Divergence in the anti-apoptotic signalling pathways used by nerve growth factor and basic fibroblast growth factor (bFGF) in PC12 cells: rescue by bFGF involves protein kinase Cδ

Maribeth M. WERT[1] and H. Clive PALFREY[2]

Department of Neurobiology, Pharmacology and Physiology, University of Chicago, 947 E. 58th St, Chicago, IL 60637, U.S.A.

The mechanisms whereby nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) block apoptosis in serum-deprived PC12 cells were investigated. NGF, but not bFGF, strongly activated Akt/protein kinase B, a downstream effector of phosphoinositide (phosphatidylinositol) 3-kinase (PI 3-kinase). In addition, inhibition of PI 3-kinase by LY294002 partially blocked inhibition of apoptosis by NGF, but not that by bFGF, suggesting divergence in NGF and bFGF anti-apoptotic signalling pathways. Both growth factors strongly activated mitogen-activated protein (MAP) kinases, but blockade of signalling through this pathway, either by the expression of dominant-negative Ras or by treatment with the MAP kinase/ERK kinase (MEK) inhibitor U0126, partially inhibited only bFGF, but not NGF, anti-apoptotic signalling. Use of isoform-specific protein kinase C (PKC) inhibitors such as bisindoylmaleimide-I and Gö 6983 suggested that PKCδ is a likely component of bFGF trophic signalling. A role for PKCδ was confirmed in PC12 cells expressing a dominant-negative PKCδ fragment, in which reversal of apoptosis by bFGF was partially blocked. The PKCδ signal was not mediated by the MAP kinase cascade, as bFGF activation of this pathway was not affected in cells expressing the dominant-negative PKCδ fragment. Full inhibition of bFGF anti-apoptotic signalling occurred when both the PKCδ and Ras/MAP kinase pathways were inhibited. Together, these data demonstrate that inhibition of apoptosis by bFGF in PC12 cells operates differently from that mediated by NGF, requiring the addition of signals from both the Ras/MAP kinase and PKC signalling pathways.

Key words: Akt, dominant-negative, growth factors, MAP kinase, protein kinase C inhibitors.

INTRODUCTION

Apoptosis, or programmed cell death, is an endogenous cell suicide program which has critical roles during development and mature tissue homoeostasis. In the nervous system a wide variety of trophic factors ensure the survival of mature neurons throughout the lifespan of the organism, and the mechanisms by which factors such as the neurotrophins and other polypeptides subvert the apoptotic programme are currently under intensive study. The signalling pathways responsible for anti-apoptotic effects have been partially characterized; for example, activation of Akt/protein kinase B and the mitogen-activated protein kinases (MAP kinases) (or extracellular-signal-regulated kinases) ERK1 and ERK2 has been linked to the suppression of cell death in cells of neural lineage (e.g. see [1,2]). Once an apoptotic cascade is initiated, effector proteins, such as those of the caspase family, direct the breakdown of the cell (reviewed in [3,4]).

The PC12 pheochromocytoma cell line has been used for many years to study mechanisms of growth and trophic factor action, particularly that of nerve growth factor (NGF) [5]. Besides the two NGF receptors TrkA and $p75^{NTR}$, PC12 cells also express receptors for a number of growth factors and hormones, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and insulin. These receptor tyrosine kinases exert different physiological effects: e.g. NGF and bFGF may promote differentiation towards the sympathetic neuron phenotype, while EGF does not. Consequently, downstream signalling from these receptors must exhibit divergent characteristics that determine the final physiological outcome. NGF and bFGF, but not EGF, apparently induce a prolonged activation of the Ras/MAP kinase pathway, and this sustained activation has been implicated as a factor in the promotion of neurite outgrowth by growth factors [6–8]. By contrast, NGF and EGF strongly activate phosphoinositide (phosphatidylinositol) 3-kinase (PI 3-kinase), while bFGF does so only weakly [9,10].

Another common feature of the action of NGF and bFGF in PC12 cells is that both factors enhance cell survival under conditions of serum deprivation. While NGF and bFGF may induce neurite outgrowth by a similar mechanism, it is presently unclear whether they use similar anti-apoptotic signals. NGF has been shown previously to utilize the PI 3-kinase pathway to block apoptosis following its withdrawal from NGF-differentiated PC12 cells or sympathetic neurons [1,11], but little work has been done to elucidate the signals involved in the inhibition of apoptosis by bFGF. Studies in endothelial cells suggest that protein kinase C (PKC) may be involved in the inhibition by bFGF of radiation-induced apoptosis [12], but prevention by bFGF of tumour necrosis factor-α-mediated apoptosis in L929 fibroblasts requires activation of the Ras/MAP kinase pathway [13]. Here we show that, in PC12 cells, bFGF anti-apoptotic signalling does not involve the PI 3-kinase cascade, but requires the additive participation of both the PKC and Ras/MAP kinase pathways. These results demonstrate that multiple anti-apoptotic signalling pathways can coexist within a single clonal cell type.

Abbreviations used: bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; FRS, bFGF receptor substrate; MAP kinase, mitogen-actvated protein kinase; MEK, MAP kinase/ERK kinase; NGF, nerve growth factor; PI 3-kinase, phosphoinositide (phosphatidylinositol) 3-kinase; PKC, protein kinase C; TBS, Tris-buffered saline.

[1] Present address: Beth Israel Deaconess Medical Center, Department of Pathology, 330 Brookline Ave., RN-216, Boston, MA 02215, U.S.A.

[2] To whom correspondence should be addressed (e-mail hpalfrey@midway.uchicago.edu).

© 2000 Biochemical Society

EXPERIMENTAL

Materials

NGF (2.5 S) and bFGF were from Collaborative Biomedical Products and R&D Systems respectively. Antibodies against pan-ERK and against PKC isoforms α, β, γ, δ, ϵ, θ, ι and λ were from Transduction Laboratories. Antibodies against Akt and PKCμ were from Santa Cruz Biotechnology. A rabbit polyclonal anti-phosphoAkt antibody (pSer-473) was obtained from New England Biolabs, and a sheep polyclonal anti-(phosphoMAP kinase) antibody (pTyr-180) was from Upstate Biotechnology Inc. Horseradish peroxidase-conjugated secondary antibodies were obtained from Sigma (anti-rabbit, anti-goat), Promega (anti-mouse) or Jackson Immunologicals (anti-sheep). The MAP kinase/ERK kinase (MEK) inhibitor U0126 was obtained from Promega, and the PI 3-kinase inhibitor LY294002 was from BioMol. All other reagents were of research grade from standard sources.

