Nerve growth factor promotes the survival of sympathetic neurons through the cooperative function of the protein kinase C and phosphatidylinositol 3-kinase pathways

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The signaling pathways activated by nerve growth factor (NGF) that account for its ability to promote the survival of neurons are not completely understood. Phosphatidylinositol 3-kinase (PI3K) is critical for the survival of several cell types, including neurons. To determine whether additional signaling pathways cooperate with PI3K to promote survival, we examined other pathways known to be activated by NGF. NGF activated protein kinases C (PKCs) in sympathetic neurons, and pharmacologic PKC activation rescued neurons from apoptosis induced by the withdrawal of NGF. Inhibition of PKCs did not inhibit the survival of NGF-maintained neurons. Similarly, inhibition of PI3K caused only a modest attrition of neurons in the presence of NGF. In contrast, the simultaneous inhibition of both PKCs and PI3K induced the apoptotic death of NGF-maintained sympathetic neurons. Inhibition of both PI3K and PKCs promoted the expression and phosphorylation of the proapoptotic transcription factor c-Jun, indicating that these pathways inhibit programmed cell death at the stage of proapoptotic gene expression. In culture conditions under which PI3K inhibition alone kills NGF-maintained neurons, PKC inhibition also led to a significant loss of viability, indicating that both pathways are required. Therefore, PKC and PI3K, regardless of the culture conditions, cooperate to promote the NGF-dependent survival of sympathetic neurons.

Nerve growth factor (NGF) can maintain the survival of sympathetic neurons in vitro and is required for their target-dependent survival in vivo (1, 2). NGF promotes survival via activation of its receptor tyrosine kinase, TrkA (3, 4). However, the signaling pathways that account for the survival-promoting activities of NGF are not completely understood. Although phosphatidylinositol 3-kinase (PI3K) is important for the NGF-dependent survival of sympathetic neurons (5), whether PI3K is the only required signaling pathway is unresolved. Under certain culture conditions, PI3K appears to be necessary and sufficient for NGF-dependent survival (6–8), whereas under different circumstances PI3K is dispensable (9–11). Therefore, additional signaling pathways probably function in the NGF-dependent survival of sympathetic neurons.

The protein kinase C (PKC) family of serine/threonine kinases encompasses at least 12 members that participate in cellular functions ranging from proliferation to apoptosis (12, 13). The PKC family can be separated into three groups based on their regulation. The conventional PKCs (α, βI, βII, and γ) are activated by DAG and phorbol esters and also require calcium, whereas the novel PKCs (δ, ε, η, θ, μ, and ν) require DAG or phorbol esters for activation but are insensitive to calcium. The atypical PKCs (ζ and ι/λ) are not sensitive to DAG, phorbol esters, or calcium. PKCζ and PKCι/λ, although they are activated by DAG and phorbol esters, lack the autoinhibitory pseudosubstrate domain and are therefore probably regulated quite differently than other novel PKCs and are frequently termed PKDs to distinguish them. Growth factor receptors regulate PKCs via the activation of phospholipase C-γ. Phospholipase C-γ, upon binding to phosphorylated Tyr785 in TrkA, is itself phosphorylated and activated (14), hydrolyzing phosphatidylinositol 4,5-bisphosphate to produce DAG and inositol trisphosphate and thus activating PKCs. Growth factor receptors also influence PKC activity through the phosphorylation of the activation loop of PKC by phosphoinositide-dependent kinase-1 (15, 16).

Although PKCs have been implicated in the regulation of survival, their function varies depending upon the cell type and apoptotic signal (17, 18). PKC-α, -β, -ε, and -ι can function as suppressors of apoptosis, whereas PKC-δ and -θ appear to be proapoptotic in function. PKC-δ appears to be involved in apoptosis induced by several stimuli and is cleaved by caspase-3 to generate a catalytically active fragment (19). Whether PKCs function in the trophic factor-dependent survival of neurons is unclear. Tyr785 of TrkA, and thus phospholipase C-γ, is required for neurite outgrowth stimulated by NGF in the sympathetic neuron-like cell line PC12 but is not required for survival (20, 21). Atypical PKCs, however, may function in the survival of PC12 cells via activation of NF-κB (22). We examined to what extent PKC signaling is involved in the survival of sympathetic neurons and here provide evidence that PKCs have a cooperative function in NGF-dependent survival along with PI3K.

