel and Zehelin (27).

Phosphopeptide mapping was performed with 2 ug Staphylococcus Aureus protease V8 (SAP; Miles lab., U.K.) per gel according to Cleveland et al. (28).

Radio immuno assay (RIA): Samples of total PC12 cell homogenates (NGF+ and NGF-) were analysed by B-50 RIA as described by Oestreicher et al. (21).

Immunofluorescence: For immunofluorescence, PC12 cells were grown on poly-L-lysine (50 ug/ml) coated culture dishes (Costar, Cambridge, MA) for 4 days with or without NGF. Fixation and immunoincubations were performed at room temperature. Cells were fixed for one hour in 2% paraformaldehyde/50 mM phosphate buffer, pH 7.4, containing 0.1 M lysine and 0.2% sodium perjodate (29). Fixed cells were rinsed 3 times for 10 minutes in PBS, containing 0.2% (v/v) Triton X-100 and 0.01% sodium azide (PBS-TX) and subsequently incubated for 24 hours with affinity purified anti-B-50 IgGs (antiserum 8420, dilution 1:25) in PBS, containing 0.2% BSA and 0.1% Triton X-100 (PBS-BT). After three washes with PBS-TX, cells were incubated with fluorescein-conjugated swine-anti-rabbit antibodies (Dako, Denmark; dilution 1:100) in PBS-BT for one hour. Excess antibody was removed by extensive rinsing in PBS-TX and cells were mounted in 0.1 M Tris/ HCl, pH 8.5, supplemented with 25% glycerol, 10% (w/v) Mowiol 4-88 (Hoechst AG, Frankfurt, FRG) and 0.1% (w/v) 1,4 di-aza-bi-cyclo (2,2,2) octane (Janssen, Beerse, Belgium). Immunofluorescence was examined with a Leitz Orthoplan microscope, equipped with a Ploem epi-illuminator.

## RESULTS

### Identification of B-50 in PC-12 cells

Endogenously phosphorylated PC12 homogenates contain a 48 kDa phosphoprotein band migrating to the same position as purified [$^{32}$P]-labelled B-50 on 11% SDS-PAGE (Fig. 1a). Immunostaining with anti-B-50 IgGs of Western blots of PC12 homogenates reveals a single band (Fig. 1b), coinciding with the 48 kDa