PC12 cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) bovine calf serum. PC12 cells expressing dominant-negative Ras or its vector were obtained from G. M. Cooper (Dana Farber Cancer Center, Boston, MA, U.S.A.). PC12 cells expressing dominant-negative PKCδ (identified as v1δ1 in [14] and as 'δ dom-neg in the present paper'), overexpressing full-length PKCδ ['δ (ox)'] or transfected with the vector (c1 in [14]; 'vector' in the present paper) were generously donated by Dr Robert Messing (University of California, San Francisco, CA, U.S.A.), and were grown as above with the addition of 0.2 mg/ml G418 (Promega) to the medium.

Viability and apoptosis assays

PC12 cells were plated on to collagen-coated coverslips and allowed to attach overnight. The cells were then washed twice with sterile Tris-buffered saline (TBS) and incubated in serum-free DMEM plus growth factors or inhibitors as indicated for each experiment. In most experiments serum deprivation was for 24 h, after which the cells were incubated for 10 min with 1 μg/ml bisbenzimide, washed once, and then inverted over a dye mixture containing 15 μg/ml fluorescein diacetate and 4.6 μg/ml propidium iodide in Hanks balanced salt solution. Condensed apoptotic nuclei were scored by bisbenzimide staining, and live/dead cells were estimated with fluorescein diacetate/propidium iodide staining. In general, these two measures agreed well with each other, and only the data from bisbenzimide staining are shown here. Data were analysed for statistical significance using Student's t-test.

Akt assay

PC12 cells were plated at 2×10^6 cells/6 cm dish. The next day the cultures were washed twice with sterile TBS and incubated in 2 ml of serum-free DMEM for 4 h at 37 °C to reduce basal phosphorylation levels. The cells were then treated with either 100 ng/ml NGF or 10 ng/ml bFGF (saturating concentrations for the respective receptors) for various lengths of time, and then washed twice with ice-cold TBS and disrupted in 300 μl of lysis buffer [1 % Nonidet P40, 150 mM NaCl, 5 mM EDTA, 20 mM Tris/HCl (pH 7.4), 40 μM leupeptin, 200 μM PMSF, 5 mM NaF, 1 mM vanadate and 1 mM sodium pyrophosphate]. The lysates were collected into Microfuge tubes and incubated on ice for 15 min to effect complete cell lysis. Particulate material was pelleted by microcentrifugation at 13000 g for 15 min at 4 °C. Protein concentrations in the supernatant were determined using Coomassie Blue binding, and 200 μg of protein was incubated with 0.8 μg of an anti-Akt IgG overnight on ice; 20 μl of a Protein G–agarose bead suspension was then added and the mixture was incubated for 1 h at 4 °C. The beads were pelleted by centrifugation at 13000 g for 1 min and then washed three times with ice-cold lysis buffer (without inhibitors), once with ice-cold water, and once with room-temperature kinase assay buffer (20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 1 mM dithiothreitol). The pellet was resuspended in 40 μl of kinase assay buffer containing 5 μg of histone 2B (100 μg/ml final concentration). The reaction was initiated by the addition of 5 μM ATP (containing 10 μCi of [γ-^{32}P]ATP), and continued for 15 min at room temperature. The reaction was terminated by the addition of 50 μl of 2 × sample buffer, and the sample was boiled for 2 min. The beads were pelleted and the supernatant proteins were separated by SDS/12.5 %-PAGE. Histone bands were identified by staining and then quantified by liquid-scintillation counting after drying.

Immunoblotting

PC12 cells were serum-deprived for 4 h to reduce basal phosphorylation levels, and then treated with growth factors and/or inhibitors as indicated for each experiment. Following treatment, the cells were washed twice with ice-cold TBS and then samples were prepared as described for the Akt assay. Equal amounts of protein (80 μg, unless otherwise indicated) were separated by electrophoresis and then transferred to a nitrocellulose membrane. The membrane was blocked for 1 h in 5 % (w/v) non-fat dry milk in 10 mM Tris (pH 7.4)/150 mM NaCl/0.1 % Tween-20 (TBST) and then with the relevant primary antibody (diluted in blocking buffer) either overnight at 4 °C (phospho-specific antibodies) or for 1 h at room temperature (all others). Following extensive washing with TBST, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h, washed extensively, and then developed using ECL® (Amersham). Some blots were stripped in 62.5 mM Tris/HCl (pH 6.8)/2 % SDS/100 mM 2-mercaptoethanol for 30 min at 50 °C and then re-probed with a second antibody.

PKC translocation assay

PC12 cells were plated at 8×10^6 cells/10 cm tissue culture dish, and on the following day were washed twice with TBS and incubated in serum-free DMEM for 4 h at 37 °C. Cells were treated with 100 ng/ml NGF, 10 ng/ml bFGF or 100 nM PMA ('TPA') for 2 min, then washed twice with ice-cold TBS, scraped into 1 ml of ice-cold lysis buffer (20 mM Tris, pH 7.5 at 4 °C, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 1 mM orthovanadate, 5 mM NaF, 200 μM PMSF and 40 μM leupeptin) and lysed with ten strokes of a Dounce homogenizer. The homogenate (800 μl) was centrifuged at 1000 g for 5 min to pellet a nuclear fraction, and then the supernatant was centrifuged at 100000 g for 1 h to produce crude membrane and cytosolic fractions. The pellet was washed twice with TBS and resuspended in 800 μl of lysis buffer by sonication. Samples, equivalent to 150 μg in total before fractionation, were separated by SDS/10 %-PAGE, transferred to nitrocellulose and blotted with anti-PKCδ antibodies, as described above.