EXPERIMENTAL PROCEDURES

Materials—LY294002 and GF109203X were obtained from Biomol (Plymouth Meeting, PA); Bryostatin 1 and PMA were from Calbiochem; and boceaspartyl fluoromethyl ketone (BAF) was obtained from Enzyme Systems Products (Livermore, CA). These compounds were dissolved in

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RESULTS

NGF Promotes PKC Signaling in Sympathetic Neurons—To determine whether NGF treatment induced PKC activity in sympathetic neurons, 6-day in vitro neurons that had been deprived of NGF were stimulated with NGF for various times. The neurons were then detergent-extracted to harvest total cellular proteins, and the extracts were subjected to phospho-PKC (P-PKC) substrate immunoblotting with an antibody that specifically recognizes proteins phosphorylated on PKC consensus sites (Fig. 1A). NGF treatment induced the rapid phosphorylation of a number of proteins on PKC consensus sites, and their phosphorylation was sustained for hours (Fig. 1A). These same proteins were robustly phosphorylated after sympathetic neurons were treated with PMA, a phorbol ester that potently activates conventional and novel PKCs (Fig. 1A), indicating that the P-PKC-substrate antibody detected bona fide PKC substrates rather than substrates of unrelated kinases. To determine the extent to which activators of PKCs can promote the phosphorylation of these proteins, sympathetic neurons that were briefly deprived of NGF were treated with either PMA or bryostatin 1, another potent PKC activator, for various times. The phosphorylation of PKC substrates was monitored by P-PKC substrate immunoblotting of whole cell extracts produced from these neurons. Both PMA and bryostatin 1 promoted robust, but transient, PKC activity in sympathetic neurons (Fig. 1B). In fact, sustained PMA and bryostatin 1 treatment resulted in the down-regulation of the phosphorylation of PKC substrates over time (Fig. 1B). These results further confirm that the proteins that are phosphorylated by NGF treatment (Fig. 1A) are, indeed, downstream substrates of phorbol ester-sensitive PKCs. PKCs are phosphorylated on several residues that regulate their catalytic activity (16). Because PKC substrates are phosphorylated after NGF stimulation, we investigated whether NGF promoted the phosphorylation of PKCs by using antibodies that specifically detect conventional PKCs when phosphorylated on Thr638/641, an autophosphorylation site (P-auto-PKC) that stabilizes the kinase core of the enzyme, or when phosphorylated on the hydrophobic Ser660 phosphorylation site (P-hydro-PKC). The hydrophobic Ser660 site is also autophosphorylated and appears to stabilize the enzyme (25). Phosphorylation of both Thr638/641 and Ser660 was enhanced by NGF treatment in sympathetic neurons (Fig. 1A), although the increased phosphorylation of Ser660 was delayed compared with Thr638/641. However, in the absence of NGF treatment, a significant amount of PKC was still phosphorylated at these residues (Fig. 1A), suggesting that other factors besides NGF account for much of the PKC phosphorylation.
PKCs when phosphorylated on the hydrophobic Ser<sup>660</sup> phosphorylation site recognize phosphorylated PKC consensus sites ([100 ng/ml for the time indicated], or PMA (100 ng/ml) for 15 min and then detergent-extracted. The neurons were then treated with medium alone (MA) for 5 min, with NGF (50 ng/ml) for the time indicated, or with PMA (100 ng/ml) for 15 min and then detergent-extracted. The whole-cell extracts were immunoblotted with antibodies that specifically recognize phosphorylated PKC consensus sites (P-PKC Sub), conventional PKCs when phosphorylated on the hydrophobic Ser<sup>660</sup> phosphorylation site (P-hydro-PKC), or conventional PKCs when phosphorylated on Thr<sup>638/641</sup>, an autophosphorylation site (P-auto-PKC). To confirm the equal loading of protein, actin immunoblotting was done as a control (bottom panel). B, sympathetic neurons that were maintained in NGF for 5 days in vitro were treated with either bryostatin 1 (100 nm) or PMA (100 ng/ml) for the time indicated. A similar analysis was then performed as described in A. In addition, the total amount of conventional PKCs was determined by using antibodies that specifically recognize PKCa, PKCb1, PKCi1, or PKCS (Total PKC). The experiments in this figure were performed on three or four independent cultures with similar results.

