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Initiation and maintenance of NGF-stimulated neurite outgrowth requires activation of a phosphoinositide 3-kinase

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SUMMARY

Application of nerve growth factor (NGF) to PC12 cells stimulates a programme of physiological changes leading to the development of a sympathetic neuron like phenotype, one aspect of which is the development of a neuronal morphology characterised by the outgrowth of neuritic processes. We have investigated the role of phosphoinositide 3-kinase in NGF-stimulated morphological differentiation through two approaches: firstly, preincubation with wortmannin, a reputedly specific inhibitor of phosphoinositide kinases, completely inhibited initial morphological responses to NGF, the formation of actin filament rich microspikes and subsequent neurite outgrowth. This correlated with wortmannin inhibition of NGF-stimulated phosphatidylinositol(3,4,5)trisphosphate (PtdInsP$_3$) and phosphatidylinositol(3,4)bisphosphate (PtdIns(3,4)P$_2$) production and with inhibition of NGF-stimulated phosphoinositide 3-kinase activity in anti-phosphotyrosine immunoprecipitates. Secondly, the overexpression of a mutant p85 regulatory subunit of the phosphoinositide 3-kinase, which cannot interact with the catalytic p110 subunit, also substantially inhibited the initiation of NGF-stimulated neurite outgrowth. In addition, we found that wortmannin caused a rapid collapse of more mature neurites formed following several days exposure of PC12 cells to NGF. These results indicate that NGF-stimulated neurite outgrowth requires the activity of a tyrosine kinase regulated PI3-kinase and suggest that the primary product of this enzyme, PtdInsP$_3$, is a necessary second messenger for the cytoskeletal and membrane reorganization events which occur during neuronal differentiation.

Key words: Neurite outgrowth, Nerve growth factor, PC12 cell, PI3-kinase, Wortmannin

INTRODUCTION

Neurotrophic factors promote the survival and differentiation of a variety of neurons in the central and peripheral nervous systems. Neurotrophic factors include NGF, brain derived neurotrophic factor (BDNF), neurotrophin 3/4 (NT3/4), fibroblast growth factor (FGF), and the interleukin-like factor ciliary neurotrophic factor (CNTF) (Glass and Yancopoulos, 1993; Thoenen, 1991). Studies with the phaeochromocytoma cell line, PC12, a well characterized neuronal model (Greene and Tischler, 1982), have provided much information about the molecular components of neurotrophic factor signal transduction. NGF application to PC12 cells induces morphological and biochemical changes resulting in differentiation to a sympathetic neuron like phenotype. In response to NGF, cells stop dividing, extend neurites, acquire action potentials, and express a variety of characteristically neuronal genes, including voltage gated sodium channels, neurotransmitter synthetic enzymes and components of the neuronal cytoskeleton (Halegoua et al., 1991). NGF-stimulated neurite outgrowth is characterized within minutes by the increased expression of filamentous actin containing microspikes, followed by the extension of neuritic processes with growth-cone like structures at their tips which, in the presence of growth factor, continues for several days (Connolly et al., 1979; Paves et al., 1988). Numerous proteins and second messengers have been implicated in the signalling cascade leading from the NGF receptor to the acquisition of a neuronal phenotype. However, the precise mechanisms of signal transduction involved in neurite outgrowth and differentiation have yet to be determined.

Many receptors for growth and trophic factors couple to the stimulation of tyrosine kinase activity as the initial step in their signal transduction cascade (Schlessinger and Ullrich, 1992). In PC12 cells, NGF binds to and activates a high affinity receptor, the TrkA proto-oncogene (p140^{trk}), leading to the stimulation of an intrinsic tyrosine kinase activity (Maher, 1988; Hempstead and Chao, 1991; Kaplan et al., 1991; Klein et al., 1991) and the subsequent accumulation of tyrosine phosphorylated proteins (Maher, 1988). The low affinity NGF receptor is also present in PC12 cells and is likely to be a component of

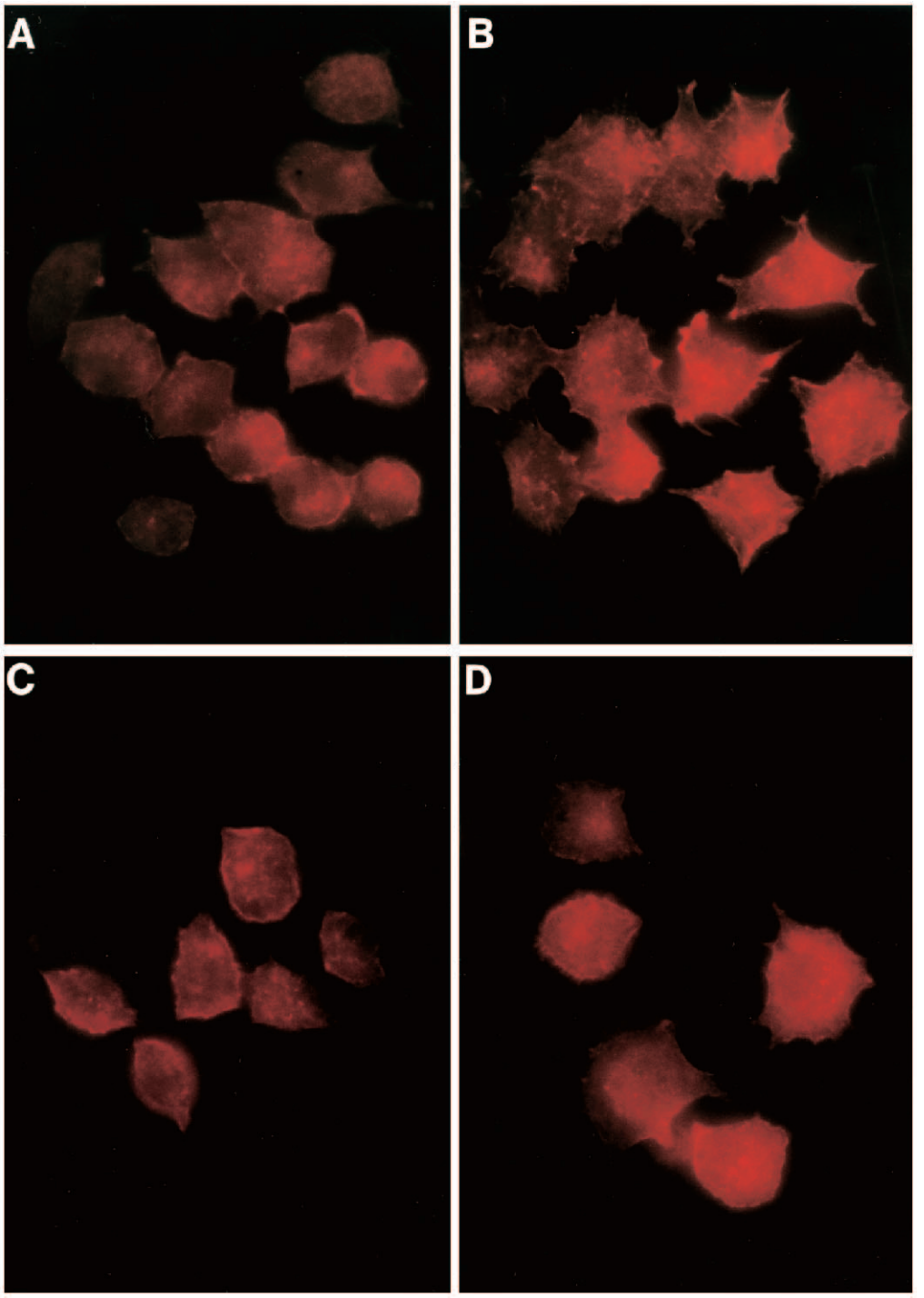

Fig. 1. Wortmannin inhibits the initiation of NGF-stimulated neurite outgrowth. PC12 cells were cultured in complete medium on poly-L-lysine coated glass coverslips for 24 hours. Cells were then treated with DMSO (0.1%; A and B) or wortmannin (100 nM; C and D) for 5 minutes prior to the addition of BSA (A and C) or NGF (100 ng/ml; B and D) for 5 minutes. Cells were then fixed, permeabilized, stained with TRITC-phalloidin and the actin cytoskeleton was visualized by fluorescence microscopy utilising a ×100 oil immersion objective.