RESULTS

Rescue of serum-deprived PC12 cells by NGF and bFGF is differentially affected by inhibition of PI 3-kinase

Activation of PI 3-kinase is involved in the inhibition of apoptosis in a number of cell types ([1,15]; see [16] for a review), so we used

© 2000 Biochemical Society

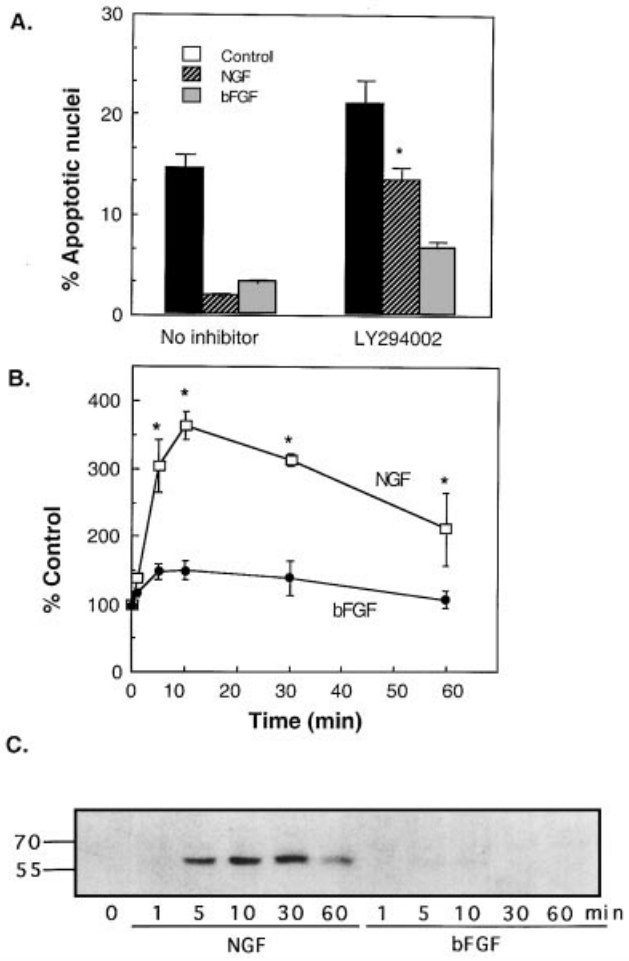

Figure 1 PI 3-kinase and Akt are involved in the inhibition of apoptosis in PC12 cells by NGF, but not that by bFGF

(**A**) PC12 cells were treated with 10 μM LY294002 in the presence or absence of 100 ng/ml NGF or 10 ng/ml bFGF in serum-free media, and 24 h later were scored for apoptotic nuclei. Results are means $\pm$ S.E.M. ($n = 5$); $^*P < 0.0001$ for growth factor alone compared with growth factor + LY294002. LY294002 itself caused a slight rise in apoptosis that was significant to $P = 0.046$. (**B**) PC12 cells were treated for 0–60 min with either 100 ng/ml NGF or 10 ng/ml bFGF. Akt was immunoprecipitated and an *in vitro* kinase assay was performed using histone 2B as substrate. Activity was quantified by liquid-scintillation counting of the histone bands from a stained and dried SDS/12.5%-PAGE gel, and is presented as a percentage of the basal activity in the absence of growth factor. Results are means $\pm$ S.E.M. ($n = 4$); $^*P < 0.0001$ by single-factor analysis of variance (relative to untreated cells). (**C**) PC12 cells were treated for 0–60 min with either 100 ng/ml NGF or 10 ng/ml bFGF. Lysate protein samples were blotted with an anti-phosphoAkt antibody (1:1000), followed by an anti-rabbit horseradish peroxidase-conjugated secondary antibody, and the blot was developed by ECL®. Total Akt levels were similar in each lane (results not shown).

the relatively specific inhibitor LY294002 [17] to examine the role of PI 3-kinase in the rescue of serum-deprived cells by NGF and bFGF. Cultures were treated with 10 μM LY294002 in the presence or absence of growth factors at the time of serum withdrawal. This concentration of LY294002 was found to effectively block the PI 3-kinase-dependent activation of Akt by NGF (results not shown; cf. [1,18] for similar data in PC12 cells). The inhibition of PI 3-kinase by LY294002 resulted in a 60% decrease in rescue of the cells by NGF, while having only a weak, statistically insignificant, effect on inhibition of apoptosis by bFGF (Figure 1A).

NGF, but not bFGF, activates Akt

The failure of PI 3-kinase inhibition to affect rescue by bFGF led us to examine whether this pathway is significantly activated by bFGF. Earlier work had shown only a slight activation of PI 3-kinase by bFGF in PC12 cells [9], similar to results in other cell types [19]. Consequently we examined whether the kinase activity of Akt, a downstream effector of PI 3-kinase [16,20], was increased by bFGF. PC12 cells were treated for 0–60 min with either NGF or bFGF, followed by immunoprecipitation of Akt and an *in vitro* kinase assay using histone 2B as a substrate (Figure 1B). While NGF treatment caused a strong, sustained increase in Akt kinase activity, bFGF treatment resulted in a weak and transient increase in enzyme activity. To corroborate these results, the phosphorylation state of Akt, reflective of its activation [21], was determined using an antibody that recognizes pSer-473 in Akt. As with the assay of Akt activity, NGF treatment gave rise to a strong, sustained elevation of Akt phosphorylation, while bFGF treatment had little effect on basal phosphorylation levels (Figure 1C). These experiments confirm that bFGF activates PI 3-kinase and Akt only feebly, suggesting that this factor must utilize other signalling pathways to exert its anti-apoptotic effect.

Role of the Ras/MAP kinase pathway in the inhibition of apoptosis by bFGF

Treatment of PC12 cells with either NGF or bFGF results in a transient peak followed by a sustained plateau activation of MAP kinase [22]. Several studies suggest that this pathway is involved in differentiation [6–8,23], although one study has also implicated it in the NGF block of apoptosis [24]. To look at the role of MAP kinase activation in the inhibition of apoptosis we used U0126, an inhibitor of the MAP kinase activator MEK [25]. We confirmed that U0126 was more potent than PD98059 in blocking MAP kinase activation in PC12 cells (cf. [26]) and did not result in an increase in background apoptosis, as seen with PD98059 (cf. [27]). Pretreatment of PC12 cells with 50 μM U0126, which completely blocked activation of ERK1 and ERK2 by NGF and bFGF, had no effect on the inhibition of apoptosis by NGF, but significantly inhibited rescue of the cells by bFGF (Figure 2A). These results suggest that activation of MEK and MAP kinase is involved in the anti-apoptotic action of bFGF, but not that of NGF.

As an independent measure of the role of the Ras/MAP kinase pathway, we asked whether inhibition of Ras function would significantly alter rescue of the cells by either NGF or bFGF. In agreement with the pharmacological data, the ability of bFGF to prevent apoptosis was reduced in PC12 cells expressing a dominant-negative RasN17 mutant, while rescue by NGF was unaffected (Figure 2B; cf. [1]). We confirmed that activation of MAP kinase by both NGF and bFGF was blocked in these cells (Figure 2C). Therefore the prevention of apoptosis by bFGF is due, at least in part, to activation of the Ras/MAP kinase cascade, whereas this pathway seems to play little role in antagonism of apoptosis by NGF under the conditions of these experiments.