observed in sympathetic neurons. Similar to the effect of phorbol ester treatment on PKC substrate phosphorylation, PKC phosphorylation on Thr<sup>638/641</sup> and Ser<sup>660</sup> was down-regulated in the prolonged presence of PMA and bryostatin 1 (Fig. 1B). Furthermore, the level of conventional PKCs themselves were down-regulated with PMA and bryostatin 1 treatment (Fig. 1B), similar to a previous report ([26]). This down-regulation of PKCs is because of the degradation of PKCs after their activation, and sustained phorbol ester treatment is sometimes used as a means of inhibiting PKC activity in cells.

In addition to phorbol esters, some PKC isoforms are regulated by PI3K via phosphorylation of Thr<sup>509</sup> in the PKC activation loop by phosphoinositide-dependent kinase-1 ([27, 28]). To determine whether PI3K regulated NGF-dependent PKC activity in sympathetic neurons, neurons were treated with the PI3K inhibitor LY294002, and PKC activity was monitored by P-PKC substrate immunoblotting of whole cell extracts produced from these neurons. PI3K inhibition blocked the phosphorylation of one PKC substrate that was ~32 kDa, whereas the other PKC substrates were unaffected (Fig. 2A). In contrast, treatment of NGF-maintained sympathetic neurons with GF109203X, a PKC inhibitor that is somewhat selective for conventional PKCs, blocked the phosphorylation of the majority of PKC substrates in sympathetic neurons, although some proteins were minimally altered (Fig. 2A). Similar to previous experiments, PMA promoted a greater initial phosphorylation of PKC substrates than NGF did. NGF deprivation for 2 h led to the dephosphorylation of only the 32-kDa protein, and other PKC substrates were unaffected at this early time point (Fig. 2A). To determine whether PKC inhibition led to the inhibition of the PI3K or MAPK pathways, the phosphorylation of Akt and extracellular signal-regulated kinases was examined by immunoblotting these extracts with antibodies specific to the active, phosphorylated forms of Akt and extracellular signal-regulated kinases. As expected, LY294002 inhibited Akt phosphorylation but did not inhibit extracellular signal-regulated kinase phosphorylation (Fig. 2A). Inhibition of PKCs with GF109203X did not alter Akt phosphorylation and changed extracellular signal-regulated kinase phosphorylation only modestly (Fig. 2A). Taken together, these experiments indicate that PI3K regulated only a small portion of PKC activity in neurons (clearly discernible only in the 32-kDa substrate) and that the PKC inhibitor GF109203X blocked most PKC phosphorylation events in sympathetic neurons. Furthermore, PKC inhibition in sympathetic neurons did not significantly impair either the MAPK or PI3K pathways.

Because PKCs are reported to regulate the activity of RTKs such as the epidermal growth factor receptor ([29, 30]), we determined whether the inhibition of PKCs in sympathetic neurons altered TrkA autophosphorylation. NGF-maintained sympathetic neurons were treated with either GF109203X or vehicle alone and were then lysed, and TrkA phosphorylation was assessed by anti-phosphotyrosine immunoblotting TrkA immunoprecipitates from these lysates. GF109203X treatment did not alter TrkA autophosphorylation or the level of TrkA protein, even after as long as 2 days of PKC inhibition (Fig. 2A). Therefore, conventional PKCs did not regulate the autophosphorylation or stability of TrkA in sympathetic neurons.

PKC Activity Protects Sympathetic Neurons from Apoptosis Induced by NGF Deprivation—Because NGF induced PKC activity in sympathetic neurons, we examined whether PKCs could promote their survival. Dissociated sympathetic neurons were deprived of NGF for 24 h or deprived of NGF in the presence of the PKC activator PMA or bryostatin 1. The numbers of surviving neurons were then counted and compared. NGF deprivation led to the apoptosis of ~70% of neurons within 24 h, and both PMA and bryostatin 1 inhibited significantly the death of these neurons (Fig. 3A). The survival effects of PMA and bryostatin 1 were, however, modest and transient. After an additional 24 h of NGF deprivation, neither PMA nor...
bryostatin 1 caused any enhancement of survival (data not shown), similar to a previous report (31). This temporary effect of PKC activation on survival is probably caused by the transient nature of the effect of these two compounds on PKC activity (Fig. 1B).

