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Overexpression of atypical PKC in PC12 cells enhances NGF-responsiveness and survival through an NF-κB dependent pathway

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Abstract

Removal of atypical PKC blocks NGF-induced differentiation of PC12 cells.[1] We now examine the consequences that overexpression of atypical PKCs had upon NGF responses. PC12 cells were stably transfected with either PKC-ι or PKC-ζ. Overexpression of atypical PKCs markedly enhanced NGF-induced neurite outgrowth as well as enhanced NGF-stimulated JNK kinase. Cotransfection of HA-JNK1 along with increasing concentrations of PKC-ι, resulted in dose-dependent phosphorylation of GST c-Jun (1–79). NGF treatment of PC12 cells resulted in activation of NF-κB. In comparison, overexpression of atypical PKC-ι was by itself sufficient to activate NF-κB and shift the kinetics of NGF-induced κB activity. Furthermore, transfection of full-length antisense PKC-ι blocked basal and NGF-stimulated NF-κB. Differentiated and undifferentiated PC12 cells overexpressing atypical PKC-ι were protected from serum deprivation-induced cell death. Collectively, these findings demonstrate that atypical PKC-ι lies in a pathway that regulates NF-κB and contributes to both neurotrophin-mediated differentiation and survival signaling.

Keywords: NGF; PC12 cells; PKC isoforms; atypical PKC; neuronal differentiation

Abbreviations: PKC, Protein kinase C; NF-κB, nuclear factor kappa beta; NGF, nerve growth factor; PC12, pheocromocytoma cells; MAPK, mitogen activated protein kinase; JNK, Jun N-terminal kinase

Introduction

Protein kinase Cs (PKCs) are a group of ubiquitously expressed serine/threonine kinases which have been implicated in a wide variety of cellular processes, including nerve growth factor (NGF) responses in pheochromocytoma (PC12) cells. Molecular cloning has established that PKC is a multigene family composed of 12 structurally related isoforms which have different tissue distribution, as well as, cofactor and substrate specificities.[2] Based upon structural features the PKC family can be divided into three related groups: classical/conventional PKC (cPKCs) (α, $\beta_{I,II}$, and γ) that are activated by calcium/diacylglycerol and tumor-promoting phorbol esters; novel/nonconventional PKC (nPKCs) (δ, ε, η, μ and θ) that are activated by diacylglycerol and phorbol esters but insensitive to calcium; and atypical PKCs (aPKCs) (ζ and ι/λ) that are insensitive to both diacylglycerol and calcium and neither bind nor are activated by phorbol esters. PKC isoforms are hypothesized to play distinct roles within the context of the cells in which they are expressed.

The precise role and placement of individual PKC isoforms within the NGF signaling cascade has been unclear and controversial due to coexpression of multiple isoforms. Thus, PKC's position within neurotrophin signaling pathways has been understudied. Upon binding of NGF to PC12 cells, diacylglycerol is generated,[3] followed by PKC activation.[4,5] Phorbol esters are PKC activators and mimic certain biological activities of NGF in PC12 cells.[6,7] Likewise, certain NGF-specific transcripts are induced in response to PKC activation.[8,9] A requirement for PKC as part of the induction pathway leading to NGF-stimulated neurite outgrowth has also been documented. The PKC inhibitor sphingosine blocks NGF-induced neurite outgrowth in PC12 cells,[10] and microinjection of PKC antibodies inhibits NGF-induced neurite outgrowth and c-*fos* expression.[8] Collectively, these studies support a role for PKC in mediating NGF's effects. However, down-regulation of PKC with chronic phorbol ester treatment,[11] resulting in removal of classical and nonclassical PKC pools, has no effect on NGF-induced neurite outgrowth[12] nor NGF-induced early and secondary responsive gene expression.[13,14] Based upon these observations we suggested that the cPKC and nPKC isoforms were not required for NGF-mediated differentiation responses. We further demonstrated that NGF activated the phorbol ester insensitive/atypical PKC-ζ isoform[12] and its removal attenuated NGF responsiveness.[1] Thus, the primary response of PC12 cells to NGF is characterized by a signaling pathway that is phorbol ester-insensitive and involves atypical PKCs.

We reasoned that overexpression of atypical PKCs might enhance NGF-responsiveness, since NGF effects were attenuated by removal of atypical PKC isoforms.[1] In the present study, we generated PC12 cell lines that overexpress atypical ζ- to ι-PKC to investigate their role in NGF signaling. We found that overexpression of PKC-ι activates NF-κB and enhances PC12 cell survival. Our results demonstrate that atypical PKCs contribute to both

neurorophin-mediated differentiation and survival signaling through a pathway that involves JNK and NF-κB.

Results

Overexpression of atypical PKC enhances NGF responsiveness

To investigate the interaction of atypical PKC in the context of NGF signaling both PKC-ζ and PKC-ι/λ were over expressed in PC12 cells (Figure 1A). Both plasmids have a neo-selectable marker that confers resistance to geneticin. Cells were selected for their ability to grow in the presence of geneticin and pooled into a mass population of stable transfectants. Overexpression of dominant negative constructs of either PKC-ζ or -ι resulted in cell death, demonstrating a critical need for these isoforms in cell survival, which is consistent with previous findings.[15,16] The level of isoform expression was determined by the presence of protein. As determined by immunoblotting, atypical PKC-ι was expressed ninefold whereas PKC-ζ was expressed threefold greater in the transfectants compared to the parental counterpart. To address possible perturbation in the expression of other PKC isoforms brought on by overexpression of atypical PKC, we examined PKC-α, PKC-δ, and PKC-μ as representative members of both classical and nonconventional isoforms. Overall, no dramatic changes in the levels of the other isoforms were noted. However, overexpression of PKC-ι consistently led to a slight reduction in the levels of PKC-α (Figure 1B). To determine if the transduced enzyme was active, PC12 cells were stimulated with NGF for 15 min, a time previously shown to stimulate maximum activation of aPKC in PC12 cells.[12] Atypical PKC activity was measured using an immunecomplex kinase assay with myelin basic protein as an exogenous substrate (Figure 2). Overexpression enhanced basal activity of PKC-ι. Furthermore, NGF-stimulated aPKC activity was increased in the cells overexpressing PKC-ι. We also examined the subcellular localization of PKC-ζ and PKC-ι in the overexpressors. Immunoreactivity for both isoforms was found throughout the cytoplasm with localized areas of intense staining observed in the perinuclear region of the cell, possibly in the region of the Golgi apparatus (data not shown).