Involvement of PKC in bFGF anti-apoptotic signalling

As inhibition of either Ras or MEK only partially blocked rescue of PC12 cells by bFGF, we reasoned that a second signal emanating from the bFGF receptor, separate from the Ras/MAP kinase signal, might also contribute to the anti-apoptotic effects of bFGF. Previous work has shown that bFGF activates various members of the PKC family [28,29], and that PC12 cells express

© 2000 Biochemical Society

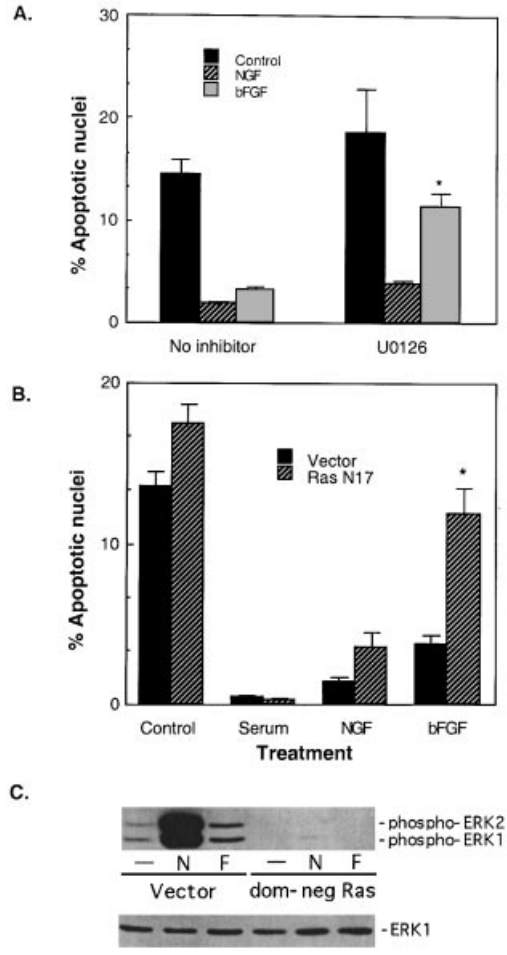

Figure 2 Effect of inhibition of Ras or MEK on NGF and bFGF anti-apoptotic signalling

(**A**) Following serum withdrawal, PC12 cells were treated with 50 μM U0126 in the presence or absence of 100 ng/ml NGF or 10 ng/ml bFGF and scored for apoptotic nuclei 24 h later. Results are means $\pm$ S.E.M. ($n = 4$); $^*P < 0.0001$ for growth factor alone compared with growth factor + U0126. (**B**) PC12 cells constitutively expressing either vector alone or dominant-negative RasN17 were left untreated or were treated with 10% serum, 10 ng/ml NGF or 1 ng/ml bFGF (lower concentrations of growth factors were used here so as not to 'overwhelm' the dominant-negative phenotype) following serum withdrawal; 24 h later the cells were scored for apoptotic nuclei. Data are means $\pm$ S.E.M. ($n = 5$–7); $^*P < 0.005$ for vector compared with RasN17 cells. (**C**) Cells expressing vector or dominant-negative (dom-neg) Ras were treated with either NGF (N) or bFGF (F) and then immunoblotted with an anti-phosphoERK antibody (upper panel), stripped and then re-probed with a pan-ERK antibody (lower panel) to demonstrate equal loading.

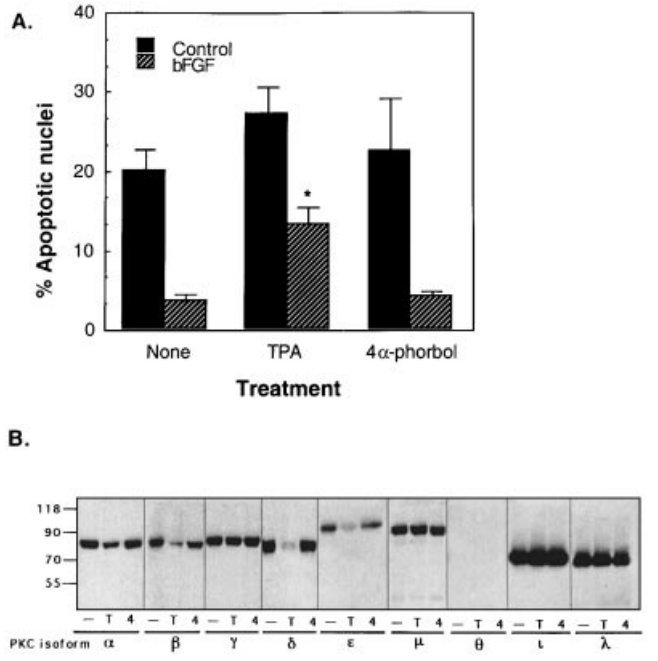

Figure 3 Down-regulation of PKC and its effect on bFGF-induced inhibition of apoptosis and PKC isoform expression

(**A**) PC12 cells were pretreated for 24 h with the phorbol ester PMA (TPA; 100 nM) or its negative control compound 4α-phorbol (100 nM). Following serum withdrawal, cells were treated with 10 ng/ml bFGF in the continued presence of the inhibitors for 24 h. Apoptotic nuclei were then scored in the usual manner. Data are means $\pm$ S.E.M. ($n = 5$–7); $^*P < 0.05$ for growth factor alone compared with growth factor + inhibitor. (**B**) PC12 cells were left untreated (—) or were incubated for 24 h with either 100 nM PMA (T) or 100 nM 4α-phorbol (4). Extracts from these cells (80 μg of protein) were then separated by SDS/PAGE and immunoblotted with PKC-isoform-specific antibodies followed by ECL® detection. Positions of molecular-mass markers are indicated on the left.

the PKC isoforms α, β_I, β_{II}, γ, δ, ϵ, ζ, ι, λ and μ [28]. Chronic treatment with the tumour-promoting phorbol esters down-regulates some isoforms of PKC; therefore, as a first step in determining a possible role for PKC in anti-apoptotic signalling, PC12 cells were pretreated for 24 h either with the phorbol ester PMA or with the negative control compound 4α-phorbol. Following serum withdrawal, inhibition of apoptosis by bFGF was partially blocked in cells that had been chronically treated with PMA, whereas the negative control had no effect (Figure 3A), suggesting that one or more PKC isoforms plays a role in inhibition of apoptosis by bFGF.