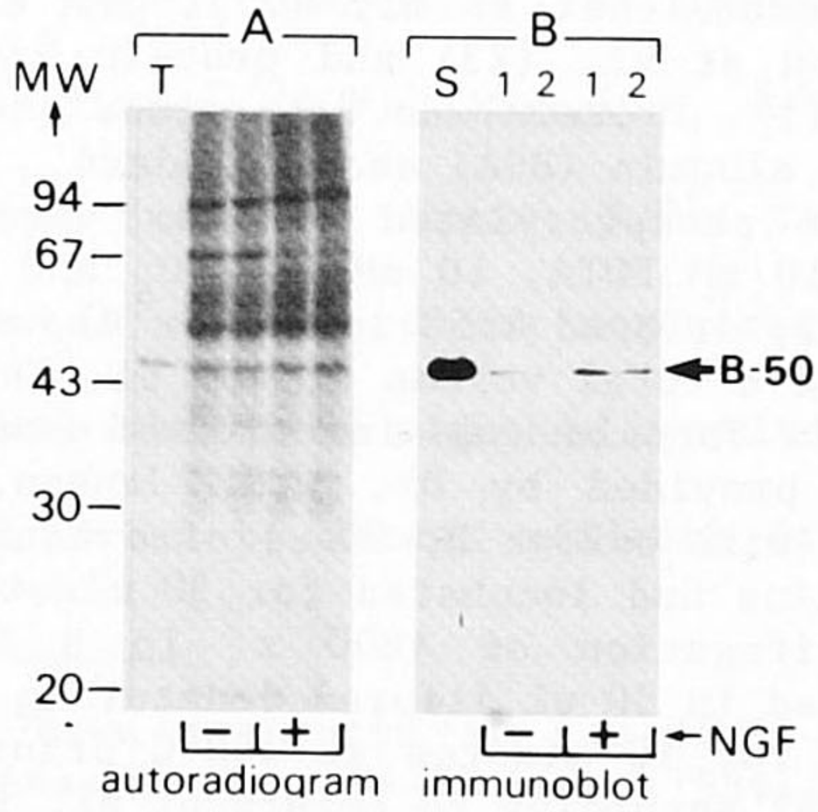


Fig. 1. The effect of NGF-exposure on endogenous B-50 phosphorylation and B-50 immunoreactivity in PC12 cell homogenates. Cells were grown for two days in the presence (+) or absence (-) of 100 ng/ml NGF. Endogenously phosphorylated homogenate proteins were separated on 11% SDS-PAGE and subjected to autoradiography (A) or to immunoblotting with anti-B-50 IgGs (B). MW: molecular weight markers; T: purified [$^{32}$P]-labelled B-50 tracer; S: 10 ug SPM protein; lanes 1: 20 ug protein; lanes 2: 10 ug protein.