To address changes in the responsiveness of the cells to NGF brought about by overexpression of atypical PKC, we first examined changes in morphological parameters. PC12 cells expressing either PKC-ι or PKC-ζ displayed neurite spikes (Figure 3A–C). NGF-dependent neurite outgrowth was markedly increased in the cells overexpressing aPKC isoforms (Figure 3D–F, Table 1). Overexpression of either aPKC isoform enhanced the numbers of cells responding to NGF, as well as, the average length of neurite elaborated by individual cells. The NGF response was examined over time as well. Differentiation occurred more rapidly in cells overexpressing aPKC isoforms compared to parental counterparts. The overexpressors extended neurites at a rate 30% greater than did their parental counterparts (data not shown). Furthermore, pretreatment of the overexpressors cells with 1 μM PMA for 48 h prior to addition of NGF failed to reduce enhancement of neurite outgrowth, thus documenting that the enhancement response was linked to the expression of the PMA-insensitive PKC isoforms PKC-ι

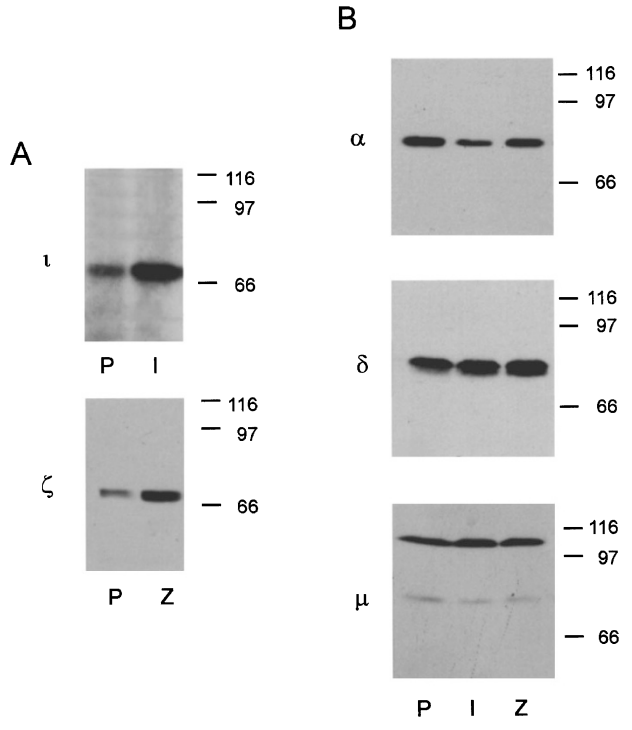

Figure 1 Overexpression of atypical PKC isoforms in PC12 cells. (**A**) Immunoblot analysis of cell lysates (80 μg) prepared from either parental PC12 cells (P) or those stably transfected with expression vectors coding for PKC-ι (I) or -ζ (Z) were analyzed with isoform specific antibody to either PKC-ι or -ζ. (**B**) Immunoblot analysis of the same cell lysates P, I or Z with antibodies to cPKC-α, nPKC-δ or -μ

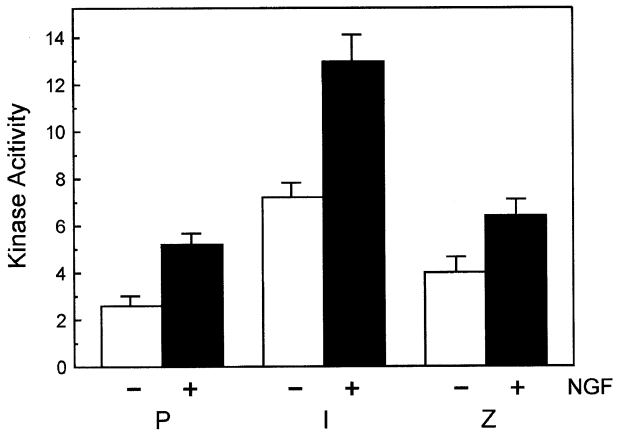

Figure 2 NGF-dependent activation of aPKC. Parental PC12 cells (P) or those stably transfected with expression vectors coding for PKC-ι (I) or -ζ (Z) were stimulated with 50 ng/ml NGF for 15 min. aPKC activity was immunoprecipitated with isoform-specific antibody to either PKC-ι or -ζ. Activity was determined by immunecomplex kinase assay with MBP as substrate. The autoradiogram was scanned and the relative changes in the phosphorylation of MBP was determined. Shown is the mean $\pm$ S.E. of 3–5 determinations

and PKC-ζ. Flow cytometric analysis corroborated the morphological observations. In the absence of NGF, increases in the G1 population of the cell cycle were observed for cells overexpressing aPKC, whereas NGF treatment resulted in further enhancement of the G1 population of cells, indicating that a greater proportion of the cell population had responded to NGF. The cells expressing PKC-ι were more robust in their responsiveness to NGF, in addition to possessing greater NGF-stimulated PKC-ι activity, compared to those expressing PKC-ζ. Therefore we elected to conduct more detailed experiments using cells overexpressing PKC-ι.

PKC-ι is linked to a pathway involving JNK

To gain insight into the possible signaling pathway that mediates aPKC=s ability to enhance NGF responsiveness, we tested whether PD98059 or SB203580 would abrogate the enhancement properties brought about by overexpression of PKC-ι. PD98059 is a well characterized, specific inhibitor of the MAP kinase/ERK pathway, blocking MEK,[17]whereas SB203580 is an inhibitor of p38.[18] To investigate the role of JNK, cells were treated with Curcumin, a recently described, potent inhibitor of JNK.[19] Additionally, cells were treated with either Wortmannin or LY294002, specific inhibitors of PI3 kinase.[20] Inhibition of either MAP kinase, p38 of PI3 kinase by these inhibitors have been shown to inhibit NGF-induced neurite outgrowth.[17,18] Overexpression of PI3 kinase, an activator of PKC-ι,[21] has been shown to induce neurite outgrowth through a JNK-dependent pathway.[22] Therefore, we reasoned that overexpression of PKC-ι may override the effects of the inhibitor and thus enable us to pinpoint which pathway might mediate enhancement of NGF effects brought about by PKC-ι overexpression. Comparing effects the various inhibitors had upon NGF-induced neurite outgrowth in the parental PC12 cells to those cells overexpressing PKC-ι, differential sensitivity to inhibitors that affected the JNK

Table 1 NGF-dependent neurite outgrowth in aPKC transfected cells

Cell line	% Cells with neurites	Neurite length (μM)	% Cells G1[b]
PC12	58±2	52±3	16.7
PKC-ι	93±2[a]	72±1[a]	73.3
PKC-ζ	95±4[a]	68±5[a]	57.0

[a]Significantly different compared with parental PC12 cells but not different between each other (ANOVA). [b]Ten thousand cells were analyzed for distribution of cells thoughout the cell cycle, shown are the percentage of cells in G1. Cells were cultured for 7 days with 50 ng/ml NGF on rat tail collagen coated plates. The percentage of cells with neurites was calculated from 5–7 experiments. Neurite lengths were from 100 neurites per condition in a representative experiment repeated two times with similar results

Table 2 Effect of inhibitors on PKC-ι stimulated enhancement of NGF effects

	% NGF-treatment[a]	
Treatment	**Parental**	**Iota**
NGF 50 ng/ml	100 (60±4)	100 (88±6)
NGF+30 μM PD98059	13 (8±3)	14 (12±9)[c]
NGF+10 μM SB203580	42 (25±7)	41 (36±8)[c]
NGF+10 μM Curcumin	9 (5.5±5)	101 (89±6)[b]
NGF+50 μM LY294002	20 (12±4)	25 (22±6)[c]
NGF+100 nM Wortmannin	33 (20±3)	40 (35±4)[c]

[a]The data are normalized to the % NGF treatment effect of either the parental cells or those overexpressing PKC-ι. Shown in parenthesis are the % cells with neurites for a given treatment effect. [b]Cells overexpressing PKC-ι were significantly different ($P<0.05$) as compared to the parental counterpart determined by Student's *t*-test. [c]Cells overexpressing PCK-ι were not significantly different ($P<0.05$) as compared to the parental counterpart determined by Student's *t*-test. PC12 cells were treated with various inhibitors as indicated. The inhibitors were added for 1 h prior to the addition of NGF for 5 days (50 ng/ml). The percentage of cells with neurites was calculated from three experiments