the NGF receptor complex (Hempstead and Chao, 1991). A central regulator of both neurite outgrowth and differentiation in PC12 cells is the proto-oncogene Ras (Bar-Sagi and Feramisco, 1985; Noda et al., 1985; Hagag et al., 1986; Szeberenyi et al., 1990). Several proteins have been demonstrated to be involved in the pathway between the TrkA receptor and Ras activation, including the tyrosine kinase Src (Alema et al., 1985; Kremer et al., 1991) and the 'adaptor' proteins, Shc, Crk and Grb-2 (Rozakis-Adcock et al., 1992; Matsuda et al., 1994), which have also been shown to regulate Ras in other systems by stimulating a Ras guanine nucleotide exchange factor, Sos (Buday and Downward, 1993; Egan et al., 1993; Gale et al., 1993; Olivier et al., 1993; Rozakis-Adcock et al., 1993). The protein components shown or proposed to lie downstream of Ras in PC12 cells include Raf, Map kinase kinase and Map kinase, which have been reported to be regulated or phosphorylated in response to NGF (Oshima et al., 1991; Wood et al., 1992). Expression of activated forms of Src, Ras, Raf and Map kinase kinase in PC12 cells can drive neurite outgrowth in the absence of NGF and expression of inactivated forms can block NGF-stimulated neurite outgrowth (Alema et al., 1985; Wood et al., 1993; D'Arcangello and Halegoua, 1993; Cowley et al.,

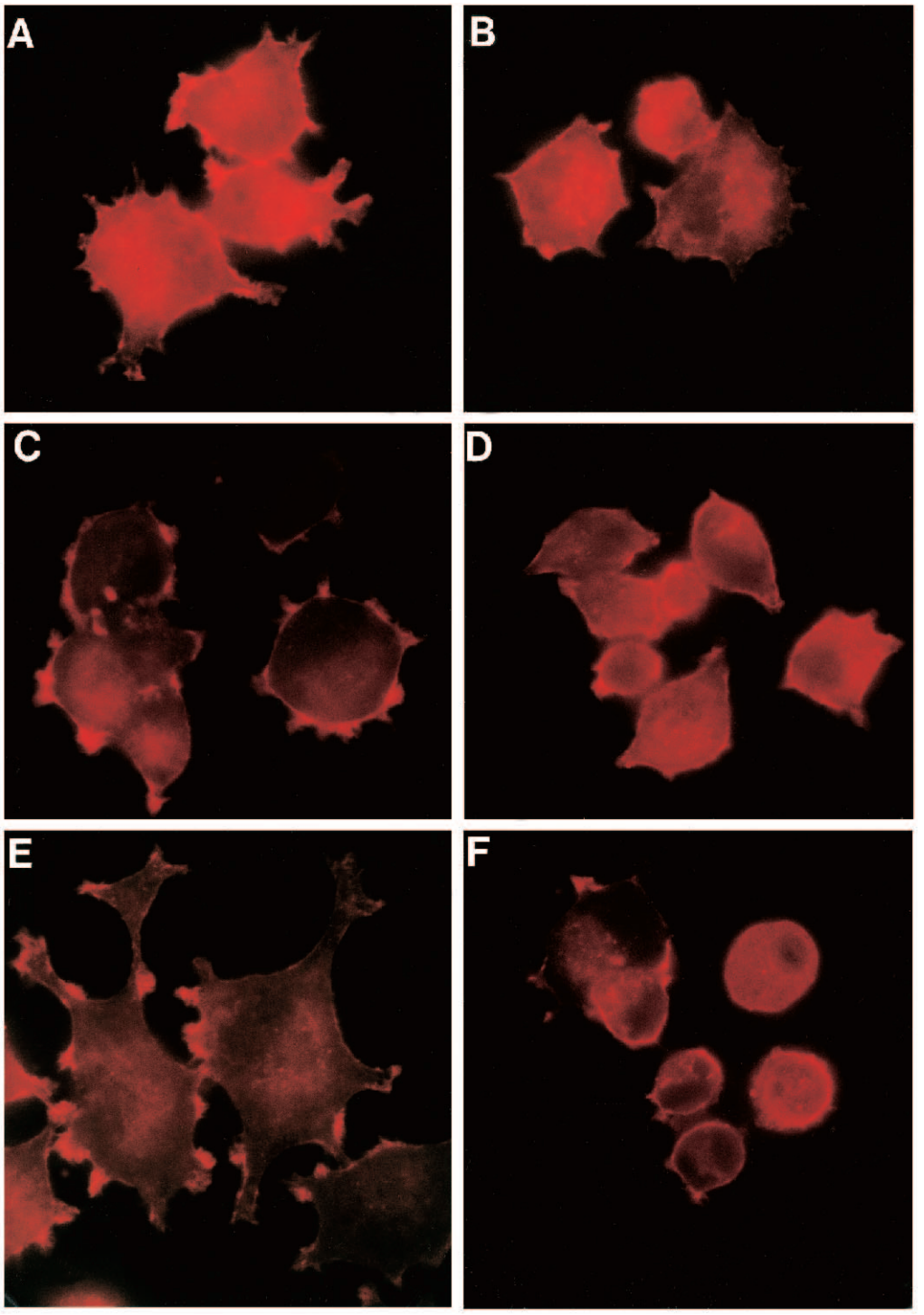

Fig. 2. Effect of wortmannin on neurite outgrowth in response to NGF. Cells were treated with DMSO (A,C,E) or wortmannin (100 nM; B,D,F) for 5 minutes followed by NGF (100 ng/ml) for 30 minutes (A and B), 3 hours (C and D) or 12 hours (E and F; for longer incubations, medium, NGF, DMSO and wortmannin were aspirated and replaced at 30 minute intervals as appropriate). Cells were prepared as described above (Fig. 1.) for observation of the actin cytoskeleton by fluorescence microscopy utilising a ×100 oil immersion objective.

1994). However, the time course of differentiation induced by activated oncogenes differs from that driven by NGF (D'Arcangello and Halegoua, 1993). Furthermore, as a number of other signalling molecules are activated in response to NGF, including the phosphoinositide specific phospholipase Cγ (PI PLCγ) and phosphoinositide 3-kinase (PI3-kinase: Kim et al., 1991; Vetter et al., 1991; Raffioni and Bradshaw, 1992; Soltoff et al., 1992; Carter and Downes, 1992; Ohmichi et al., 1992), other signals, in addition to those mediated by the Ras pathway, may be involved in regulating neurite outgrowth.

Recent studies in non-neuronal cells have implicated PI3-kinase in the regulation of membrane and cytoskeletal rearrangements occurring in response to growth factor stimulation (Wymann and Arcaro, 1994; Wennstrom et al., 1994; Kotani et al., 1994). Phosphatidylinositol(3,4,5)trisphosphate ($PtdInsP_3$) is the primary in vivo product of the receptor stimulated PI3-kinase resulting from phosphorylation of phosphatidylinositol(4,5)bisphosphate ($PtdIns(4,5)P_2$; Stephens et al., 1991; Hawkins et al., 1992). In PC12 cells, NGF addition leads to the rapid activation of PI3-kinase and production of

PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (Raffioni and Bradshaw, 1992; Soltoff et al., 1992; Carter and Downes, 1992). A number of isoforms of PI3-kinase have been identified and cloned, which consist of an SH2 domain containing regulatory subunit, of 85 or 50 kDa, combined with a 110 kDa catalytic subunit (Otsu et al., 1991; Escobedo et al., 1991; Skolnik et al., 1991; Hiles et al., 1992). By analogy with other systems (Carpenter et al., 1993; Backer et al., 1992), it is likely to be the binding of paired Src homology 2 (SH2) domains in the p85 regulatory subunit to appropriate phosphotyrosine containing sequences in either the TrkA receptor or a tyrosine-phosphorylated substrate which leads to the activation of the p110 and thus to PtdInsP_3 and PtdIns(3,4)P_2 production. An immunologically distinct, receptor independent, mammalian PI 3 kinase has also been characterized. This enzyme is specific for phosphatidylinositol, producing only PtdIns(3)P, the exact function of which is currently unknown (Stephens et al., 1994). Similarly, the target of the PtdInsP_3 produced on tyrosine kinase receptor stimulation of the PtdIns(4,5)P_2 preferring PI3-kinase is unknown.