To narrow the range of PKC isoforms that may be involved in the trophic effect, we determined which isoforms were affected by chronic PMA treatment (Figure 3B). Only PKC isoforms α, β, δ and ϵ showed decreased levels following chronic phorbol ester treatment (the partial decrease with the α and β isoforms was probably due to treatment being for only 24 h), suggesting that one or more of these isoforms might be relevant to the trophic effect. The levels of PKC isoforms γ, μ, λ, ζ and ι were unaffected, indicating that these isoforms are unlikely to be involved in anti-apoptotic signalling. Based on these data, we then tested a panel of purportedly isoform-selective PKC inhibitors for their ability to antagonize bFGF inhibition of apoptosis, using NGF as a negative control (Figure 4A). Rescue of the cells by bFGF was partially blocked by both Gö 6983 (inhibits PKC α, β, γ and δ subtypes at the concentration used) and rottlerin (δ-selective; [30]), but was unaffected by bisindoylmaleimide-I (inhibits isoforms α, β and γ at the concentration used [31]). These results mitigate against a role for PKCα, β or γ, leaving PKCδ as a possible candidate for mediating the effects of bFGF. However, this conclusion is tempered by the ambiguous nature of purely pharmacological experiments. For example, rottlerin treatment elicited non-specific cell cytotoxicity (Figure 4A, control), which was evident even at the reported IC_{50} for the compound's effect on PKCδ (5 μM; results not shown). In addition, the abolition of NGF-mediated anti-apoptotic signalling by Gö 6976 might have been attributable to the inhibition of PKCμ. However, it has been demonstrated recently that Gö 6976 acts as a potent direct inhibitor of the Trk-family receptor tyrosine kinases ([32]; M. M. Wert, unpublished work); thus the effect of this drug on rescue

© 2000 Biochemical Society

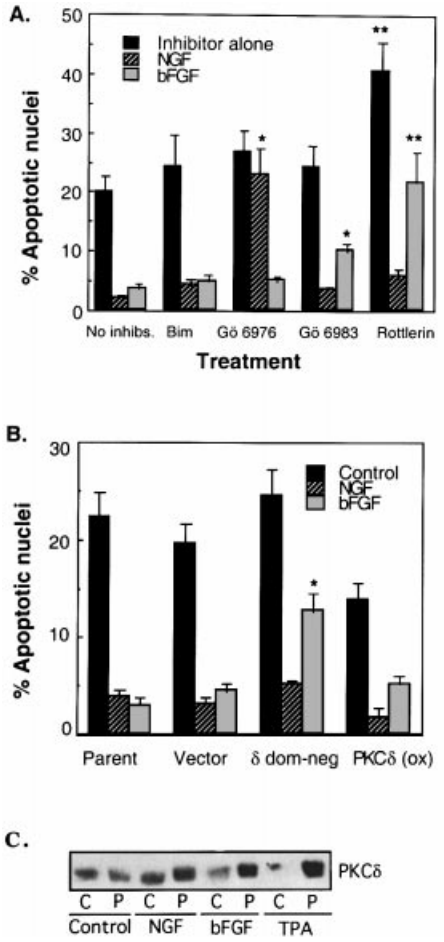

Figure 4 Effects of isoform-specific PKC inhibitors on the anti-apoptotic effect of bFGF

(**A**) Following serum withdrawal, PC12 cells were treated with isoform-specific PKC inhibitors in the presence or absence of NGF or bFGF. The treatments are, from left to right: growth factor alone; 300 nM bisindoylmaleimide-I (Bim), which inhibits the α, β and γ isoforms ($n = 5$); 100 nM Gö 6976, which inhibits the α, β and μ isoforms ($n = 7$–8); 100 nM Gö 6983, which inhibits α, β, γ and δ isoforms ($n = 7$–8); and 20 μM rottlerin, which inhibits the δ isoform ($n = 6$–7). All data are means $\pm$ S.E.M.; $^{*}P \leqslant 0.001$, $^{**}P < 0.01$ for no inhibitor compared with growth factor + inhibitor. Note the increase in apoptotic nuclei found in cells treated with rottlerin alone. (**B**) Parental PC12 cells, PC12 cells transfected with vector alone, a dominant-negative inhibitory fragment of PKCδ (δ dom-neg) or full-length PKCδ [PKCδ (ox)] were serum deprived and treated with either 100 ng/ml NGF or 10 ng/ml bFGF for 24 h, and scored for apoptotic nuclei. Data are means $\pm$ S.E.M. ($n = 6$); $^{*}P \leqslant 0.001$ compared with vector controls. (**C**) PC12 cells were treated for 2 min with 100 ng/ml NGF, 10 ng/ml bFGF or 100 nM PMA (TPA), and separated into crude cytosolic (C) and particulate (P) fractions. Extracts of each fraction were then immunoblotted with an anti-PKCδ antibody. Note the increased presence of membrane-associated enzyme in the treated samples. A typical experiment, which was repeated three times, is shown.

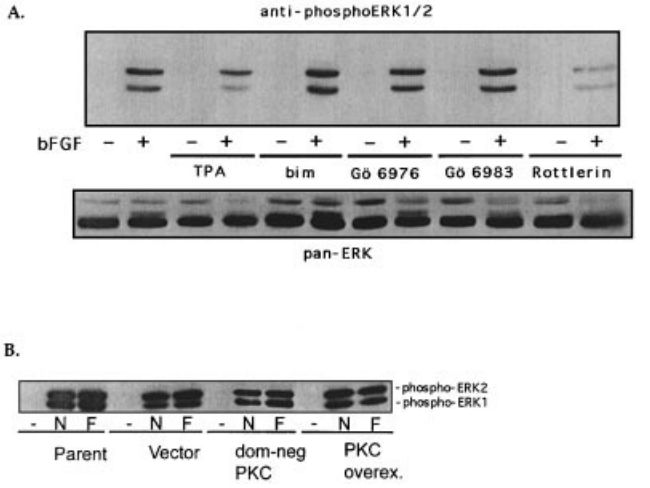

Figure 5 Effect of PKC inhibition on MAP kinase activation

(**A**) PC12 cells were pretreated for 24 h with PMA (TPA) or for 30 min with the indicated inhibitor (bim, bisindoylmaleimide-I), and then treated for 5 min with 10 ng/ml bFGF. Cell lysate protein samples were separated by SDS/10 %-PAGE and then blotted with an anti-phosphoERK1/2 antibody, stripped, and re-probed with a pan-ERK antibody. (**B**) Parent PC12 cells or PC12 cells expressing dominant-negative (dom-neg) PKCδ, full-length PKCδ (overex.) or its vector were treated for 0 or 5 min with either 10 ng/ml NGF or 10 ng/ml bFGF, and then lysate proteins (75 μg/lane) were subjected to immunoblotting with an anti-phosphoERK1/2 antibody.

from apoptosis by NGF is likely to stem from inhibition of TrkA kinase activity rather than antagonism of PKCμ.