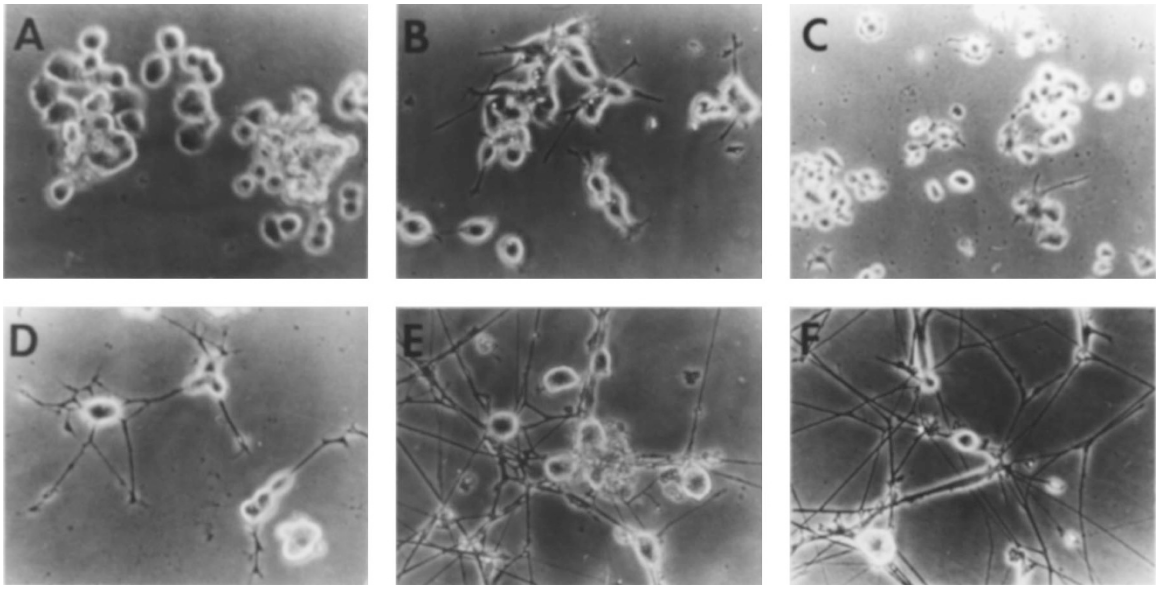

Figure 3 Overexpression of atypical PKC enhances neurite outgrowth. PC12 cells were photographed 7 days post addition of 50 ng/ml NGF: parental cells (**A** and **D**), PC12 cells stably transfected with PKC-ι (**B** and **E**), and PC12 cells stably transfected with PKC-ζ (**C** and **F**)

pathway was observed (Table 2). Overexpression of PKC-ι has a profound effect on the sensitivity of the cells to the JNK inhibitor, Curcumin. These findings reveal that PKC-ι=s enhancement of NGF responsiveness is mediated, in part, by a pathway linked to JNK.

Further studies were undertaken to characterize possible alteration in TrkA receptor, PI3 kinase, as well as, SHC, JNK and MAP kinase. Since aPKC isoforms enhanced NGF-dependent neurite outgrowth through a pathway dependent upon PI3 kinase and JNK, we predicted that aPKC might alter the magnitude or duration of the signal associated with NGF activation of this pathway. To test this hypothesis, we measured NGF-stimulated tyrosine phosphorylation of TrkA, pp110 PI3 kinase, SHC, Jun N-terminal kinase (JNK), pp44/42 mitogen activated protein (MAP) kinase and p38 kinase in cells overexpressing PKC-ι (Figure 4). Overexpression of PKC-ι did not significantly alter NGF-stimulated increases in TrkA nor SHC. Interestingly, overexpression consistently suppressed NGF-induced p38 activity. The basal phosphotyrosine content of pp110 PI3 kinase was higher in the overexpressors and NGF did not appear to stimulate any changes in PI3 kinase activity. In addition, overexpression of PKC-ι altered the pattern of NGF-induced JNK activation, which was amplified in magnitude and occurred in an earlier time frame than that observed in the parental counterpart. The