Evidence as to the role of PI3-kinase in stimulated neurite outgrowth is currently equivocal. A study utilizing PDGF/TrkA chimaeric receptor mutants lacking specific tyrosine phosphorylation sites suggested that PI3-kinase recruitment to TrkA is not required for growth factor stimulated neurite outgrowth (Obermeier et al., 1994) and a similar conclusion was drawn following exogenous expression of PDGF receptor mutants in PC12 cells (Vetter and Bishop, 1995; Vaillancourt et al., 1995). In contrast, however, a study utilizing the reputedly specific inhibitor of PI kinase(s), wortmannin, indicates that one of these enzymes may be necessary for the stimulation of neurite outgrowth (Kimura et al., 1994). In this report, we have utilised complementary pharmacological and molecular biological approaches to address the question, is a receptor activated PI3-kinase involved in NGF mediated neurite outgrowth and maintenance in PC12 cells? Our results demonstrate that a tyrosine kinase regulated PI3-kinase is specifically required for neurite outgrowth in response to NGF.

MATERIALS AND METHODS

Measurement of the effect of wortmannin on neurite outgrowth in PC12 cells

PC12 cells were routinely cultured in RPMI 1640 supplemented with 10% horse serum, 5% fetal bovine serum, 50 i.u./ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine (complete medium). For investigation of morphology, PC12 cells were plated on poly-L-lysine coated (100 μg/ml) 22 mm × 22 mm glass coverslips at a density of 2×10^5 cells/ml in complete medium. To determine the effect of wortmannin on neurite maintenance, NGF was added to a final concentration of 100 ng/ml and cells were cultured for a further 36-40 hours prior to use. To investigate the effect of wortmannin on early events in neurite outgrowth, PC12 cells were cultured in complete medium for 24 hours prior to treatment with either wortmannin or DMSO followed by the addition of 0.1 μg/ml BSA in phosphate buffered saline (PBS) with or without 100 ng/ml NGF at appropriate times. Due to the instability of wortmannin in aqueous solutions, media were aspirated and replaced with fresh containing wortmannin or DMSO at 30 minute intervals throughout long incubations. To terminate incubations media were aspirated and the cells were washed once with ice-cold PBS and then fixed and permeabilized by the addition of room temperature (25°C) 3.7% paraformaldehyde, 0.01% glutaraldehyde and 0.2% Triton X-100 in PBS. After 20 minutes in fix, the cells were washed three times with PBS and blocked overnight in PBS containing 5% goat serum (Sigma) and 0.1% BSA. To visualize filamentous actin, cells were incubated for 1 hour with TRITC-phalloidin (50 ng/ml, Sigma) in block, washed by dipping successively in PBS and water, and mounted on slides in Vectashield (Vector Labs, Burlingame, CA, USA) for fluorescence microscopy.

Measurement of ^{32}P labelled lipids in PC12 cells

Lipid labelling and analysis in intact cells was described previously (Jackson et al., 1992), with the following modification: PC12 cells were cultured in Wheaton 'Shorty-Vials' (Jencons Scientific Ltd, Leicester, UK) coated with 100 μg/ml poly-L-lysine at an initial density of 1×10^6 cells/vial in starvation medium (RPMI 1640 supplemented with 1% horse serum, 50 i.u./ml penicillin and 50 μg/ml streptomycin and 2 mM glutamine). After 16 hours, cells were washed twice with Hepes buffered RPMI 1640 salts (HBRS: 111 mM NaCl, 5.37 mM KCl, 11.9 mM $NaHCO_3$, 0.41 mM $MgSO_4$, 0.307 mM $CaCl_2$, 25 mM Hepes, 11.1 mM glucose, pH 7.4, containing 0.1% fatty acid free BSA) and labelled in 400 μl HBRS with 250 μCi/ml [^{32}P]Pi (Amersham, Bucks, UK) at 37°C for 90 minutes. Cells were washed twice with room temperature HBRS to remove unincorporated label and then incubated for 2 minutes at 37°C prior to addition of vehicle (DMSO) or 100 nM wortmannin. Cells were then incubated at 37°C for a further 10 minutes prior to addition of BSA or NGF (100 ng/ml; giving a final volume of 400 μl salts/vial). Cells were then incubated for 5 minutes further prior to addition of 1.5 ml chloroform:methanol (1:2) stop mixture. Subsequently lipids were extracted, deacylated, ^{32}P-labelled headgroups separated by ion exchange HPLC, and quantified by liquid scintillation counting. To correct for variations in cell number between bottles, individual ^{32}P values were normalized to the mean total ^{32}P content in all lipids.

Measurement of PI3-kinase activity in anti-phosphotyrosine immunoprecipitates

PI3-kinase activity measurements were essentially as described (Jackson et al., 1992). Briefly, 7×10^6 cells/10 cm poly-L-lysine coated plate were incubated 16 hours in starvation medium. Cells were then preincubated with or without 100 nM wortmannin for 10 minutes at 37°C, followed by BSA or NGF treatment (100 ng/ml) for 5 minutes. The medium was removed and cell lysates (1 ml/plate) were prepared as previously described. In the wortmannin treated samples, 100 nM wortmannin was maintained in all buffers throughout the immunoprecipitations and assay. The PI3-kinase activity in the immunoprecipitates was assayed for 5 minutes at 30°C in a modified kinase assay buffer (20 mM β-glycerophosphate, 0.8 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 30 mM NaCl, 5 mM $MgCl_2$, 60 μg/ml phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL, USA), 3 μM ATP containing 30 μCi [γ-^{32}P]ATP, Amersham, USA). Assays were stopped, lipids extracted, separated on TLC and quantified using a phosphorimager (Molecular Dynamics, CA, USA).

Measurement of intracellular [Ca^{2+}] in Fura-2 loaded PC12 cells

PC12 cells that had been maintained on poly-L-lysine coated tissue culture plastic in complete medium were resuspended by trituration. Cells were pelleted by centrifugation (600 ***g***, 4 minutes at 25°C) and then resuspended at approximately 2×10^6/ml in HBRS, cells were again pelleted by centrifugation and then resuspended in the same medium with 2 mM Fura-2/AM and 0.024% pluronic F-127 (Calbiochem, Cambridge, UK) to aid its dispersion. Cells were allowed to load in this medium at room temperature with occasional agitation for 50 minutes. Cells were then washed twice, by centrifugation and resuspension, with HBRS. Finally, the cells were resuspended at a density of 1.3×10^7/ml in this medium and kept at room temperature

for a further 20 minutes prior to use. $[Ca^{2+}]_i$ measurements were made using 300 μl of this cell suspension in a Perkin-Elmer LS-50 fluorescence spectrometer and were essentially as previously described (Barry and Cheek, 1994a). Calibration of $[Ca^{2+}]_i$ values was achieved by successive permeabilization of cells with saponin (50 mg/ml) to give f_{max} and subsequent addition of $MnCl_2$ (to 8 mM) to give f_{min} $[Ca^{2+}]_i$ values were calculated as described by Grynkiewitz et al. (1985).