To investigate further the role of PKCδ in the prevention of apoptosis by bFGF, we took a genetic approach. In PC12 cell lines expressing a dominant-negative PKCδ fragment, derived from the first variable domain of the enzyme, the translocation and activation of the full-length PKC isoform is selectively blocked (cell line v1δ1 in [14]; see also [33]). If PKCδ were involved in the delivery of an anti-apoptotic signal from either bFGF or NGF receptors, then the effects of these factors should be reduced in these cells. Indeed, we found significant inhibition of bFGF anti-apoptotic signalling following serum withdrawal in the same cell line (δ dom-neg in Figure 4B), but NGF anti-apoptotic signalling was unaffected. However, simple over-expression of the full-length isoform alone was insufficient to inhibit apoptosis following serum withdrawal [PKCδ (ox) in Figure 4B].

Finally, if PKCδ participates in bFGF signalling, it should be possible to demonstrate activation of the kinase by growth factor treatment of cells. One method of detecting PKC activation is translocation of the protein from the cytosol to the membrane, and we therefore used this measure to confirm that bFGF activates PKCδ. PC12 cells were treated with bFGF (using NGF and PMA as controls), separated into cytosolic and crude post-nuclear membrane fractions, and then assayed by immunoblotting with a PKCδ-specific antibody (Figure 4C). All three treatments resulted in the translocation of a portion of PKCδ from the soluble fraction to a crude post-nuclear particulate fraction, confirming that bFGF does activate PKCδ under the conditions of our experiments. Taken together, these data support the view that bFGF anti-apoptotic signalling occurs, in part, via activation of PKCδ.

Effect of PKC inhibition on MAP kinase activation

Since bFGF anti-apoptotic signalling encompasses both the Ras/MAP kinase and PKC signalling cascades, we sought to determine whether there was a direct interaction between these apparently separate pathways. To do this we first examined the effect of the isoform-specific PKC inhibitors on activation of MAP kinase by bFGF (Figure 5A). Gö 6983, a fairly specific inhibitor of PKCδ, did not affect MAP kinase phosphorylation, while chronic PMA treatment or rottlerin, which are less specific in their effects, caused a decrease in MAP kinase phosphorylation. To clarify these contradictory results, we again utilized the PC12 cells expressing the dominant-negative PKCδ fragment or full-

© 2000 Biochemical Society

Table 1 Additive effects of inhibition of PKCδ and Ras on bFGF anti-apoptotic signalling

Following serum withdrawal, PC12 cells expressing either RasN17 or empty vector were treated with 100 nM Gö 6983 alone or together with 10 ng/ml NGF or 1 ng/ml bFGF for 24 h. Cells were then scored for apoptotic nuclei. Data are means ± S.E.M. ($n = 6$–8); $^*P \leqslant 0.005$ for vector compared with RasN17.

	Apoptotic cells (%)		
Cell type	Gö 6983	NGF + Gö 6983	bFGF + Gö 6983
Vector control ($n = 6$)	14.4 ± 0.5	2.1 ± 0.5	10.4 ± 0.7
RasN17 ($n = 8$)	19.5 ± 2.3	2.7 ± 0.6	20.7 ± 2.5*

length PKCδ. Confirming the results with Gö 6983, MAP kinase activation in response to NGF or bFGF was unaffected in these cell types (Figure 5B). Together, these data suggest that the Ras/MAP kinase and PKCδ anti-apoptotic signals act independently rather than via cross-talk of one pathway with the other.

Additive effects of Ras/MAP kinase and PKCδ inhibition in anti-apoptotic signalling

To assess whether the two anti-apoptotic signals were acting additively, we treated dominant-negative Ras-expressing PC12 cells with 100 nM Gö 6983 in the presence or absence of bFGF. Antagonism of both Ras and PKCδ in the dominant-negative Ras cells exerted a significantly greater effect on bFGF anti-apoptotic signalling than Ras inhibition alone. Similarly, PKCδ inhibition of bFGF anti-apoptotic signalling in the vector cells was significantly different from the PKCδ/Ras inhibition in the dominant-negative expressing cells (Table 1). These results suggest that bFGF anti-apoptotic signalling occurs by the addition of Ras/MAP kinase- and PKCδ-dependent signals.

DISCUSSION

The action of trophic factors in the nervous system is important for the maintenance of normal cellular function both during development and in the mature organism. NGF support of serum-deprived undifferentiated and NGF-differentiated PC12 cells, as well as of primary sympathetic neurons, have been useful paradigms to define the pathways involved in trophic factor survival signalling [1,2,11,34–36]. The PI 3-kinase/Akt pathway has emerged as a central effector in the anti-apoptotic effects of NGF [1,11] and is involved in the response to other growth factors in various cell types [15,16]. While it is apparent that some factors work by different routes in other cells, it is less clear whether different growth factors can activate separate pathways to rescue the same cell type. NGF and bFGF have similar effects on PC12 cells: both induce morphological and biochemical differentiation and block apoptosis following serum deprivation. However, while common signalling events may be involved in the differentiation response, these polypeptides inhibit apoptosis via distinct mechanisms. As demonstrated previously, inhibition of apoptosis by NGF stems predominantly from activation of the PI 3-kinase/Akt pathway, but we show here that the trophic action of bFGF does not involve Akt, but instead requires both MAP kinase and PKCδ signalling.

Our initial experiments confirmed that bFGF only weakly and transiently stimulates the PI 3-kinase pathway in PC12 cells [9], in that its activation of Akt was poor compared with that by NGF. As bFGF also activates both the Ras/MAP kinase cascade and PKC signalling, we focused our attention on these pathways as possible mediators of the anti-apoptotic effects of this trophic factor. Interference with either Ras or MEK signalling was sufficient to partially block inhibition of apoptosis by bFGF, while having no effect on rescue by NGF. While inhibition of Ras or MEK activity partially blocked the inhibition of apoptosis by bFGF, it did not completely eliminate it, suggesting the intriguing possibility of a second anti-apoptotic signal generated by bFGF. Using three independent approaches, we showed that the remaining signal appears to come from activation of a specific PKC isoform by bFGF.