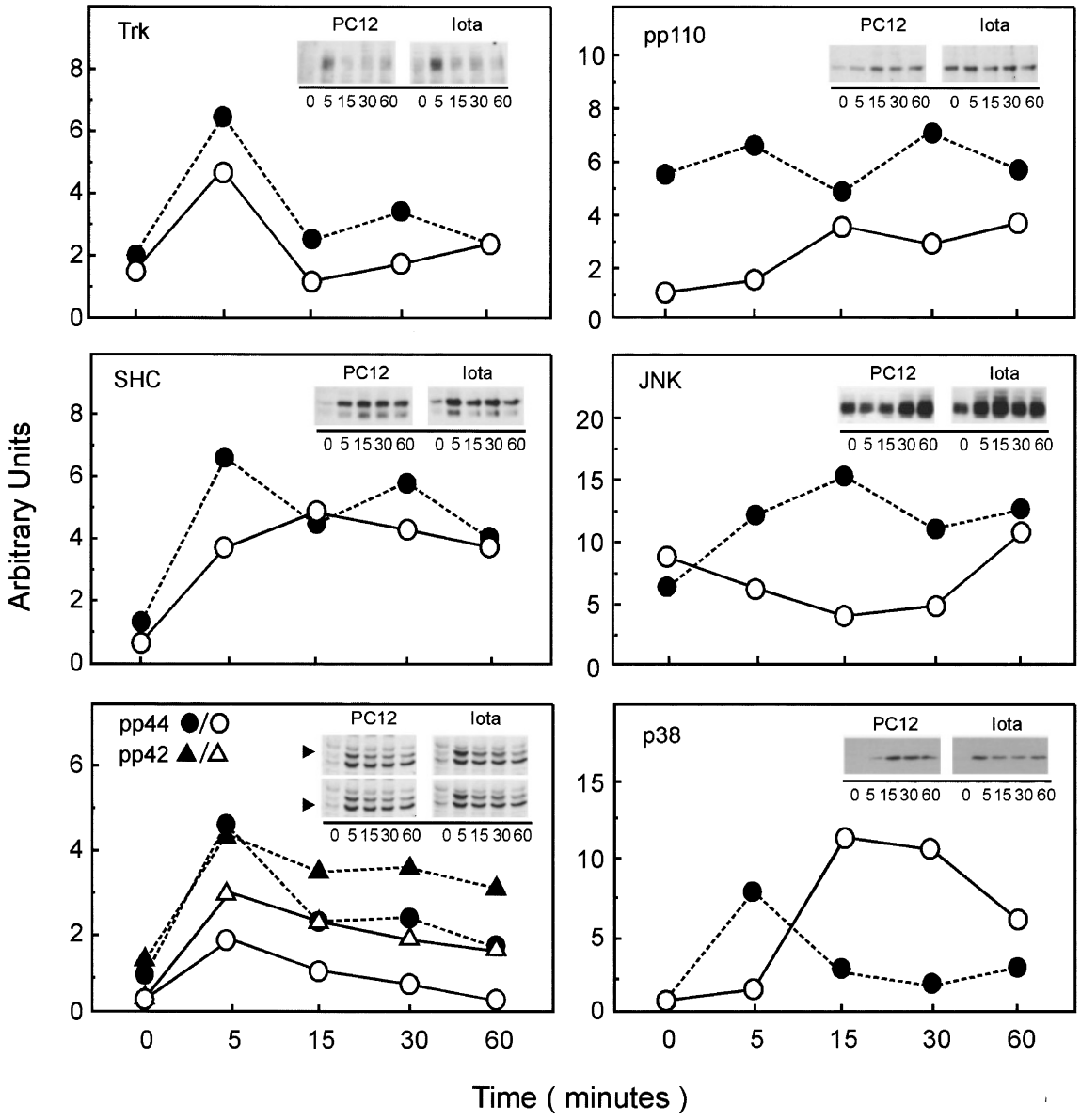

Figure 4 Overexpression of atypical PKC enhances tyrosine phosphorylation. PC12 cells (P: ' –') or those stably overexpressing PKC-ι (I:! – !) were treated as indicated with 100 ng/ml NGF for 0 – 60 min. Cell lysates were prepared and immunoprecipitated with G410 ptyr antibody conjugated to agarose and immunoblotted as indicated with antibody to gp140 TrkA, pp110 PI3 kinase or SHC. Whole cell lysates (80 μg) were immunoblotted post-NGF treatment with phosphospecific antibodies to either JNK or MAP kinase (pp44, pp42). Similar results were obtained in two other experiments

overexpressors also displayed slightly enhanced increases in pp44 MAP kinase.

Since a major consequence of PKC-ι overexpression was to alter NGF-induced JNK activation patterns, the relationship of PKC-ι to JNK was examined further. HA-JNK1 was transiently cotransfected along with pCMV PKC-ι in 293T cells. Recombinant JNK 1 was then immunoprecipitated from cell lysates and included in an immunecomplex kinase assay with GST-c Jun (1–79) as substrate. Increasing concentrations of PKC-ι resulted in JNK-stimulated c-Jun phosphorylation (Figure 5). Thus, these results demonstrate that PKC-ι lies upstream of JNK and parallel the observation obtained in PC12 cells overexpressing PKC-ι.

Overexpression of atypical PKC enhances activation of NF-κB and survival

In other cell types, atypical PKCs have previously been shown lie in a pathway leading to the activation of NF-κB.[23–30] In PC12 cells, little, if any, information is available regarding the kinetics of NF-κB activation in response to NGF. Thus, we first characterized parameters associated with NGF-induced activation of NF-κB in PC12 cells. We first sought to validate assay of NF-κB using the commercial kit and establish baseline parameters for NGF induction in PC12 cells (Figure 6A). Addition of 100-fold excess of unlabeled cold NF-κB oligonucleotide probe (Figure 6A) inhibited binding of the upper band detected in the EMSA assay. This band was unaffected by inclusion of 100-fold excess of an AP-1 oligonucleotide. Omission of labeled κB oligonucleotide in the binding reaction resulted in an absence of DNA-protein interaction. Collectively, these parameters establish the validity of the κB gel shift assay in our hands. To identify proteins involved in the binding, extracts were incubated with antibody to p65 prior to EMSA assay. We were unable to detect super-shifted product and conclude that the epitope

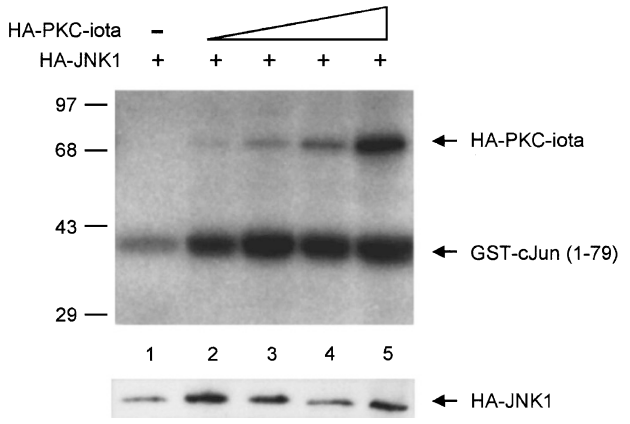

Figure 5 PKC-ι lies upstream of JNK. Cells were transfected with 0.5 μg HA-JNK1 plus 0, 0.5, 1, 2 or 3 μg of HA-PKC-iota. The empty vectors were used to normalize the amount of transfected DNA. Immunecomplex kinase assays were performed 44 h post-transfection with anti-HA (12CA5) antibodies using GST-c Jun (1-79) as substrate. Equivalent expression levels of HA-JNK1 was verified by immunoblotting using anti-HA (12CA5). Similar results were obtained in two other experiments

which the antibody recognizes overlaps with the binding site of the oligo probe. Similar findings have been previously reported using this oligonucleotide sequence and p65 antibody.[31,32] The localization of NF-κB was also examined immunocytochemically using specific antibodies against the p65 subunit. Upon stimulation with NGF, p65 was detected in the nucleus of a majority of the cells after 3 h treatment (data not shown). Thus, the immunocytochemical findings likewise confirmed activation of NF-κB by NGF.

Previous studies have implicated activation of NF-κB in the NGF survival response,[31,33] as well as, survival of PC12 cells to presenilin-1 induced apoptosis.[34] Likewise, atypical PKCs have been shown to play a role in the activation of NF-κB.[23–30] Therefore, we hypothesized that overexpression of atypical PKCs might enhance NGF-induced activation of NF-κB and prolong the survival of PC12 cells in serum-free or NGF-deprived situations. To test this idea, NF-κB activation, IκBα phosphorylation and degradation were measured in both the parental cells and those overexpressing PKC-ι. These measurements were conducted simultaneously with cells seeded at the same density; analysis was conducted with the same amount of protein, the same batch of labeled oligo and exposure to X-ray film conducted at the same period of time, to ensure quantitative measure of differences between the transfected cells and the parental counterpart. Treatment of PC12 cells with NGF resulted in activation of NF-κB, which peaked by 6 h (Figure 6B). By comparison, the cells overexpressing PKC-ι had higher basal activity of NF-κB and the kinetics of NGF induction was shifted to an earlier time-frame. Regulation of κB is due to phosphorylation and subsequent degradation of IκBα, paralleled by translocation of p65 into the nucleus. The activation of NF-κB was thus compared to phosphorylation and degradation of IκBα post-NGF stimulation. Increased Ser-32 phosphorylation of IκBα occurs concomitant to activation of NF-κB.[35] Likewise, NGF-mediated changes in Ser-32 phosphorylation observed in the parental cells occurred in a time-frame parallel to enhanced DNA binding activity (Figure 6C). However, by comparison, NGF-induced Ser-32 phosphorylation observed in the cells overexpressing PKC-ι was enhanced and sustained. The degradation of IκBα induced by NGF began to take place by 6 h treatment (Figure 6D), concomitant with activation (Figure 6B and C). However, degradation of IκBα was blocked in cells overexpressing PKC-ι. Unlike other systems where the turnover of IκBα occurs rather rapidly,[36] in PC12 cells IκBα remained fairly stable during the early phases of NGF treatment (Figure 6D). These findings confirm that activation of NF-κB was coincident with phosphorylation and subsequent degradation IκBα.