Transfection of PC12 cells with a mutant regulatory subunit of phosphoinositide 3-kinase

Cells for Lipofectamine transfections were plated on poly-L-lysine coated coverslips as described above. After 12-24 hours cells were washed once with serum and antibiotic free RPMI-1640 and then incubated in 0.8 ml of this medium supplemented with 0.2 ml Optimem containing DNA/Lipofectamine (Gibco-BRL Paisley, UK) complexes. For each 35 mm dish transfected, 6 μl of Lipofectamine reagent was mixed with 5 μg of appropriate DNA, either pCMV1R-Δp85 encoding the mutant Δp85 subunit under a cytomegalovirus promoter (see Wennstrom et al., 1994), or luciferase in the same vector, pCMV1R-luc. After 5 hours transfection 1 ml of RPMI-1640 containing 20% horse serum, 10% foetal bovine serum, without antibiotics and with 100 ng/ml NGF where appropriate, was added to each dish. Cells were then incubated for a further 10 hours prior to fixation/permeabilisation and blocking as described above. Cells were then stained for filamentous actin and p85 expression levels. Primary incubation was for 1 hour with an antibody against the α-p85 subunit of PI3-kinase, 1/20 dilution of P13020 (Transduction Labs, Lexington, KY, USA) and 200 ng/ml TRITC-phalloidin in block. Coverslips were then washed by sequential dipping in PBS and incubated with FITC-goat anti-mouse IgG (1:125 dilution; Sigma) and TRITC-phalloidin in block for 1 hour. For detection of luciferase, coverslips were incubated with a 1/250 dilution of a rabbit polyclonal anti-luciferase antisera (Promega, Madison, WI, USA) with TRITC-phalloidin in block followed by incubation with a 1/200 dilution of FITC-coupled anti-rabbit IgG antibody (Jackson Immuno-Research, West Grove, PA, USA) in the continued presence of TRITC phalloidin. Coverslips were then washed and mounted for fluorescence microscopy as described above. For electroporation, PC12 cells were trypsinized and suspended in normal culture medium prior to centrifugation and resuspension in RPMI 1640 at a density of 1×10^7/ml. An aliquot of cells (400 μl) were transferred to an electroporation cuvette containing 30 μg of pCMV1R-Δp85 or pCMV1R-luc. Cuvettes were cooled on ice for 5 minutes prior to electroporation (Bio-Rad Genepulser II: 450V, 250 μF). Cells were held on ice for 5 minutes after electroporation and then diluted to 2×10^5 cells/ml in normal growth medium and plated onto poly-L-lysine coated 22 mm × 22 mm glass coverslips. After 30 minutes for cell attachment, complete medium with or without 100 ng/ml NGF was added to a total volume of 2 ml/well of a six-well plate. After 6-9 hours growth, cells were fixed/permeabilized, blocked and then stained for filamentous actin and p85 expression levels as above.

RESULTS

Wortmannin inhibits NGF-stimulated neurite outgrowth

Addition of NGF to PC12 cells gave rise to a rapid alteration in cell morphology. Compared with unstimulated cells (Fig. 1A) 5 minutes after stimulation many cells showed spreading and had more short filamentous actin rich cell surface protrusions (Fig. 1B). Following 30 minutes of stimulation, cells had spread further, surface processes were more elaborate and clusters of actin rich filopodia had begun to form at the end of the short processes (Fig. 2A). During the next 2-3 hours there was a steady increase in the number of microspikes in individual clusters, in the number of clusters per cell and in the number of cells showing such clusters (Fig. 2C). Neurite growth proceeded until by 12 hours many cells possessed neurites of up to a cell body in length (Fig. 2E). To determine whether the PI3-kinase inhibitor wortmannin affects these responses, cells were pretreated with 100 nM wortmannin prior to stimulation with NGF. Wortmannin significantly inhibited the appearance of actin filament containing processes after 5 minutes of NGF treatment (Fig. 1D), prevented the appearance of filopodial clusters at 30 minutes and 3 hours (Fig. 2B and 2D), and further inhibited the development of neurites following 12 hours incubation with NGF (Fig. 2F: fresh medium containing wortmannin, DMSO and NGF was added at 30 minute intervals throughout this period, see below). Wortmannin only partially inhibited cell spreading in response to NGF at 5 or 30 minutes (Figs 1D and 2B). However, following 3 hours wortmannin treatment, cells showed substantially less spreading (Fig. 2D) and by 12 hours many treated cells had become rounded (Fig. 2F). The time course of neurite outgrowth we have observed is similar to that previously described for PC12 cells (Connolly et al., 1979; Paves et al., 1988) and though slower is very similar to the process of outgrowth described for primary cultures of sympathetic neurones (Smith, 1994), which is also sensitive to inhibition by wortmannin (I.J.B. and A.B.T., in preparation). Wortmannin inhibition of filopodial outgrowth was quantified following 5 minutes of NGF stimulation (for direct comparison with PI3-kinase measurements, see below) and is shown in Fig. 3. In the wortmannin treated cells there was a small reduction in the number of cells displaying processes in the absence of NGF and a substantial inhibition of the NGF-stimulated response. Inhibition of process formation showed dose dependency with an IC_{50} of approximately 20 nM (data not shown), which is in good agreement with previously reported values for wortmannin inhibition of tyrosine kinase activated forms of PI3-kinase (Yano et al., 1993; Wymann and Arcaro, 1994).

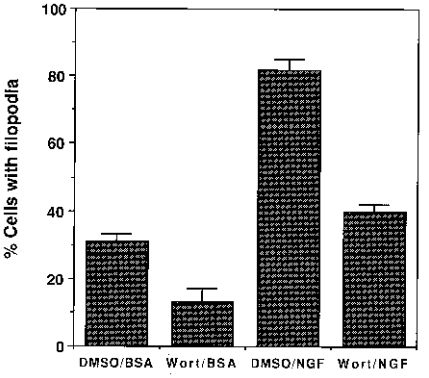

Fig. 3. Effect of wortmannin on NGF-stimulated filopodial extension. Cells were treated with DMSO or wortmannin (Wort; 100 nM) for 10 minutes followed by BSA or NGF (100 ng/ml) for 5 minutes after which cells were prepared for visualization of the actin cytoskeleton by fluorescence microscopy (Fig. 1). The percentage of cells showing actin rich filopodia or other actin containing cell surface projections was quantified and is illustrated.