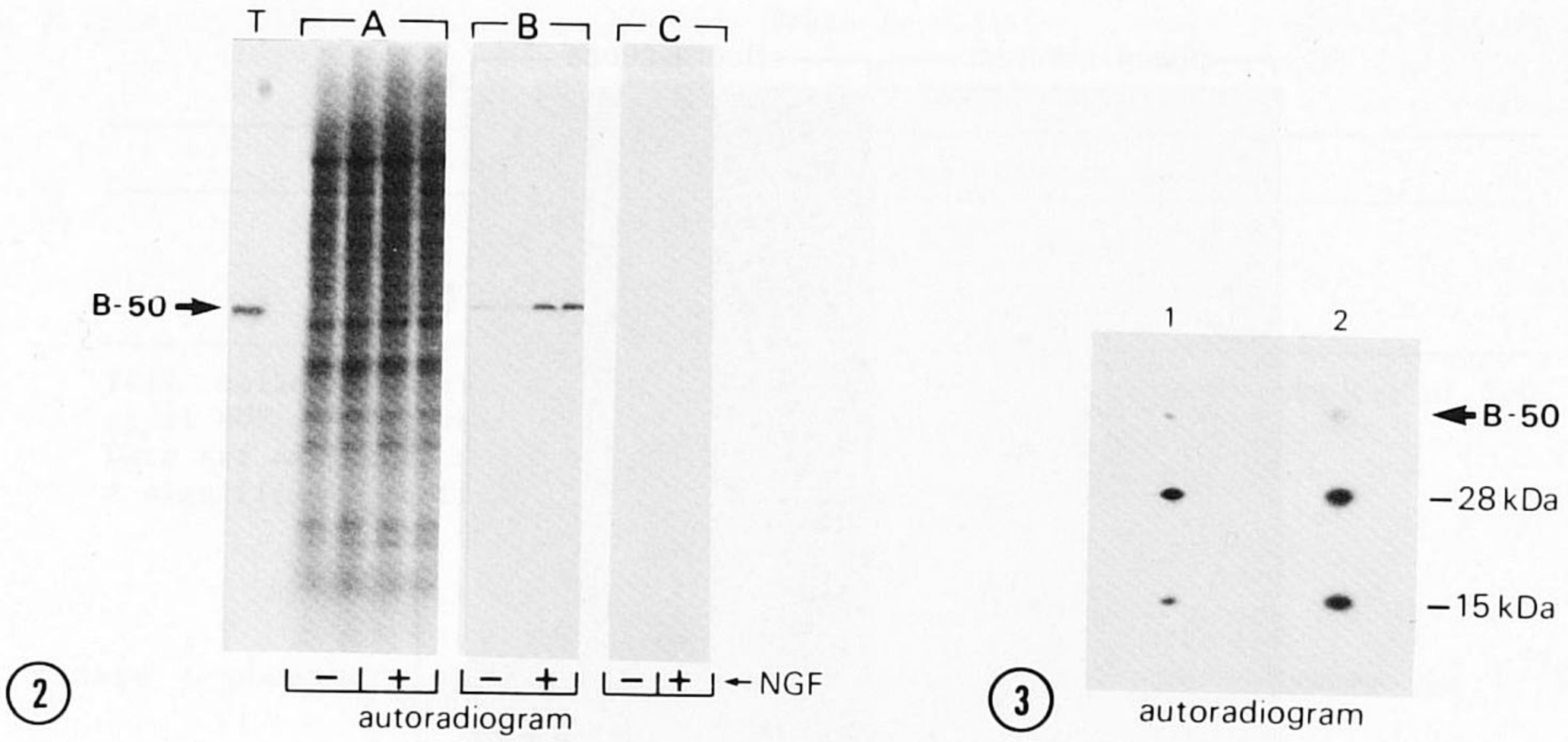


Fig. 2. Autoradiogram showing the effect of NGF-exposure on B-50 phosphorylation in immunoprecipitates from PC12 homogenates. Cells were grown for two days in the presence (+) or absence (-) of 100 ng/ml NGF. Fifteen ug homogenate protein was phosphorylated with added protein kinase C (A), prior to immunoprecipitation with anti-B-50 (B) or pre-immune (C) antiserum.
T: purified [$^{32}$P]-labelled B-50 tracer.

Fig. 3. Limited proteolysis with SAP of B-50 immunoprecipitates from [$^{32}$P]-labelled rat brain SPM (1) and PC12 cell homogenate (2). Equal amounts of SPM and PC12 immunoprecipitates (based on [$^{32}$P]-incorporation in B-50) are used.

phosphoprotein on the autoradiogram. Control incubations with pre-immune IgGs are negative (results not shown). Immunostaining of Western blots after 2 dimensional protein separation shows that this band contains only one cross-reacting protein with an IEP of 4.5, an IEP which is identical to that of purified B-50 (3) and of B-50 in SPM (23) (results not shown).

Immunoprecipitation of B-50-like proteins from protein kinase C phosphorylated PC12 homogenates (Fig. 2a), using rabbit anti-B-50 serum, reveals the presence of a single phosphoprotein, comigrating with purified [$^{32}$P]-labelled B-50 on 11% SDS-PAGE (Fig. 2b). No phosphoproteins are precipitated with pre-immune IgGs (Fig. 2c). Limited proteolysis of the immunoprecipitates with SAP produces two major phosphopeptide fragments, with apparent molecular weights of 28 kDa and 15 kDa, respectively (Fig. 3). This phosphopeptide map of the PC12 48 kDa phosphoprotein is identical to that obtained with immunoprecipitated B-50 from SPM (Fig. 3).