These studies indicate that PKC-ι modulated NGF-induced NF-κB responses and may lie within a pathway regulating NF-κB. Thus, experiments were undertaken to confirm that PKC-ι lies within the pathway leading to activation of NF-κB. PC12 cells were transiently transfected with a full length antisense construct to PKC-ι.[37] Diminished levels of PKC-ι blocked NGF-induced activation of NF-κB (Figure 7). Collectively, these studies demonstrate that PKC-ι lies within the NF-κB signaling

pathway and resides as a component of a prosurvival signaling network.

Modulation of NF-κB has been shown to modulate PC12 cell survival[31] and removal of NF-κB blocks NGF-mediated survival of sympathetic neurons.[33] Consequently, studies were conducted to test whether PKC-ι overexpression might alter the survivability of cells deprived of serum (Figure 8). At 48 h post removal of serum, the overexpressors displayed enhanced survivability compared to their parental counterpart. Protection was only provided in this narrow window, whereas by day 4, the overexpressors displayed the same cell death response as did the parental counterpart. Likewise, studies were conducted to examine the ability of PKC-ι overexpression to modulate survival of NGF-differentiated PC12 cells deprived of NGF. A significant difference was observed in the response of the parental PC12 cells compared to the overexpressors and their ability to survive in the absence of NGF.

Discussion

In the present study, we utilized PC12 cells stably overexpressing atypical PKC isoforms to gain insight into their function in NGF responses. In other systems overexpression of atypical PKC has yielded various results. For example, in U937 cells PKC-ζ enhanced cell differentiation.[38] Overexpression of PKC-ζ in rat mesangial cells enhanced COX-2 expression and inducible nitric oxide synthase.[39] PKC-ι overexpression has been shown to protect cells from UV-induced cell death,[40] as well as, okadaic acid- and taxol- induced apoptosis.[37] Moreover, overexpression of atypical PKC in NIH3T3 cells has been shown to block PAR4-induced apoptosis.[15] Whereas, increased PAR4 expression results in enhancement of the neurotoxic effects of amyloid beta peptide.[41] Although some cell specific responses have been noted during overexpression, collectively these findings document a role

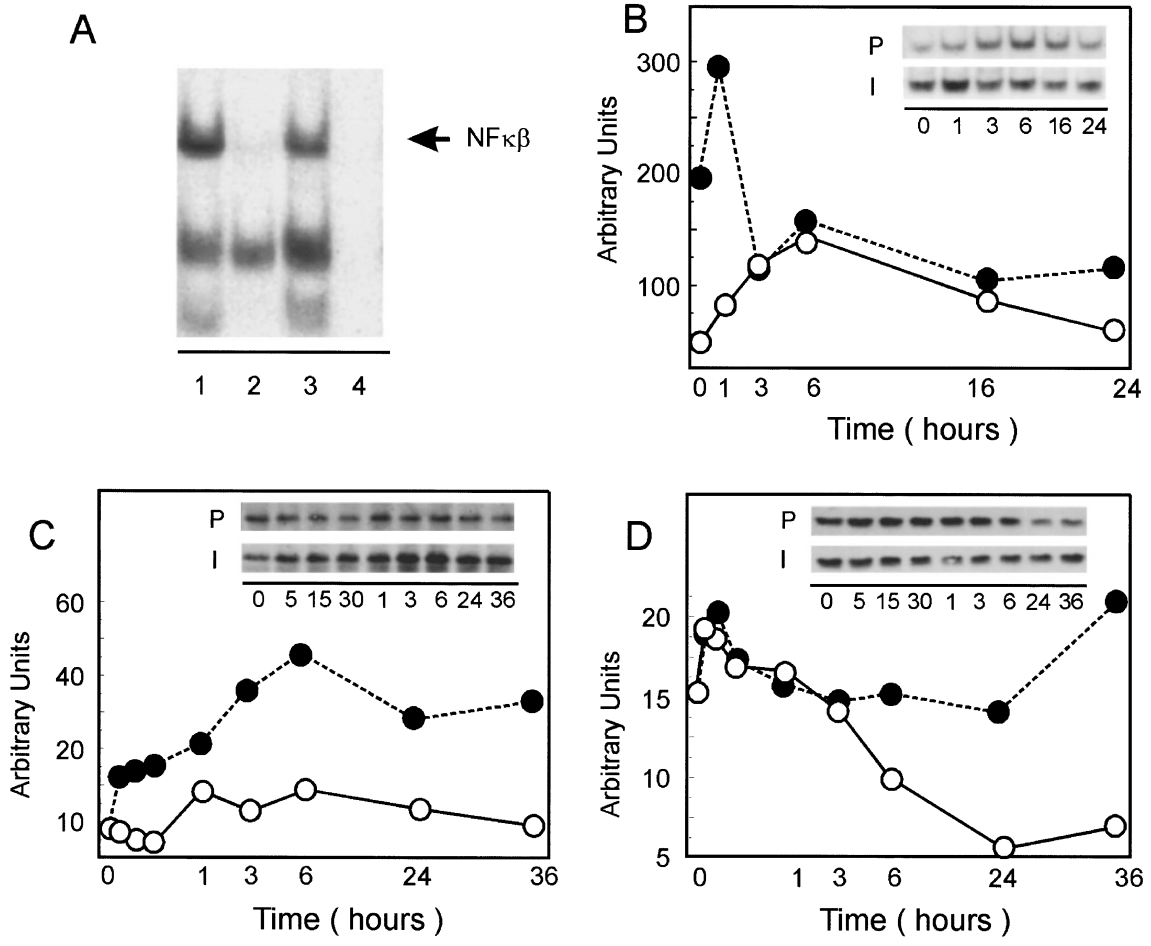

Figure 6 Overexpression of PKC-ι resulted in enhanced NF-κB activity. (**A**) Control for EMSA binding assays. Extracts were prepared and incubated with ^{32}P-labeled oligonucleotide encompassing the κB motif as described in 'Materials and Methods'. Lane 1: NGF- stimulated extract; Lane 2: excess κB oligo; Lane 3: excess AP-1 oligo; Lane 4: no oligo. (**B**) NGF treatment results in enhanced DNA binding activity of NF-κB. Parental cells ('–') or cells overexpressing PKC-ι (!–!) were treated for 0 – 24 h as indicated with 100 ng/ml NGF followed by EMSA analysis to examine activation of NF-κB. (**C**) NGF treatment results in phosphorylation of IκBα. Post-treatment with NGF, lysates were prepared and analyzed by immunoblotting with phosphospecific antibody to Ser-32 of IκBα. (**D**) NGF treatment results in degradation of IκBα. Post-treatment with NGF, lysates were prepared and analyzed by immunoblotting with antibody to IκBα. Similar results were obtained in three other experiments