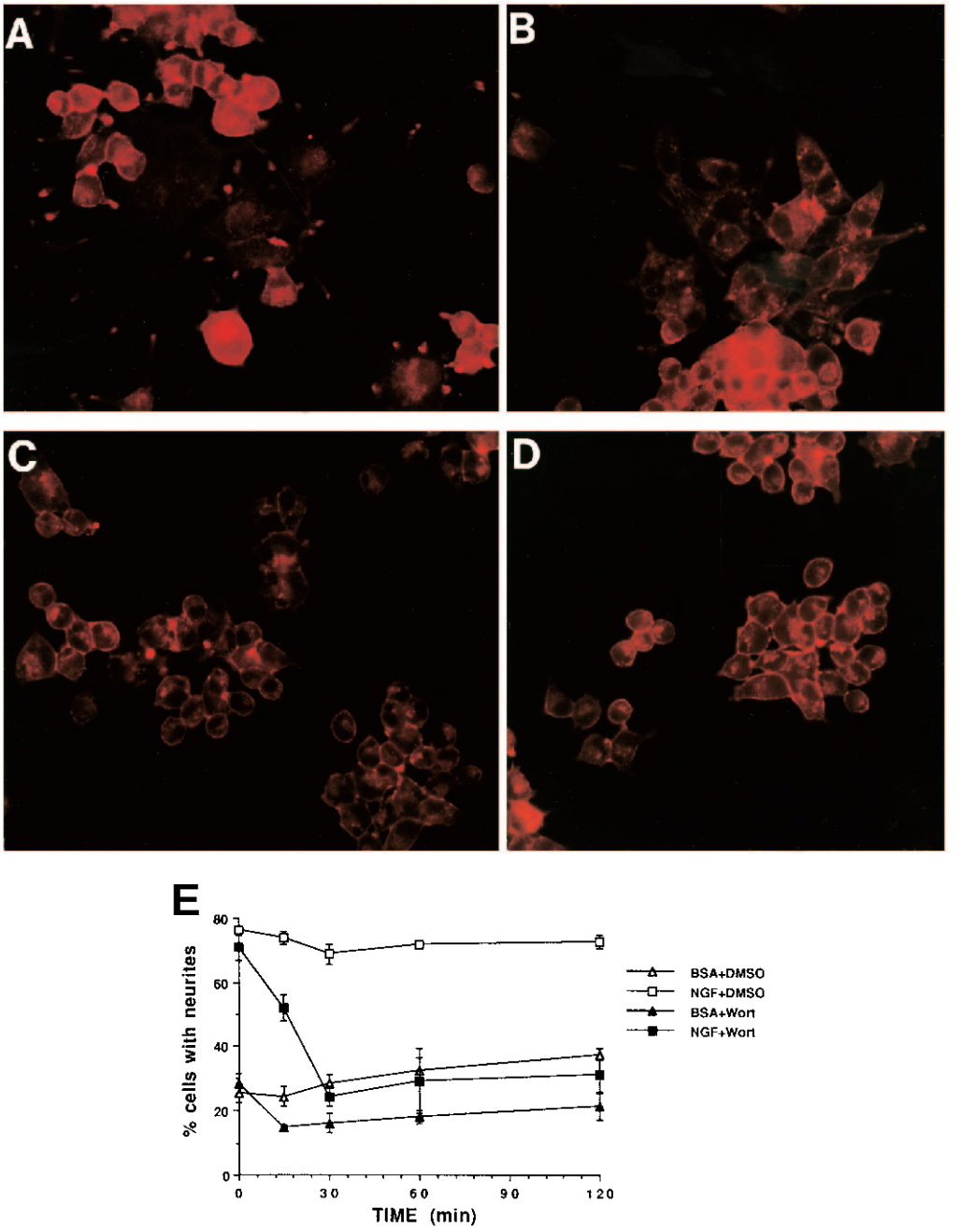

Fig. 4. Effect of wortmannin on maintenance of NGF-stimulated neurites. PC12 cells were plated on poly-L-lysine coated glass coverslips and cultured, in complete medium, in the presence of NGF (100 ng/ml) for 48 hours. Cells were then treated with DMSO (A) or wortmannin (100 nM; B,C,D) for 15 minutes (B), 30 minutes (C) or 1 hour (D), and for longer time points the medium was replaced at 30 minute intervals with fresh medium containing NGF, wortmannin or DMSO. Cells were then fixed, permeabilized and stained with TRITC-phalloidin for visualization of the actin cytoskeleton by fluorescence microscopy utilising a ×40 objective. (E) Quantification of the effects of wortmannin on neurite maintenance. Cells were treated with DMSO or wortmannin in the presence or absence of NGF, with an additional 2 hour time point and observed by phase-contrast microscopy. Data shown are the mean percentage of cells showing neurites ± s.d. from two separate experiments in which duplicate counts of at least 500 cells were performed for each time and condition.

NGF dependent neurite maintenance is inhibited by wortmannin

Following 48 hours exposure to NGF, PC12 cells possessed neurites (approximately 1-2 cell bodies in length) terminating in actin rich filopodial clusters reminiscent of small growth cones (Fig. 4A). To determine if PI3-kinase is involved in this 'maintenance' phase of the neurite response, cells with NGF-stimulated neurites were treated with wortmannin. Addition of 100 nM wortmannin caused a rapid loss of actin filament clusters from the tips of neurites (within 15 minutes; Fig. 4B), with a progressive loss of neurites and actin filament clusters between 30 and 60 minutes. During this time patches of actin staining appeared within the cell bodies, often in association with the cytoplasmic face of vacuolar structures (Fig. 4C and D; similar actin associated vacuolar structures follow wortmannin inhibition of neurite outgrowth; see Fig. 2F). Vacuoles also occur, albeit to a much lesser extent, in control cells (not shown). The kinetics of vesicle appearance closely matched those of neurite loss and if wortmannin was not replenished in the culture medium, the kinetics of vesicle disappearance paralleled the recovery of neurites.

The effect of wortmannin was quantified by counting neurites under phase contrast microscopy (Fig. 4E). Wortmannin had a maximal effect (70% inhibition; see Fig. 4C and D) by 30 minutes after addition, with a $t_{1/2}$ of approximately 10 minutes (Fig. 4E). As observed above there was a low basal level of neurite outgrowth in non-NGF treated cells, which also appeared to be sensitive to wortmannin (Fig. 4E).

Neurite collapse in response to wortmannin is substantially more rapid than that resulting from NGF withdrawal (Seeley and Greene, 1983; Greene and Tischler, 1976). This is to be expected

Table 1. Dose dependency of wortmannin inhibition of neurite maintenance

[Wortmannin] (nM)	% Inhibition of maximal (±s.d.)
0	0
10	22±11
30	55±4
60	63±2
100	74±3

PC12 cells were grown on poly-L-lysine coated 6-well plates in the presence of NGF for 48 hours and then exposed to various concentrations of wortmannin for 30 minutes prior to fixation. Cells with processes were assessed by phase contrast microscopy. Data are expressed as percentage inhibition of the maximum number of cells showing processes. Data are mean ± s.d. from four independent counts, of at least 200 cells in each condition, in one experiment and is representative of two separate experiments.

as wortmannin, as an inhibitor of second messenger production, will produce a blockade of receptor signalling substantially more rapidly and completely than would result from withdrawal of NGF from the medium. Further, the effect of wortmannin treatment is reversible, indicating that the collapse of neurites is not due to induction of apoptosis or a toxic effect of wortmannin (cells remain viable after 24 hours of wortmannin treatment in FCS containing medium; data not shown and see Kimura et al., 1994). As was observed in the studies of neurite outgrowth inhibition, wortmannin caused neurite collapse with an IC_{50} of approximately 20 nM (see Table 1), consistent with specific inhibition of a tyrosine kinase regulated PI3-kinase.

Wortmannin inhibits NGF-stimulated PI3-kinase activity

To assess the effect of wortmannin on NGF-stimulated PI3-kinase, two independent techniques, in vivo labelling of phospholipids, and in vitro assay following immunoprecipitation were employed. In the first assays, cells were incubated with ^{32}P to label the phospholipid pool, stimulated with NGF, and then the lipids were extracted, deacylated and analyzed by HPLC. Addition of NGF to intact, ^{32}P labelled, PC12 cells, produced increased levels of [^{32}P]PtdIns(3,4,5)P_3, [^{32}P]PtdIns(3,4)P_2 and [^{32}P]phosphatidic acid (PA: see Fig. 5). The most dramatic response measured was a 5-fold increase in the levels of [^{32}P]PtdInsP_3, the molecule proposed to be the primary second messenger product of receptor activation of PI3-kinase (Stephens et al., 1991; Hawkins et al., 1992). The increases in [^{32}P]PtdIns(3,4)P_2 and [^{32}P]PA in response to NGF were relatively small, only 42% and 25% above basal, respectively. The levels of the other inositol lipids analyzed, [^{32}P]PtdIns(4,5)P_2, [^{32}P]PtdIns(4)P and [^{32}P]PtdIns(3)P, remained unchanged following NGF treatment. Pretreatment of cells with wortmannin completely abolished the NGF-stimulated increases in [^{32}P]PtdIns(3,4,5)P_3 and [^{32}P]PtdIns(3,4)P_2 levels, indicating that wortmannin inhibited the activated PI3-kinase responsible for PtdInsP_3 and PtdIns(3,4)P_2 production. Wortmannin treatment also reduced the levels of [^{32}P]PtdIns(3)P in both the unstimulated and NGF-stimulated cells. This result suggests that in unstimulated cells, a pool of [^{32}P]PtdIns(3)P exists whose production is dependent on a wortmannin sensitive PI3-kinase. However, the production of [^{32}P]PtdIns(3)P was not stimulated by NGF. Wortmannin had