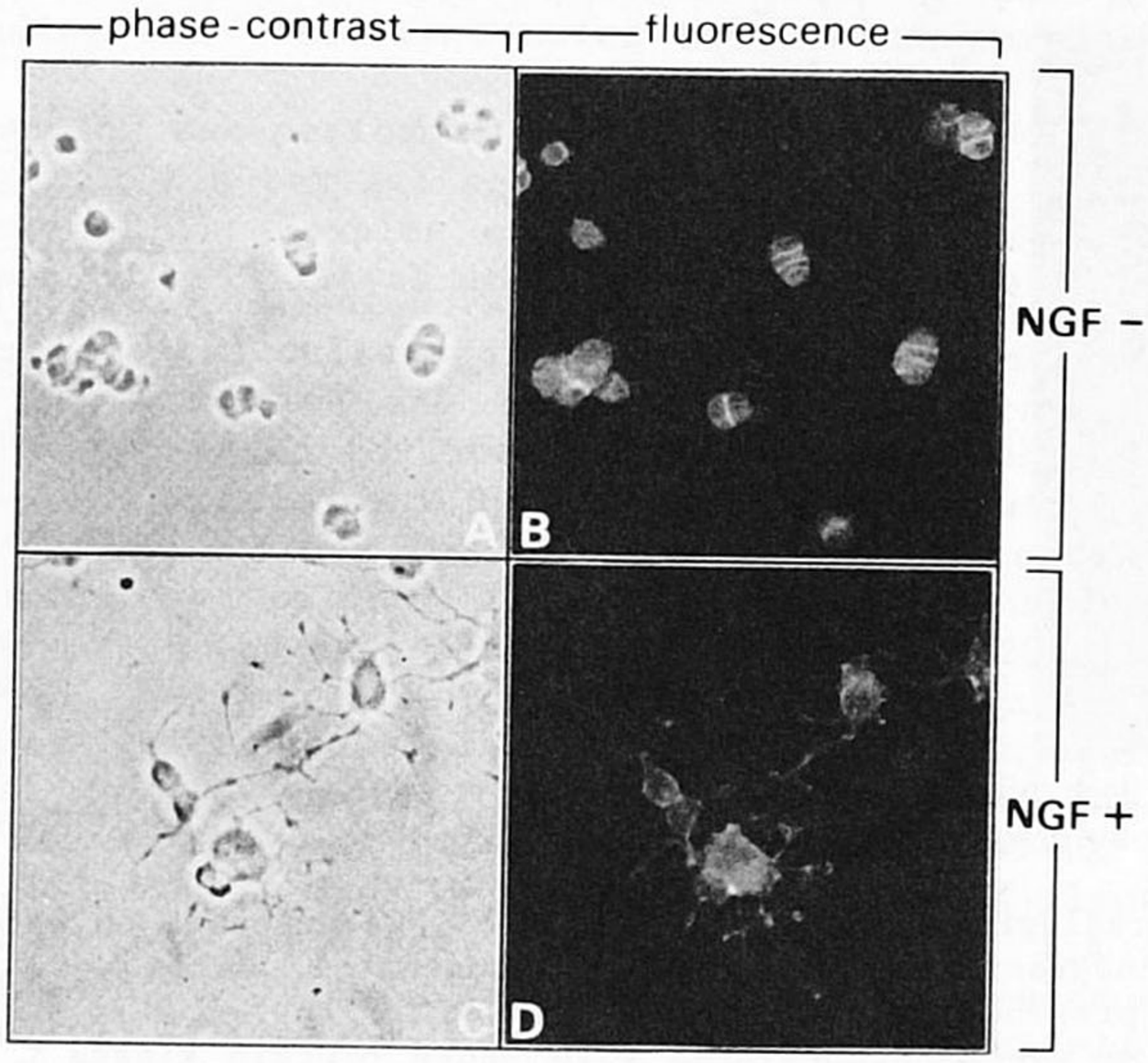


Fig. 4. Phase-contrast (a,c) and B-50 immunofluorescence (b,d) photomicrographs of untreated (a,b) and four days NGF-exposed (c,d) PC12 cells. Original magnification 250 x.

## Effect of NGF-treatment on B-50

Untreated PC12 cells are small and spherical cells (Fig. 4a). Exposure to NGF for 4 days induces the outgrowth of neurites with clearly visible growth cones at their tips (Fig. 4c). B-50 immunofluorescence is detectable in untreated (Fig. 4b) as well as in NGF-treated PC12 cells (Fig. 4d). In undifferentiated PC12 cells B-50 immunofluorescence is most pronounced at the cell membrane. NGF-treated cells show bright granular staining at the plasma membrane of the cell body and in the neurites, with brightest fluorescence in the growth cones. The cytoplasm of the cell bodies and the neurites stains only faintly. Secondary antibody controls do not show any fluorescence (results not shown).