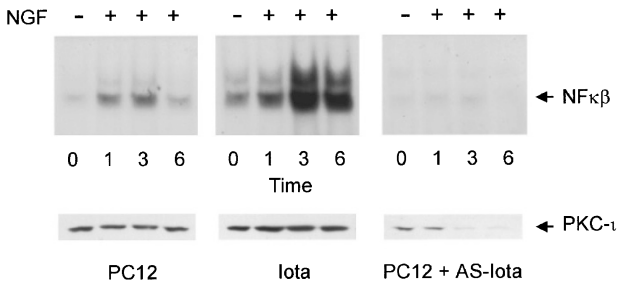

Figure 7 PKC-ι is required for activation of NF-κB. (**A**) PC12 cells, cells overexpressing PKC-ι or cells transiently transfected with a full length antisense construct to PKC-ι were treated for 0, 1, 3 and 6 h with NGF as indicated followed by EMSA analysis to examine activation of NF-κB. Levels of PKC-ι expression were determined by Western blotting an aliquot of the cell lysate with antibody to PKC-ι

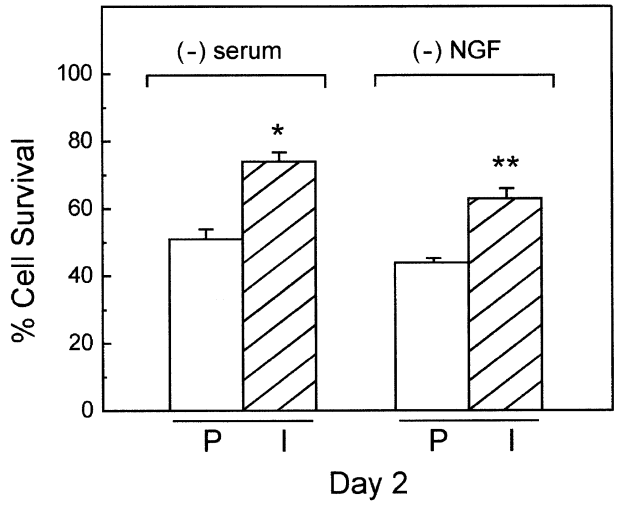

Figure 8 Overexpression of aytpical PKC enhances cell survival. PC12 cells (P) or those overexpressing PKC-ι (I) were switched to serum-free media for 48 h as indicated (-serum). Alternatively the cells were treated with 50 ng/ml NGF for 10 days and switched to an NGF-deprived/serum-free media for 48 h Cell viability was determined by trypan blue exclusion. Overexpression of PKC-ι protected both naive and NGF-differentiated PC12 cells from cell death. Overexpressors were significantly different ($P<0.01$) than parental cells as determined by Student's paired *t*-test

for atypical PKCs a regulator of cell growth, differentiation and survival signaling.

Our data demonstrate that NGF responses are amplified by the presence of excess atypical PKC, concomitant with amplification of NGF-induced activation of JNK. Although most work characterizing the JNK pathway has focused on cellular responses to stress, other growth factors such as insulin, that provide prosurvival signals, also lead to activation of JNK.[20,42] Likewise, activation of Sek1, the upstream activator of JNK, has been shown to protect thymocytes from apoptosis.[43] In PC12 cells previous studies have documented activation of JNK kinase by NGF, independent of changes in pp42/44 MAP kinase.[44] Overexpression of PKC-ι was observed to enhance basal PI3 kinase and amplify JNK responses in the absence of dramatic changes in pp42/44 MAP kinase. These findings suggest that PI3 and JNK kinases may lie within a common signaling cascade regulated by aPKC. The placement of both JNK and PI3 kinase within a common pathway would be in keeping with the ability of dominant negative mutants of JNK to inhibit neurite outgrowth in PC12 cells induced by activated PI3 kinase.[22] Atypical PKCs may act as a MEK kinase[24] and this could explain how overexpression of PKC-ι might modulate JNK, MAPK and p38 pathways. MEK kinase is the upstream activator of JNK, whereas JNK kinase also activates p38.[45] Similarly overexpression of MEK kinase can activate MAPK.[45] Thus, PKC-ι acting as a MEK kinase could modulate the three pathways and thus suggests that some degree of crosstalk exists between the pathways, as evidenced by the suppression of NGF-induced p38 activity in the overexpressors.

Interestingly, we observed constitutively active phosphorylation of pp110 in the cells overexpressing PKC-ι. Previously, it has been reported that constitutively active pp110 activates JNK.[46,47] Moreover, aPKC has been shown to associate with pp110 PI3 kinase.[48] Thus, PKC-ι may modulate pp110 and contribute further to activation of JNK. Liu *et al*.[49] have shown that JNK activation is not linked to apoptosis and NF-κB protects against apoptotic responses. Moreover, activation of NF-κB has recently been shown to occur through a pathway likewise regulated by PI3 kinase.[50] However, in other systems NF-κB activation can occur in the absence of JNK,[51] suggesting cell-type specificity.

Since the phosphorylated lipid products of PI3 kinase activate PKC-ι,[21] it still remains to be determined whether PI3 kinase is a common second messenger linking PKC-ι to both differentiation and prosurvival signaling pathways. On the one hand, removal of PI3 kinase or PKC-ι blocks NGF-induced differentiation[1,52,53] likewise overexpression of PI3 kinase has been reported to promote neurite outgrowth.[54] Whereas, PI3 kinase has also been shown to modulate survival of PC12 cells.[55] Since NF-κB activation by NGF is dependent upon PI3 kinase (M.W. Wooten and M.L. Seibenhener, unpublished findings), it appears that both NGF-antiapoptotic signaling and NGF-activation of NF-κB involve PI3 kinase. Thus, further studies will be aimed toward the teasing apart the relationship between PKC-ι, PI3 kinase and the mapping of their position relative to downstream activation of NF-κB, cell survival and differentiation. It is always possible that overexpression may drive pathways different than those under control of normal receptor regulation. However, placement of PKC-ι upstream of JNK is supported by data demonstrating that inhibition of JNK abrogated NGF-induced neurite outgrowth, whereas overexpression of PKC-ι compensated for this effect. Second, cotransfection of increasing amounts of PKC-ι lead to JNK induced phosphorylation of c-Jun. Collectively, our findings demonstrate that JNK lies upstream of PKC-ι and that PKC-ι contributes to activation of NF-κB.

Atypical PKC has previously been linked with sphingomyelinase activation of the κB-promoter[27] and regulation of

NF-κB activity.[23,26] Moreover, it has recently been shown that low doses of ceramide activate PKC-ι, JNK and NF-κB providing a prosurvival signal.[56] The NGF receptor is composed of two proteins, p75 and gp140 TrkA. Selective activation of NF-κB by NGF has been reported to occur through the p75 component of the receptor.[57] This would be in keeping with NGF induced sphingomyelin hydrolysis by p75.[58] However, upon expression of both receptors, p75 and TrkA, as is the case for PC12 cells, NGF fails to induce sphingomyelin hydrolysis.[58] Thus, it is likely that other second messengers contribute to activation of NF-κB by NGF and that both receptor components cooperate in some fashion toward activation of NF-κB.

The precise pathway(s) which culminate in activation of NF-κB by NGF has yet to be fully unraveled. Although changes in ser-phosphorylation of IκBα occurred rapidly in response to NGF, activation of NF-κB was maximal by 6 h post-treatment, followed by degradation at 16–24 h. This pattern of activation is substantially different than that observed with TNFα treatment[36] which occurs rapidly (0–60 min). This finding suggests that NGF-induced activation of NF-κB may be more complex or occurring via an alternate pathway. It is possible that tyrosine phosphorylation of IκBα in response to NGF may also play a regulatory role, since tyrosine phosphorylation has been reported to stabilize the protein from degradation.[59] The interaction of PKC-ι and tyrosine kinases is worth addressing, since overexpression of PKC-ι blocked IκBα degradation.