To compare more directly the survival-promoting ability of PKC activation with NGF, rescue experiments were performed. Rescue experiments allowed us to compare the effectiveness of PMA and NGF at preventing apoptosis of trophic factor-deprived neurons with short rescue periods, thus avoiding the transient effects of PMA on PKC activation. Sympathetic neurons were deprived of NGF for 20 or 26 h and then switched to medium containing NGF, PMA, or vehicle alone for 5 h to rescue those neurons that would have committed to die in that period. After this 5-h rescue period, all of the neurons were switched back to medium containing NGF and also maintained for an additional 5 days. The number of surviving neurons in each condition was counted and graphed as a percentage. The experiments in this figure were performed in duplicate in two or three independent cultures. Error bars, S.E.; asterisks, statistically significant differences (p < 0.05) from the leftmost condition of each subgroup.

Fig. 2. Biochemical effects of PKC inhibitors on NGF-maintained sympathetic neurons. A, NGF-maintained sympathetic neurons were treated with PMA (100 ng/ml), LY294002, GF109203X, or vehicle alone, at the concentrations listed for 2 h in the continued presence of NGF or were deprived of NGF for 2 h. Whole cell lysates were produced, and these extracts were analyzed with phospho-PKC substrate, phospho-Akt, or phosphoextracellular signal-regulated kinase immunoblotting. Protein levels in the various samples were determined with actin immunoblotting. B, sympathetic neurons were treated with either GF109203X (10 μM, right lane) or vehicle alone (left lane) for 48 h, and Nonidet P-40 extracts were produced. TrkA was immunoprecipitated (IP) from the extracts, and its level of phosphorylation was determined with phosphotyrosine immunoblotting (W). The amount of TrkA and the total level of protein in each sample were determined with Trk and actin immunoblotting, respectively. The experiments described in this figure were performed two or three times with similar results.

Fig. 3. PKC activation can rescue neurons from apoptosis induced by NGF deprivation. A, sympathetic neurons were deprived of NGF in the presence of bryostatin 1 (100 nM), PMA (100 ng/ml), or vehicle alone (medium alone) for 24 h. The neurons were fixed, and the remaining number of viable neurons was counted and graphed as a percentage of neurons from the same culture that were not deprived of NGF. B, schematic representation of the rescue paradigm performed in C. C, neurons were deprived of NGF for 20 or 26 h, or NGF alone (leftmost condition) for 2 days. The neurons deprived of NGF for 20 or 26 h were subjected to a 5-h rescue in which the anti-NGF containing medium was removed and replaced with medium containing NGF (200 ng/ml), PMA (100 ng/ml), or anti-NGF again (α-NGF). After this rescue period, all of the neurons were switched back to medium containing NGF and also maintained for an additional 5 days. The number of surviving neurons in each condition was counted and graphed as a percentage. The experiments in this figure were performed in duplicate in two or three independent cultures. Error bars, S.E.; asterisks, statistically significant differences (p < 0.05) from the leftmost condition of each subgroup.
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Given that activation of PKCs prevented the death of NGF-deprived neurons (Fig. 3), we tested whether PKCs were required for the NGF-dependent survival of sympathetic neurons. When sympathetic neurons were deprived of NGF, 80% of neurons died within 48 h (Fig. 4A). In contrast to the dramatic effects of NGF withdrawal on neuronal survival, inhibition of conventional PKCs with GF109203X in the presence of NGF had no effect on their survival (Fig. 4A). This suggests that conventional PKCs were not solely required for the survival of sympathetic neurons. PI3K inhibition of NGF-maintained sympathetic neurons has varied effects on their survival. We have reported previously that PI3K inhibition of sympathetic neurons causes a modest and delayed apoptotic death, killing 30–40% of neurons after 4 days of treatment with LY294002 at a concentration that completely blocks activation of Akt (11). We again observed these same kinetics of death when NGF-maintained sympathetic neurons were treated with LY294002 (Fig. 4A). Therefore, under our culture conditions, PI3K was not solely required for the survival-promoting effects of NGF. To determine whether PKCs and PI3K cooperate to promote the survival of sympathetic neurons, NGF-maintained neurons were treated with both LY294002 and GF109203X, and the number of surviving neurons was determined. The combined inhibition of both PKCs and PI3K caused the death of sympathetic neurons to a similar extent and with similar kinetics as does the withdrawal of NGF (Fig. 4A). As with NGF deprivation (32), the cell death caused by PKC and PI3K inhibition was completely prevented with the broad spectrum caspase inhibitor BAF (Fig. 4A), indicating that the combinatorial inhibition of these signaling pathways induced a caspase-dependent, apoptotic death similar to that caused by NGF deprivation. These results are consistent with the hypothesis that PKCs and PI3K cooperated to promote the NGF-mediated survival of dissociated sympathetic neurons.