Down-regulation of the PKC α, β, δ and ϵ isoforms in PC12 cells by chronic PMA treatment led to significant decreases in the ability of bFGF to block apoptosis following serum withdrawal. Furthermore, the use of four relatively isoform-selective pharmacological inhibitors indicated a possible role for PKCδ in bFGF anti-apoptotic signalling. Although Gö 6983 (which inhibits PKC isoforms α, β, γ and δ) partially blocked the bFGF effect, bisindoylmaleimide-I (which inhibits PKC isoforms α, β and γ) had no effect on the rescue by bFGF, arguing against a role for PKC isoforms α, β and γ, but for a role for PKCδ. Interpretation of the results with rottlerin (which inhibits PKCδ), although also supportive of a role for PKCδ in blocking apoptosis, was compromised by non-specific cytotoxic effects (in addition to its inhibition of protein kinases such as calcium/calmodulin-dependent protein kinase III [30]); thus we turned to a genetic approach to investigate further the role of PKCδ in bFGF signalling. In PC12 lines in which PKCδ activation was blocked through expression of a dominant-negative PKCδ fragment [14,33], a partial blockade of bFGF-dependent rescue was seen, confirming a role for PKCδ in bFGF anti-apoptotic signalling.

With the identification of two potential anti-apoptotic pathways in response to bFGF, the question arose as to whether trophic signalling results from an additive effect of the Ras/MAP kinase and PKCδ pathways, or whether one signalling pathway impinges directly downstream on the other to magnify its effect. Our data support the first hypothesis. Treatment of cells expressing the dominant-negative fragment of PKCδ with either NGF or bFGF had no effect on MAP kinase phosphorylation, suggesting that signalling via PKC was independent of an effect on the MAP kinase pathway. Moreover, only inhibition of both pathways, as seen when PC12 cells expressing RasN17 were treated with the PKCδ inhibitor Gö 6983, completely blocked inhibition of apoptosis by bFGF.

A broader problem exposed by our experiments is why the activation of similar pathways by NGF and bFGF yields different outcomes. For example, both growth factors activate the Ras/MAP kinase pathway as part of a differentiative signal, yet bFGF also activates it as part of its anti-apoptotic signal. Thus, when activation of PI 3-kinase by NGF is blocked by LY294002, why is NGF's unabated activation of MAP kinase insufficient to promote cell survival? One possibility is that NGF and bFGF may activate a given effector differentially, either temporally or via distinct upstream effectors. For example, the cytoplasmic adapter protein FRS-2 (bFGF receptor substrate-2) binds to the FGFR-1 receptor and acts as a docking site for multiple signalling proteins. FRS-2 is apparently required for bFGF signalling to MAP kinase, and contains phosphotyrosine-containing sequences to which Grb2 can bind [37]. By contrast, NGF signalling to the Ras/MAP kinase pathway is accomplished principally through the association of Shc with Tyr-490 on the Trk receptor tyrosine kinase domain [38,39]. FRS-2 is not required for NGF signalling, although recent work suggests that FRS-2 may actually compete with Shc for binding to Trk

© 2000 Biochemical Society

receptors [40]. Precisely how MAP kinases are activated may thus determine the kinetics and magnitude of downstream responses.

Finally, the question arises as to how the Ras/MAP kinase and PKC signals are merged to yield complete inhibition of apoptosis by bFGF. While our data suggest that the pathways do not affect each other directly, their convergence at common downstream effectors remains a possibility. For example, one candidate could be the transcription factor c-Jun, the activity of which appears to play a key role in the regulation of apoptosis [41–43]. Both Ras/MAP kinase and PKC signalling have been shown to increase c-Jun transcription [23,44], supporting its possible identity as a common downstream effector. Alternatively, bFGF may delay cell death by preventing activation of apoptosis effectors. The level at which bFGF effectors interact with the apoptotic machinery will be an interesting area for further investigation.

We thank Dr R.O. Messing for the dominant-negative and overexpressing PKCδ PC12 cells, Dr G. M. Cooper for RasN17-expressing PC12 cells, and Dr N. Hay for critical reading of the manuscript.

REFERENCES

1 Yao, R. and Cooper, G. M. (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science **267**, 2003–2006

2 Anderson, C. N. G. and Tolkovsky, A. M. (1999) A role for MAPK/ERK in sympathetic neuron survival: Protection against a p53-dependent, JNK-independent induction of apoptosis by cytosine arabinoside. J. Neurosci. **19**, 664–673

3 Cory, S. and Adams, J. M. (1998) Matters of life and death: programmed cell death at Cold Spring Harbor. Biochim. Biophys. Acta **1377**, R25–R44

4 Salvesen, G. S. and Dixit, V. M. (1997) Caspases: Intracellular signaling by proteolysis. Cell **91**, 443–446

5 Greene, L. A. (1978) Nerve growth factor prevents the death and stimulates the neuronal differentiation of clonal PC12 pheochromocytoma cells in serum-free medium. J. Cell Biol. **78**, 747–755

6 Marshall, C. J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell **80**, 179–185

7 Qiu, M.-S. and Green, S. H. (1992) PC12 cell neuronal differentiation is associated with prolonged p21^{ras} activity and consequent prolonged ERK activity. Neuron **9**, 705–717

8 Traverse, S., Gomez, N., Paterson, H., Marshall, C. and Cohen, P. (1992) Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Biochem. J. **288**, 351–355

9 Raffioni, S. and Bradshaw, R. A. (1992) Activation of phosphatidylinositol 3-kinase by epidermal growth factor, basic fibroblast growth factor and nerve growth factor in PC12 pheochromocytoma cells. Proc. Natl. Acad. Sci. U.S.A. **89**, 9121–9125

10 Carter, A. N. and Downes, C. P. (1992) Phosphatidylinositol 3-kinase is activated by nerve growth factor and epidermal growth factor in PC12 cells. J. Biol. Chem. **267**, 14563–14567

11 Crowder, R. J. and Freeman, R. S. (1998) Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J. Neurosci. **18**, 2933–2943

12 Haimovitz-Friedman, A., Balaban, N., McLoughlin, M., Ehleiter, D., Michaeli, J., Vlodavsky, I. and Fuks, Z. (1994) Protein kinase C mediates basic fibroblast growth factor protection of endothelial cells against radiation-induced apoptosis. Cancer Res. **54**, 2591–2597

13 Gardner, A. M. and Johnson, G. L. (1996) Fibroblast growth factor-2 supression of tumor necrosis factor α-mediated apoptosis requires Ras and the activation of mitogen-activated protein kinase. J. Biol. Chem. **271**, 14560–14566

14 Hundle, B., McMahon, T., Dadger, J., Chen, C.-H., Mochly-Rosen, D. and Messing, R. O. (1997) An inhibitory fragment derived from protein kinase Cϵ prevents enhancement of nerve growth factor responses by ethanol and phorbol esters. J. Biol. Chem. **272**, 15028–15035