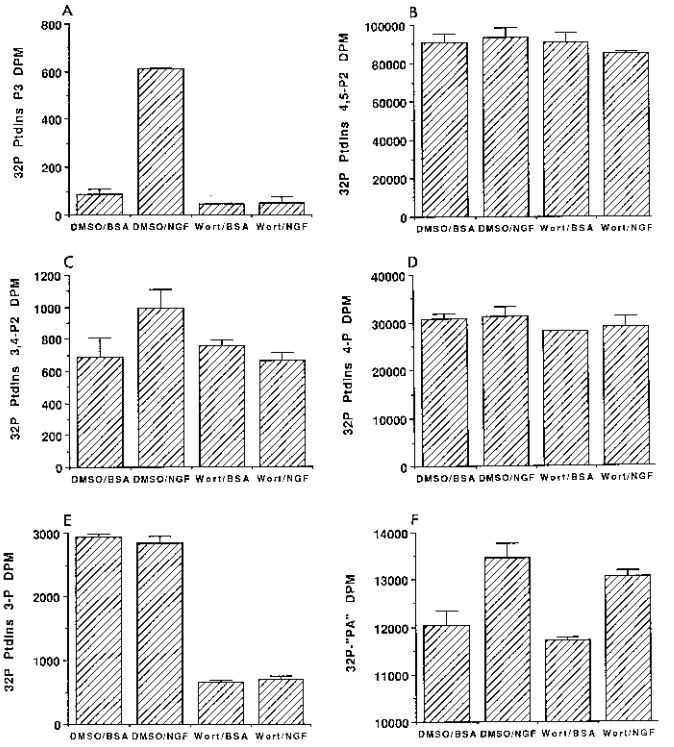

Fig. 5. Effect of wortmannin on the levels of ^{32}P-labelled lipids in PC12 cells. Cells were cultured for 16 hours in poly-L-lysine coated glass vials in starvation medium. Cells were labelled with ^{32}P-Pi for 90 minutes, washed and then incubated with DMSO or wortmannin (Wort; 100 nM) for 5 minutes prior to addition of BSA or NGF (100 ng/ml). Following a 5 minute stimulation, incubations were stopped and radiolabelled lipids extracted, deacylated, separated and quantified by liquid scintillation spectrophotometry. The data shown are the mean DPM in individual deacylated lipid fractions from duplicate vials ± s.d.

no effect on the basal or stimulated levels of [^{32}P]PtdIns(4,5)P_2 or [^{32}P]PtdIns(4)P and did not inhibit the NGF-stimulated rise in [^{32}P]PA, indicating that wortmannin specifically affected the 3-phosphorylated inositol lipids.

Binding of NGF to the TrkA NGF receptor leads to phosphorylation of the receptor and associated substrate molecules on tyrosine residues. Preparation of immunoprecipitates using anti-phosphotyrosine specific antibodies allows the identification of proteins recruited into complexes with tyrosine phosphorylated molecules. NGF stimulation of PC12 cells led to an increased level of PI3-kinase activity in anti-phosphotyrosine immunoprecipitates and this activity was abolished by the presence of wortmannin (Fig. 6). NGF also stimulated a modest increase in PI3-kinase activity in immunoprecipitates prepared using an antibody recognizing the p85 subunit of PI3-kinase, which was also wortmannin sensitive (I.J.B., T.R.J. and A.B.T., unpublished observation).

Wortmannin does not block NGF-stimulated elevations in intracellular calcium

To determine if the effects of wortmannin were specific for the inhibition of the NGF-stimulated PtdInsP_3 production, the NGF-stimulated calcium response in PC12 cells was examined. Using Fura-2 loaded PC12 cells to detect intracellular calcium

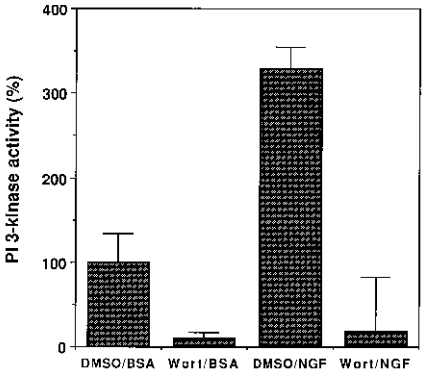

Fig. 6. Wortmannin inhibits PI3-kinase activity in anti-phosphotyrosine immunoprecipitates from PC12 cells. Cells were cultured on poly-L-lysine coated 100 mm plastic tissue culture dishes in complete medium for 24-48 hours. Cells were incubated for 5 minutes with DMSO or 100 nM wortmannin (Wort) followed by either BSA or NGF (100 ng/ml) for a further 5 minutes. Cells were lysed and tyrosine phosphate containing proteins were isolated by immunoprecipitation with PY20 anti-phosphotyrosine antibody. PtdIns kinase activity in immunoprecipitates was then determined (Materials and Methods). Results shown are mean ± s.d. (arbitrary units expressed as % of control) from duplicate samples from one experiment representative of at least three separate determinations.

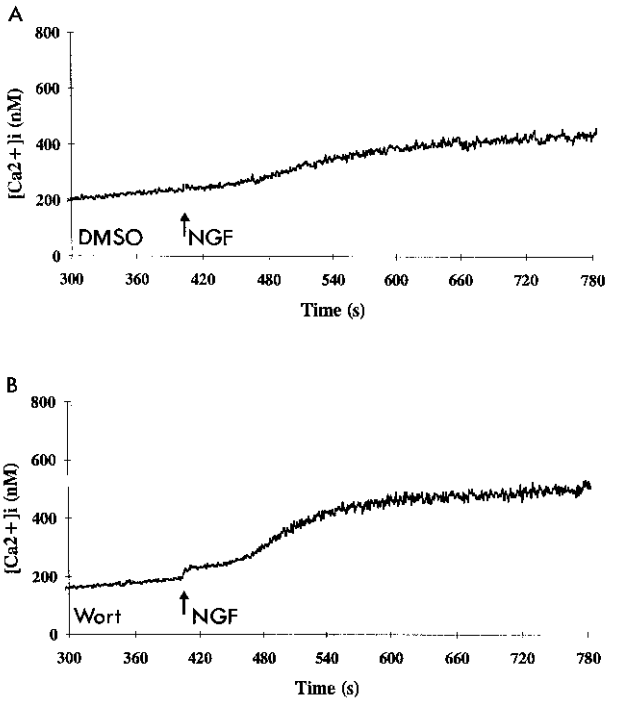

Fig. 7. Wortmannin does not inhibit NGF-stimulated elevation of intracellular $[Ca^{2+}]$. PC12 cells loaded with Fura-2/AM in suspension were incubated in a Perkin Elmer LS50 fluorescence spectrophotometer with a stirring cuvette thermostatted at 37°C. Following 3 minutes preincubation, cells were treated with DMSO (A) or wortmannin (100 nM; B) and after a further 5 minutes NGF (100 ng/ml) was added (indicated by arrow) to each. Similar $[Ca^{2+}]_i$ increases were observed in response to NGF in the presence (from 248±73 to 381±102 nM mean $[Ca^{2+}]_i \pm$ s.d.) or absence (from 228±6 to 431±86 nM mean $[Ca^{2+}]_i \pm$ s.d.) of wortmannin in three separate experiments.

responses, a slowly developing, sustained rise in cytoplasmic $[Ca^{2+}]$ in response to NGF (Fig. 7A) was consistently measured. Pretreatment of cells with wortmannin prior to the addition of NGF had no effect on the $[Ca^{2+}]_i$ response (Fig. 7B). The basis of the NGF-stimulated $[Ca^{2+}]_i$ response is not known, but may be triggered by Ins(1,4,5)P_3 generated following recruitment of PI-PLCγ to NGF-R/substrate complexes, which has itself been suggested to be essential for neurite outgrowth (Obermeier et al., 1994). Alternatively, this calcium rise may be caused by an NGF-stimulated calcium influx. That the NGF-stimulated generation of a Ca^{2+} signal was not blocked by wortmannin indicates that NGF signal transduction, in general, is not interrupted by this compound. In further support of the specific nature of the inhibition of PI3-kinase, we also noted that intracellular Ca^{2+} responses to ATP receptor-stimulation, which are mediated by PI-PLCβ (Barry and Cheek, 1994b), were unaffected by wortmannin (data not shown).