Activation of NF-κB in the nervous system can be viewed as somewhat enigmatic. On the one hand, increases in DNA binding exhibited by NF-κB are regarded as part of an inflammatory stress response leading to cell death.[60] However, NF-κB is constitutively expressed in post-mitotic neurons[61] demonstrating a prosurvival need. In addition, recent studies have shown that high constitutive activity of NF-κB mediates resistance to oxidative stress in neuronal cells[32] and resistance to apoptosis of T cell lymphoma HuT-78 cells.[62] Furthermore, a prosurvival role for NF-κB is substantiated by studies demonstrating that removal of NF-κB by either antisense oligonucleotide or proteasome inhibitor blocks the neuroprotective effects that NGF has upon PC12 cells in serum-free media.[31] Furthermore, microinjection of c-Rel has been shown to enhance survival of NGF- deprived sympathetic neurons, whereas removal of NF-κB blocks NGF-mediated survival.[33] Moreover, sAPP which has been shown to enhance NGF effects[63] counteracts the proapoptotic effects of presenilin-1 by activation of NF-κB.[34] Collectively, these findings support a role for NF-κB in modulation of prosurvival signaling in neuronal systems.

How does PKC-ι participate in NF-κB activation? It has been suggested that aPKCs might directly contribute to regulation of IκBα.[24] However, IκBα itself is not a substrate for PKCs, but is phosphorylated by casein kinase II.[64] Two IκB kinase activities have been identified that directly phosphorylate IκBα.[65] Interestingly, it has recently been shown that aPKCs ζ/ι phosphorylate IκB kinase and complex with the IκB kinase signalsome.[30] These findings clearly document that PKC-ι lies upstream of NF-κB regulating its activation. Further studies will be required to evaluate the complexing of PKC-ι with IκB kinase by NGF in PC12 cells.

In addition we speculate that cells overexpressing aPKC isoforms may secrete autocrine factors which would enhance certain signal cascades and promote neurite outgrowth. In this regard, PKC-ε expression has been shown to stimulate production of growth factors in an autocrine fashion.[66] The amyloid precursor protein (APP) gene has a κB regulatory site.[67] Thus, overexpression of aPKC might directly stimulate transcription of APP through a κB-dependent pathway resulting in accumulation of excess secretory APP in the media. In this manner, secretory APP (sAPP) acting in an autocrine fashion could promote survival through an NF-κB dependent pathway. sAPP has been previously shown to promote prosurvival signaling by activation of NF-κB,[34] as well as, neurite outgrowth of PC12 cells.[63]

In conclusion, we demonstrate: (1) that atypical PKC-ι positions itself in a pathway upstream of JNK: (2) that PKC-ι is a component of a pathway regulating NF-κB; and (3) that PKC-ι plays a role in modulating of prosurvival signaling. The relationship between PKC-ι, PI3 kinase, MAP kinase, JNK, p38 kinase, NF-κB and NGF receptor components toward survival and differentiation signaling in PC12 cells is the focus of ongoing research in our laboratory.

Materials and Methods

Materials

2.5 S NGF and EGF were purchased from Bioproducts for Science (Indianapolis, IN, USA). Monoclonal antibody to PKC-ζ, SHC and pp110 PI3 kinase was purchased from Transduction Laboratories (Lexington, KY, USA). A monoclonal antibody (12CA5) against the hemagglutinin (HA) was purchased from Boehringer Mannheim (Indianapolis, IN, USA). Secondary sheep anti-mouse horseradish peroxidase-labeled antisera, ECL reagents, Hyperfilm and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Amersham (Arlington Heights, IL, USA). Protein dye-binding reagent was purchased from Bio-Rad (Richmond, CA, USA). Poly dI-dC was obtained from Boehringer Mannheim (Indianapolis, IN, USA). Anti-p50, p65, IκBα and TrkA antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to phospho MAP kinase, phospho JNK kinase, phospho p38 kinase and phospho IκBα-Ser 32 were purchased from New England Biolabs (Beverly, MA, USA). NF-κB oligonucleotide was from Gibco BRL (Grand Island, NY, USA) or Promega (Madison, WI, USA). Antiphosphotyrosine conjugated to agarose was from UpState Biotechnology (Lake Placid, NY, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

PC12 cells were seeded onto 100 mm plates coated with rat tail collagen, grown in RPMI containing 10% heat-inactivated horse serum, 5% heat-inactivated fetal calf serum, and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin), and maintained in a 92% air : 8% CO_2 atmosphere.

Transfection and expression of atypical PKC

PC12 cells were transfected with either: (1) the full length PKC-ι cDNA, provided by Dr. Trevor Biden, Garvan Institute of Biomedical Research, Sydney, Australia;[68] (2) the full length antisense PKC-ι cDNA provided by Dr. Alan Fields, University of Texas Medical Branch, Galveston, TX, USA; (3) dominant negative mutants for PKC-ι, PKC-ζ or HA-tagged PKC-ι provided by Dr. Jorge Moscat, Universidad Autonoma de Madrid, Spain or (4) the full-length mouse PKC-ζ cDNA provided by Dr. Frederic Mushinski, Molecular Genetics Section, Laboratory of Genetics, National Cancer Institute, Bethesda, MD, USA.[69] Fifty per cent confluent PC12 cells were washed with serum-free RPMI medium without antibiotics. One to two μg of the plasmids and 2–20 μl of Lipofectin Reagent was diluted in 100 μl serum-free medium. Lipofectin Reagent was a 1 : 1 (w/w) liposome formulation of the cationic lipid N-(1-(2,3-dioleyloxy)propyl)-n,n,n,-trimmethylammonium chloride (DOTMA), and dioleoyl phosphatidylethanolamine (DOPE) in distilled water. 0.8 ml serum-free RPMI medium was added into the Lipofectin Reagent-DNA complex. The complex was mixed gently and overlaid onto the cells. After 24 h incubation at 37°C, the DNA-containing medium was replaced by normal medium containing serum and the cells were incubated for an additional 24 h at 37°C. To generate stable transfectants the cells were split 1 : 20 and selected in the presence of 800 μg/ml neomycin (G418). After 14 days, antibiotic- resistant colonies were pooled and cultured in mass. Alternatively, transient transfection was conducted with either full-length antisense PKC-ι construct[37] or HA-tagged PKC-ι[16] using Fugene 6 as described (Boehringer Mannheim). Two days post-transfection the cells were switched to a reduced serum environment and used in experiments.

Cell growth and viability analysis

PC12 cells were seeded in 24-well plates at 5000 cells per ml in complete growth media. Cell numbers were determined every other day for 8 days by measuring triplet wells. To assess the effects of overexpression on viability of differentiated neurons, PC12 cells were treated with NGF(50 ng/ml) for 10 days. The cells were washed three times with serum-free media and cultures in a NGF-deprived/serum-free environment for 48 h.[55] Both experiments were repeated three times. For analysis of cell viability, all the cells in a well were removed and were stained with trypan blue and counted. In some cases MTT reduction was used to measure viability. In brief, at the end of the experiment 50 μl of the dye MTT (3,[4,5-dimethylthiazol-2-yl-] diphenyltetrazolium bromide, 5 mg/ml) was added to each well and the plates were incubated for 3 h at 37°C. To each well 500 μl of lysis buffer (20% SDS in 50% N,N,-dimethylformamide, containing 0.5% [v : v] 80% acetic acid and 0.4% [v : v] 1 N HCl was then added to each well and the color intensity was assessed by reading the samples at 590 nm.