To test the effect of NGF-treatment on B-50, PC12 cell homogenates are analysed for B-50 phosphorylation and B-50 levels. Endogeneous B-50 phosphorylation (Fig. 1a) and B-50 immunoreactivity (Fig. 1b) are higher in NGF-treated cells than in untreated controls. The NGF-induced increase in B-50 phosphorylation is also found when phosphorylation is performed with exogenous protein kinase C (Fig. 2a). Quantitative immunoprecipitation of B-50 from protein

Table 1

EFFECT OF NGF-EXPOSURE ON B-50 LEVELS IN PC12 CELLS

| Treatment | ng B-50/mg protein |
|---|---|
| NGF- | 35.8 ± 2.0 (n=4) |
| NGF+ | 89.6 ± 7.4 * (n=4) |

PC12 cells were grown for two days in the presence (+) or absence (-) of 100 ng/ml NGF. B-50 levels of cell homogenates were assayed by RIA.
Data are expressed as mean ± S.E.M.
* significantly different ($p < 0.001$; student T-test)

kinase C-phosphorylated homogenates reveals a two-fold increase in [$^{32}$P]-incorporation into B-50 after NGF-treatment (Fig. 2b). Measurements of B-50 in PC12 homogenates by RIA show that NGF increases the B-50 content 2.5-fold (Tabel 1).

## DISCUSSION

Rat pheochromocytoma PC12 cells contain a 48 kDa phosphoprotein that shares many characteristics with the neuron-specific, protein kinase C substrate B-50: 1) the PC12 protein comigrates with purified B-50; 2) its IEP (4.5) is identical to that of rat brain B-50 (3,23); 3) the protein cross-reacts with affinity purified anti-B-50 IgGs and selectively immunoprecipitates with anti-B-50 antiserum and 4) its phosphopeptide map is identical to that of purified rat brain B-50 (30). Based on these four criteria we conclude that the 48 kDa PC12 protein is identical to rat brain B-50.

B-50 is present in undifferentiated as well as in NGF-differentiated PC12 cells. B-50 immunofluorescence at the PC12 cell membrane is in line with its plasmamembrane localization in rat brain (3,5). The strong B-50 immunofluorescence in PC12 growth cones supports the notion that B-50 may be associated with neurite outgrowth (8,10,11). This notion is further substanciated by the fact that NGF-induced neurite outgrowth in PC12 cells is paralleled by an increase in B-50 levels (Table 1). The amount of B-50 in rat brain is highest during neuronal development (31) and in peripheral nerves during regeneration (11). As B-50 is very similar if not identical to GAP43, GAP48, pp46 or F1 (12-18 and unpublished comparative studies), available evidence

from several laboratories in a number of systems thus suggests that B-50 is associated with neurite outgrowth.

At present, no information is available about a function of B-50 in neurite outgrowth or about a possible involvement of B-50 in the mechanism of action of NGF. NGF has been shown to affect the degree of phosphorylation of a number of phosphoproteins in PC12 cells (32-36). Interestingly, NGF induces an increase in the phosphorylation of B-50 as measured in PC12 homogenates. It is not clear whether this increase in B-50 phosphorylation only reflects increased B-50 levels or also changes in the degree of B-50 phosphorylation. In adult rat brain B-50 phosphorylation has been implicated in a negative feed-back control of receptor-mediated hydrolysis of polyphosphoinositides (PPI), modulated by changes in the extent of B-50 phosphorylation elicited by protein kinase C (37-40). All elements involved in such a feed-back system appear to be present in PC12 cells. These include NGF- or carbachol-induced PPI-hydrolysis (41,42), protein kinase C activation (43) and B-50 phosphorylation. However, it remains to be shown whether such a feed-back system exists in PC12 cells and whether it plays a role in the molecular mechanism of NGF-induced neurite outgrowth. Even if the degree of B-50 phosphorylation is not of relevance to its fysiological function, this cell-system is a good model in which to study the role and expression of B-50 during NGF-induced neurite outgrowth.

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