Expression of a mutant PI3-kinase regulatory subunit inhibits NGF-stimulated neurite outgrowth

The effect of wortmannin clearly demonstrated a correlation between inhibition of PI3-kinase (s) and the inhibition of neurite outgrowth and maintenance. However, using this inhibitor we cannot distinguish between a requirement for receptor stimulated PtdInsP_3 production in NGF signalling or a requirement for a basal level of PtdIns(3)P, which might be necessary for membrane trafficking during neurite outgrowth. To test the involvement of the receptor activated form of the PI3-kinase, which is responsible for PtdInsP_3 production, we have examined the effects of overexpression of a mutant form of the p85 regulatory subunit on neurite outgrowth. This mutant lacks the site of association with the p110 subunit but retains the ability to bind to tyrosine phosphorylated targets (Dhand et al., 1994; Wennstrom et al., 1994). The overexpression of this mutant acts as a dominant negative inhibitor since it competes with wild-type PI3-kinase regulatory subunits for recruitment, but does not itself activate p110 catalytic activity. A construct containing the gene for Δp85 was introduced into PC12 cells by transfection with Lipofectamine, followed by incubation with NGF for 12 hours, after which cells were fixed and stained for both filamentous actin and for levels of p85 expression. Following NGF treatment many cells exhibited filamentous actin tipped neurites of 1/2-1 cell body in length (Fig. 8A and C: these short exposure times were necessary as no overexpressing cells were detected 18 hours after transfection). Δp85 transfected cells showed substantially higher levels of p85 staining than controls (Fig. 8B and D) but none of these overexpressing cells possessed neurites or other process (Fig. 8A and C). Typically Δp85 transfected PC12 cells displayed a rounded morphology with extremely little filamentous actin being detected by TRITC-phalloidin staining (actin staining was unaffected by luciferase-overexpression, data not shown). To obtain larger numbers of transfected cells for quantitative analysis, we used electroporation to introduce constructs into PC12 cells. Analysis of these responses showed that overexpression of Δp85 led to approximately an 80% reduction in the response to NGF (Fig. 8E). To control for the effects of overexpression of foreign DNA, the same vector containing the luciferase gene in place of Δp85 (pCMV1R-luc) was transfected into PC12 cells. Luciferase expression had no effect on cell

responses to NGF (Fig. 8F; this transfection was performed in a separate series of experiments to those in Fig. 8E, hence the higher level of basal neurite initiation). The results of Δp85 expression indicate that selectively blocking receptor activated PI3-kinases leads to inhibition of neurite outgrowth.

DISCUSSION

We have shown here that inactivation of PI3-kinase by two independent mechanisms, addition of the fungal metabolite, wortmannin, or overexpression of a mutant form of the p85 regulatory subunit of PI3-kinase, results in a substantial inhibition of neurite outgrowth in PC12 cells. In biochemical assays, wortmannin inhibited NGF-stimulated increases in $PtdInsP_3$ and $PtdIns(3,4)P_2$ levels, and reduced the basal levels of PtdIns(3)P. However, it is unlikely that it is the PtdIns(3)P that regulates neurite outgrowth, as PtdIns(3)P levels did not increase in response to NGF and expression of Δp85, which inhibited neurite outgrowth as demonstrated here, did not affect PtdIns(3)P levels when measured in transfected CHO or U937 cells (Hara et al., 1994, and T.R.J., F.C., P.T.H. and L. R. Stephens, unpublished observations). Further, studies of 3-phosphorylated lipid responses to other growth factors (Hawkins et al., 1992; Jackson et al., 1992) indicate that $PtdIns(3,4)P_2$ is most probably produced as a metabolite of $PtdInsP_3$ through the action of a 5-phosphatase. That stimulation of $PtdIns(3,4)P_2$ production does not match that of $PtdInsP_3$ probably reflects the additional contribution of a 3-phosphatase to $PtdInsP_3$ metabolism, see Hawkins et al. (1992). Thus, as the primary product of receptor activation of PI3-kinase, it is $PtdInsP_3$ which is likely to be the second messenger required for NGF-stimulated neurite outgrowth.

Inhibition of neurite outgrowth following overexpression of the Δp85 subunit of PI3-kinase indicates that it is a tyrosine phosphate regulated PI3-kinase which is required for this aspect of NGF receptor signalling. Two lines of evidence suggest that it is the catalytic function of the enzyme, rather than any protein/protein interaction, which is required for neurite outgrowth. Firstly, the catalytic inhibitor wortmannin does not interfere with the recruitment of p85 or p110 subunits of PI3-kinase to appropriate tyrosine phosphate targets (Wennstrom et al., 1994; Kimura et al., 1994). Secondly, sites of other potential protein/protein interactions found within the p85 regulatory subunit (SH3, SH3 binding and BCR domains; Dhand et al. (1994) are present in Δp85 and are still recruited to appropriate phosphotyrosine targets in the absence of the p110 catalytic subunit.

Our results indicate that selective inhibition of receptor-stimulated PI3-kinase by pharmacological or genetic manipulation of PC12 cells prevents neurite outgrowth. In addition, more mature neurites formed following prolonged NGF treatment are sensitive to the inhibitor wortmannin, suggesting that neurite maintenance also requires a PI3-kinase. This data, together with the observation of Kimura et al. (1994) that production of $PtdInsP_3$ accompanies neurite elongation even after prolonged (16 hours) periods of NGF stimulation, supports our suggestion that $PtdInsP_3$ is the PI3-kinase product required in neurite maintenance as well as outgrowth.

Our results contrast with those derived from studies utilising exogenous PDGF-R or PDGF-R/TrkA chimaera mutants lacking potential sites of PI3-kinase association (Obermeier et al., 1994; Vetter and Bishop, 1995; Vaillancourt et al., 1995). Such mutants are still able to stimulate neurite outgrowth in response to growth factor, which led to the suggestion that receptor stimulation of PI3-kinase is not required for morphological differentiation of PC12 cells. However, these studies are open to the alternative interpretation that PI3-kinase activation may not require direct recruitment to the receptor, but rather may be stimulated by another pathway. Association of PI3-kinase with p110/p100 tyrosine phosphorylated proteins, which may be analogous to the insulin-receptor substrate IRS-1, has been observed in NGF-stimulated PC12 cells (Ohmichi et al., 1992). That NGF stimulation of PI3-kinase might use such an indirect mechanism of recruitment is consistent with the observations that either PI3-kinase cannot be detected in anti-TrkA receptor immunoprecipitates (Carter and Downes, 1992; Ohmichi et al., 1992), or that TrkA associated activity represents only a small proportion of that associated with tyrosine phosphorylated substrates (Soltoff et al., 1992). Thus although these receptors contain consensus sequences for PI3-kinase binding, they may be redundant to other high affinity tyrosine phosphate recruitment sites, or this activation may be bypassed by other signalling molecules such as Ras (see below).