15 Kennedy, S. G., Wagner, A. J., Conzen, S.D., Jordán, J., Bellacosa, A., Tsichlis, P. N. and Hay, N. (1997) The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev. **11**, 701–713

16 Kandel, E. S. and Hay, N. (1999) The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp. Cell Res. **253**, 210–229

17 Vlahos, C. J., Matter, W. F., Hui, K. Y. and Brown, R. F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J. Biol. Chem. **296**, 5241–5246

18 Yamamoto-Honda, R., Tobe, K., Kaburagi, Y., Ueki, K., Asai, S., Yachi, M., Shirouzu, M., Yodoi, J., Akanuma, Y. and Yokoyama, S. (1995) Upstream mechanisms of glycogen synthase activation by insulin and insulin-like growth factor-I. Glycogen synthase activation is antagonized by wortmannin or LY294002 but not by rapamycin or by inhibiting p21ras. J. Biol. Chem. **270**, 2729–2734

19 van Weering, D. H. J., de Rooij, J., Marte, B., Downward, J., Bos, J. L. and Burgering, B. M. T. (1998) Protein kinase B activation and lamellipodium formation are independent phosphoinositide 3-kinase-mediated events differentially regulated by endogenous Ras. Mol. Cell. Biol. **18**, 1802–1811

20 Coffer, P. J., Jin, J. and Woodgett, J. R. (1998) Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem. J. **335**, 1–13

21 Alessi, D. R., Andjelkovíc, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B. A. (1996) Mechanisms of activation of protein kinase B by insulin and IGF-1. EMBO J. **15**, 6541–6551

22 Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H. and Yancopoulos, G. D. (1991) ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell **65**, 663–675

23 Szeberényi, J., Cai, H. and Cooper, G. M. (1990) Effect of a dominant inhibitory Ha-*ras* mutation on neuronal differentiation of PC12 cells. Mol. Cell. Biol. **10**, 5324–5332

24 Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. and Greenberg, M. E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science **270**, 1326–1330

25 Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F. et al. (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J. Biol. Chem. **273**, 18623–18632

26 Kamakura, S., Moriguchi, T. and Nishida, E. (1999) Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus. J. Biol. Chem. **274**, 26563–26571

27 Parrizas, M., Saltiel, A. R. and LeRoith, D. (1997) Insulin-like growth factor 1 inhibits apoptosis using the phosphatidylinositol 3′-kinase and mitogen-activated protein kinase pathways. J. Biol. Chem. **272**, 154–161

28 Wooten, M. W., Zhou, G., Wooten, M. C. and Seibenhener, M. L. (1997) Transport of protein kinase C isoforms to the nucleus of PC12 cells by nerve growth factor: association of atypical ζ-PKC with the nuclear matrix. J. Neurosci. Res. **49**, 393–403

29 Ohmichi, M., Zhu, G. and Saltiel, A. R. (1993) Nerve growth factor activates calcium-insensitive protein kinase C epsilon in PC-12 rat pheochromocytoma cells. Biochem. J. **296**, 767–772

30 Gschwendt, M., Muller, H. J., Kielbassa, K., Zang, R., Kittstein, W., Rincke, G. and Marks, F. (1994) Rottlerin, a novel protein kinase inhibitor. Biochem. Biophys. Res. Commun. **199**, 93–98

31 Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursir, E. and Loriolle, F. (1991) The bisindoylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J. Biol. Chem. **266**, 15771–15781

32 Behrens, M. M., Strasser, U. and Choi, D. W. (1999) Gö 6976 is a potent inhibitor of neurotrophin-receptor intrinsic tyrosine kinase. J. Neurochem. **72**, 919–924

33 Gerstin, Jr, E H., McMahon, T., Dadgar, J. and Messing, R. O. (1998) Protein kinase Cdelta mediates ethanol-induced up-regulation of L-type calcium channels. J. Biol. Chem. **273**, 16409–16414

34 Mesner, P. W., Epting, C. L., Hegarty, J. L. and Green, S. H. (1995) A timetable of events during programmed cell death induced by trophic factor withdrawal from neuronal PC12 cells. J. Neurosci. **15**, 7357–7366

35 Park, D. S., Stefanis, L., Yan, C. Y. I., Farinelli, S.E. and Greene, L. A. (1996) Ordering the cell death pathway. J. Biol. Chem. **271**, 21898–21905

36 Pittman, R. N., Wang, S., DiBenedetto, A. J. and Mills, J. C. (1993) A system for characterizing cellular and molecular events in programmed neuronal cell death. J. Neurosci. **13**, 3669–3680

37 Kouhara, H., Hadari, Y. R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I. and Schlessinger, J. (1997) A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. Cell **89**, 693–702

38 Kaplan, D. R. and Miller, F. D. (1997) Signal transduction by the neurotrophin receptors. Curr. Opin. Cell Biol. **9**, 213–221

39 Obermeier, A., Lammers, R., Wiesmüller, K.-H., Jung, G., Schlessinger, J. and Ullrich, A. (1993) Identification of Trk binding sites for SHC and phosphatidylinositol 3′-kinase and formation of a multimeric signaling complex. J. Biol. Chem. **268**, 22963–22966

40 Meakin, S. O., MacDonald, J. I. S., Gryz, E. A., Kubu, C. J. and Verdi, J. M. (1999) The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA. J. Biol. Chem. **274**, 9861–9870

© 2000 Biochemical Society

41 Courtney, M. J., Akerman, K. E. O. and Coffey, E. T. (1997) Neurotrophins protect cultured cerebellar granule neurons agains early phase of cell death by a two-component mechanism. J. Neurosci. **17**, 4201–4211
42 Ham, J., Babij, C., Whitfield, J., Pfarr, C. M., Lallemand, D., Yaniv, M. and Rubin, L. (1995) A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. Neuron **14**, 927–939
43 Clark, W. and Gillespie, D. A. F. (1997) Transformation by v-Jun prevents cell cycle exit and promotes apoptosis in the absence of serum growth factors. Cell Growth Differ. **8**, 371–380
44 Hirai, S., Izumi, Y., Higa, K., Kaibuchi, K., Mizuno, K., Osada, S., Suzuki, K. and Ohno, S. (1994) Ras-dependent signal transduction is indispensable but not sufficient for the activation of AP1/jun by PKC delta. EMBO J. **13**, 2331–2340

Received 27 April 2000/18 August 2000; accepted 1 September 2000

© 2000 Biochemical Society