To determine whether other means of inhibiting PKC activity also induced programmed cell death in combination with PI3K inhibition, prolonged PMA treatment of neurons was tested. Similar to GF109203X, the sustained treatment of NGF-maintained sympathetic neurons with PMA alone had no effect on their survival (Fig. 4B). In contrast, during treatment with both PMA and LY294002, considerably more neurons underwent cell death as compared with either inhibitor alone (Fig. 4B). PMA and LY294002 induced apoptosis with somewhat delayed kinetics as compared with NGF withdrawal (Fig. 4B), which was observed to a lesser extent when apoptosis was induced with LY294002 and GF109203X (Fig. 4A). The delayed kinetics of apoptosis induced by PMA and LY294002 may be explained by the observation that PMA is a less effective PKC inhibitor than is GF109203X (Figs. 1B and 2A) and that PMA does not down-regulate all phorbol ester-sensitive PKCs (26, 33, 34). In conclusion, the PI3K and PKC pathways function in a cooperative manner to promote the survival of sympathetic neurons maintained in NGF.

PKC and PI3K Are Both Required for NGF-dependent Survival of Sympathetic Neurons Maintained under Serum-free Conditions—Although our previous results indicate that PI3K is not solely required for the survival of sympathetic neurons (11), others have reported that PI3K accounts for the majority of the survival-promoting effects of NGF (6–8). In some of these studies, sympathetic neurons were maintained in me-
different between neurons maintained in serum-containing and serum-free conditions. For example, two proteins that were phosphorylated after NGF treatment under serum-containing conditions were not under serum-free conditions (denoted with asterisks, 55 and 68 kDa). The basal level of PKC phosphorylation events was greater in neurons maintained in serum as well (Fig. 5A). These data suggest that the ability of NGF to enhance PKC activity, and which substrates are phosphorylated, depends upon the culture conditions in which the neurons are maintained.

Given these results, we sought to determine the relative contribution of both the PI3K pathway and the PKC pathway in the NGF-dependent survival of sympathetic neurons maintained in serum-free medium. Sympathetic neurons were dissociated and plated identically to the previous experiments and, after 4 days in vitro, were changed to a defined, serum-free N2 medium containing the same concentration of NGF for an additional day. Similar to NGF-maintained neurons in serum-containing medium, NGF deprivation of neurons maintained in N2 medium caused the apoptotic cell death of the neurons (Fig. 5B). In contrast to neurons maintained in serum-containing medium, PI3K inhibition induced a rapid apoptotic death of NGF-maintained sympathetic neurons in serum-free medium (Fig. 5B), suggesting that PI3K was required for survival under these conditions. When NGF-dependent neurons maintained in serum-free medium were subjected to PKC inhibition with GF109203X, a significant percentage, greater than 50% by 4 days, underwent cell death (Fig. 5B), in contrast to neurons supported by NGF in serum-containing medium (Fig. 4). The treatment of neurons with both GF109203X and LY294002 under serum-free conditions did not increase the number of neurons that died, nor did it speed up the time course of their death, as compared with LY294002 treatment alone (Fig. 5B). These data indicate that both PKCs and PI3K were required for the survival of NGF-maintained sympathetic neurons sustained under defined, serum-free conditions, whereas neither of these signaling pathways were obligatory in the presence of serum.