It has recently been reported that Ras may interact with and activate PI3-kinase (Kodaki et al., 1994; Rodriguez-Viciana, 1994). Therefore, in NGF-stimulated PC12 cells, PI3-kinase may be activated by two co-operating processes: (i) regulatory subunit SH2 domains binding to tyrosine phosphorylated receptor substrates; and (ii) through interaction of Ras with the p110 catalytic subunit (Rodriguez-Viciana, 1994). Our finding that expression of a Δp85 was able to almost completely abolish NGF-stimulated neurite outgrowth, in keeping with its ability to inhibit receptor stimulated $PtdInsP_3$ production (Hara et al., 1994), supports the idea that SH2 domain dependent recruitment of PI3-kinase to sites of tyrosine phosphorylation is essential for its activation by NGF. This is consistent with reports that for the Ras interaction with p110 to stimulate PtdInsP3 production, the p110 must be complexed with p85. Further, it suggests that in vivo sensitivity to Ras requires the PI3-kinase complex to be associated with tyrosine phosphorylated target proteins.

There has been considerable speculation about the identity of the biochemical activity regulated by $PtdInsP_3$ and the nature of the $PtdInsP_3$ target. PI3-kinase has been suggested to be specifically involved in the regulation of the Akt protein kinase (Franke et al., 1995; Kohn et al., 1995), stimulation of p70 ribosomal S6-kinase (Chung et al., 1994; Cheatham et al., 1994), in regulating vesicle trafficking (Yano et al., 1993; Hara et al., 1994; Cheatham et al., 1994) and in membrane ruffling responses (Wymann and Arcaro, 1994; Wennstrom et al., 1994; Kotani et al., 1994). There are similarities between the processes of membrane ruffling and neurite outgrowth in that both involve concerted rearrangements of the plasma membrane and the subplasmalemmal cytoskeleton with the generation of characteristic actin rich structures such as filopodia and lamellipodia (Ridley et al., 1992; Trinkaus, 1994; Wang, 1985; Forscher and Smith, 1988). Growth factor stimulated membrane ruffling requires activation of the small GTPase Rac through stimulation of exchange of GDP on Rac for GTP (Ridley et al., 1992) and it has recently been shown that this is dependent on the action of PI3-kinase (Hawkins et al., 1995). Expression of mutant Rac proteins in *Drosophila* indicate that it is required for axonogen-

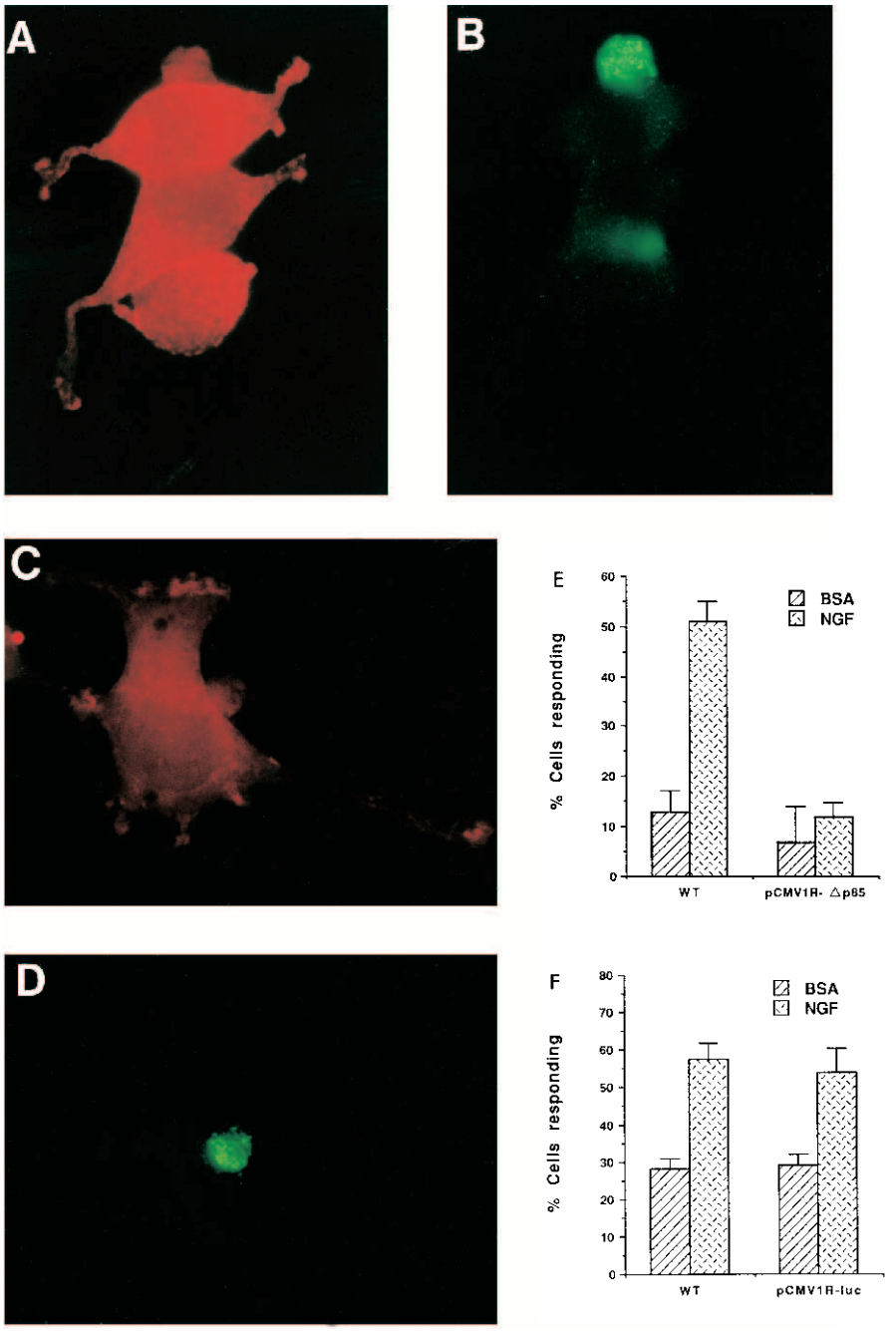

Fig. 8. Expression of a mutant PI3-kinase p85 subunit prevents NGF-stimulated neurite outgrowth. PC12 cells were transfected with a mutant p85 subunit (Δp85) or luciferase, cultured for 10 hours in the presence of NGF (100 ng/ml) and then stained either for αp85 overexpression (B and D) or with TRITC-phalloidin to visualize their actin cytoskeleton (A and C) as described in Materials and Methods. Cells were observed by fluorescence microscopy using a ×100 oil immersion objective. The proportion of cells transfected with pCMV1R-Δp85 (E), pCMC1R-luc (F) or untransfected ('wild type', WT) and showing filamentous actin containing processes was assessed and is expressed as the percentage of cells responding in the presence or absence of NGF. Data shown are from one transfection with pCMV1R Δp85 (E: without NGF, 3,050 cells; with NGF, 2,300 cells) or pCMV1R-luc (F: without NGF, 650 cells; with NGF, 750 cells). For electroporation in each transfection efficiency was between 5 and 10%, for Lipofectamine transfection efficiency was <5%; these results are representative of 2-5 separate experiments.

esis and thus support a role for this pathway in regulation of neuronal morphology in vivo (Luo et al., 1994).

In conclusion, the use of two separate means of inhibition of PI3-kinase catalytic activity, exposure to wortmannin and over-expression of a dominant negative mutant regulatory subunit together provide the first unequivocal demonstration of a role for the PI3-kinase signalling pathway in neuronal morphological differentiation stimulated by NGF. Furthermore, it suggests that the process of neurite outgrowth may share common components with morphological events in non-neuronal cells which have previously been shown to be dependent upon $PtdInsP_3$ production. Common targets of $PtdInsP_3$ regulation may include factors such as a Rac-guanine nucleotide exchange factor or cytoskeletal organising proteins, which may now be predicted to be involved in neuronal differentiation as well as regulating morphological responses in other cell types.

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