Cell cycle analysis

For flow cytometric analysis, the cells were collected by centrifugation and washed twice in PBS, followed by resuspending in 1 ml PBS.[70] Seventy per cent ethanol (−70°C), 3 ml was added dropwise while gently vortexing and the suspension was incubated on ice for 30 min. The cells were collected by centrifugation, resuspended in 1 ml PBS, and stained with 0.2 mg/ml propidium iodide treated with 1 mg/ml RNase, followed by incubation for 30 min in the dark. The stained cells (10 000 per treatment) were analyzed on a Coulter EPICS 753 flow cytometer for DNA content. The output was analyzed by Multicycle program for cell cycle analysis.

Morphology

The percentage of cells that possessed neurites was determined by counting treatments in triplicate. A minimum of 500 cells were counted within a treatment group. The percentage of cells with neurites was calculated based upon an individual cell bearing one process with an extension which was greater in length than two cell diameters. Clumped cells were not included in the scoring process.

Immunostaining

PC12 cells were plated directly onto glass cover slips that had been coated with a mixture of collagen/poly lysine (4 : 1, v/v) and treated with NGF (50 ng/ml for 3 days). The cover slips were rinsed with PBS and incubated for 3 min in 2% (v/v) paraformaldehyde in PBS and then incubated for another 3 min in 4% (v/v) paraformaldehyde in PBS. The fixed cells were blocked in PBS containing 1% BSA and 0.1% (v/v) saponin for 2 h at 27°C. Thereafter, polyclonal anti-PKC-ζ (1 : 250) was added in blocking buffer and incubated at 4°C overnight. The cover slips were rinsed three times, 5 min each followed by addition of goat anti-rabbit IgG-FITC-conjugated antibody (12 μg/ml) in blocking buffer for 2 h in the dark at 27°C. Thereafter, the coverslips were mounted in glycerol/PBS and observed using a Nikon Optishot epi-fluorescence microscope. As control, samples were processed without primary antibody, or with antibody that had been previously preincubated with peptide antigen. In either case, no background fluorescence could be detected.

Immunoprecipitation of tyrosine phosphorylated proteins and Western blotting

PC12 cells on 100 mm culture dishes were serum starved for 48 h by removing the growth media except 1 ml followed by adding 5 ml of serum-free media. The cells were stimulated with 50 ng/ml NGF, collected by centrifugation, washed in PBS and lysed in buffer containing 20 mM Tris, pH 7.6, 137 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM $NaOV_3$, 10% glycerol, 0.1% TX-100, 1 mM PMSF, 10 μg/ml aprotinin, 10 mM β-glycerophosphate and 100 μM NaF. Phosphotyrosine antibody coupled to agarose was added 20 μl beads per 1 mg of protein for 4 h. The immunocomplexes were washed extensively in buffer containing 20 mM Tris, pH 7.6, 137 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM $NaOV_3$, 10% glycerol, 0.1% TX-100, 1 mM PMSF, 10 μg/ml aprotinin and 10 mM β-glycerophosphate followed by addition of SDS–PAGE sample buffer. The proteins were separated by a 7.5% SDS–PAGE, transferred to nitrocellulose and Western blotted with antibodies to either TrkA, pp110 PI3 kinase or SHC. To detect activation patterns of either JNK, MAP or p38 kinase whole cell lysates were prepared. The proteins were separated by 12% SDS–PAGE, transferred to nitrocellulose and immunoblotted with antisera to phospho- JNK, MAP or p38 kinase. The blots were stripped and reprobed with non-phospho antibody to validate an equivalent load of protein.

Assay for atypical PKC or JNK activity

PC12 cells (100 mm dishes) were serum-starved and atypical PKC isoform specific activity was measured by immune-complex kinase assay using isoform specific antibody for either PKC-ζ or PKC-ι. The incorporation of [^{32}P]ATP stimulated by atypical PKC into MBP was used as a measure of enzyme activity.[48] Briefly, cell extracts were normalized for protein concentration and pre-cleared for 1 h at 4°C with 20 μl 50% Rabbit IgG coupled to agarose. Immunoprecipitation was performed at 4°C by incubating the lysate with 4 μg antibody per

mg of protein. Control experiments were performed by carrying out the immunoprecipitation with anti-PKC-ζ or ι antibody that had been preadsorbed with corresponding peptide (1 : 2 ratio). After extensive washing, the immunoprecipitates were resuspended in 30 μl buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 10 μM ATP, and 0.5 μCi of [γ-^{32}P]ATP and incubated for 10 min at 30°C in the presence or absence of 10 μM of an atypical PKC inhibitor peptide (Upstate Biotechnology, Inc, NY, USA). JNK activity was determined by immunecomplex kinase assay as previously described[71] using GST-cJun (1-79) as substrate. Reactions were terminated by addition of 50 μl Laemmli sample buffer, followed by 12% SDS–PAGE, staining, drying, and exposure to X-ray film overnight at -80°C. The changes in atypical PKC activity were quantitated by densitometry of MBP substrate phosphorylation.

NF-κB electrophoretic mobility shift assays (EMSA)

Cell extracts were prepared in high-salt detergent buffer (Totex) [20 mM HEPES, pH 7.9, 350 mM NaCl, 20% (w/v) glycerol, 1% (w/v) NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.1% PMSF and 1% aprotinin].[72] The cells were harvested by centrifugation, washed in ice-cold PBS and resuspended in four volumes Totex buffer, followed by incubation on ice for 30 min, and centrifugation for 5 min at 13 000 $\times$ *g* at 4°C. The protein content of the supernatant was determined and equal amounts of protein (20 μg) were added to a reaction mixture containing 20 μg BSA, 2 μg poly (d[I-C], 2 μl buffer D+(20 mM HEPES; pH 7.9; 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0l.1% PMSF), 4 μl buffer F (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, 0.1% PMSF), and 100 000 c.p.m. of a ^{32}P-labeled oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) in a final volume of 20 μl. Samples were incubated at room temperature for 25 min. For supershift assays, 2–5 μg antibody was added to the protein and allowed to incubate overnight, followed by inclusion in an assay. Excess AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) or NF-κB oligonucleotide were included as negative controls. The samples were resolved on a 6% TGE/PAGE gel. The gel was dried and exposed to X-ray film for 24–72 h. NF-κB activation was also studied by examining IκBα protein degradation and IκBα Ser-32 phosphorylation by Western blotting, as well as, nuclear localization of p65 by immunofluorescent microscopy.

Statistical analysis

Results are given as the means $\pm$ S.E. for the indicated number of independently performed experiments. Differences between the mean values were evaluated by Student's *t*-test or ANOVA.

Note added in proof

Overexpression of PKC-ι has recently been shown to attenuate amyloid B-peptide (AB) induced peroxide accumulation and oxidative damage thus providing a protective role against stress induced damage (Q. Guo, M.P. Mattson and M.W. Wooten, unpublished findings).

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