**PKCs Function Proximal to Jun Phosphorylation in the Cell Death Pathway**—To identify at which point activated PKCs block the apoptotic cascade, we examined events in the apoptotic pathway that were initiated by PKC inhibitors. To determine whether PKC and PI3K inhibition promoted the induction of apoptotic gene expression, NGF-maintained sympathetic neurons under serum-containing conditions were treated with LY294002, GF109203X, or PMA alone or in combination, for 24 h. As negative and positive controls, some NGF-maintained neurons were treated with vehicle alone, and some were deprived of NGF, respectively. After these treatments, the neurons were extracted to obtain whole-cell lysates and analyzed by immunoblotting. One event that is required for the expression of apoptotic genes necessary for programmed cell death in sympathetic neurons is the activation of the transcription factor c-Jun (35–37). Similar to previous studies, NGF deprivation of sympathetic neurons induced the phosphorylation of Jun on Ser63, a transcription regulatory site (Fig. 6). Inhibition of PI3K also induced the phosphorylation of Jun (Fig. 6), consistent with previous immunocytochemical observations (11), but this phosphorylation was considerably less than that induced by NGF deprivation after 24 h. Inhibition of PKCs with either GF109203X or PMA also induced some Jun phosphorylation on Ser55, but promoted less Jun phosphorylation than NGF deprivation induced (Fig. 6). In contrast, the simultaneous inhibition of both PKCs and PI3K induced Jun phosphorylation to the same extent as NGF withdrawal (Fig. 6). In a positive feedback mechanism, Jun regulates its own
PKCs inhibit the apoptotic pathway proximal to Jun activation. Sympathetic neurons maintained in NGF and serum-containing medium for 5 days in vitro were treated with LY294002 (50 μM), PMA (200 ng/ml), GF109203X (10 μM), both LY294002 and GF109203X, both LY294002 and PMA, or vehicle alone (NGF main) in the continued presence of NGF (condition listed above). In addition, some were deprived of NGF (anti-NGF). All neurons were additionally treated with BAF to prevent the loss of dying neurons, and after 24 h, the neurons were detergent-extracted. The extracts were subjected to Western analysis with antibodies specific for phospho-Ser63-Jun, total c-Jun, Bcl-2-interacting mediator of cell death (BIM), or actin as a loading control (antibody listed to the right). This experiment was performed on three independent cultures with similar results.

DISCUSSION

The PKC family comprises a large group of signaling molecules that participate in biological functions as diverse as neuronal plasticity and inflammation. Here we report that NGF promoted the phosphorylation and transactivation. Similar to Ser63 phosphorylation, inhibition of both PKC and PI3K activity induced Jun expression to the same extent as NGF deprivation, whereas inhibition of either PKCs or PI3K alone was much less effective (Fig. 6). To determine whether proapoptotic gene expression was affected, we monitored the expression of the BH3-only, Bcl-2 family member BIM, an event that is important for NGF deprivation-induced cell death (38, 39). Inhibition of either PKCs or PI3K alone induced little or no BIM expression, in contrast to NGF deprivation, which promoted the robust expression of BIM (Fig. 6). Inhibition of both PKC and PI3K activity promoted BIM expression to a greater extent than did inhibition of either signaling pathway alone, but simultaneous PKC and PI3K blockade caused less BIM expression than did NGF deprivation (Fig. 6). The expression of another proapoptotic family member, PUMA, was also examined by immunoblotting and had a similar profile as did BIM; simultaneous PKC and PI3K inhibition promoted much less expression of PUMA than did NGF deprivation (data not shown). Therefore, PKC acted with PI3K to inhibit the cell death pathway proximal to the early event of Jun activation and proapoptotic gene expression. The modest effects of simultaneous PKC and PI3K inhibition on the expression of proapoptotic genes such as BIM, as compared with NGF deprivation, may explain the somewhat slower kinetics of apoptosis induced by combined PKC and PI3K inhibition (Fig. 4).

| M | NGF main | anti-NGF | PMA | GF | LY294002 | P-Jun |
|---|----------|----------|-----|----|----------|-------|
| 46 | + | + | + | + | + | + |

How does PKC inhibit apoptosis? Although PKCs appear to act proximal to Jun activation, which substrates are required for this antiapoptotic function are unclear. PKD (PKCd) can associate with Jun N-terminal kinase and phosphorylate Jun on residues distinct from serines 63 and 73, thus potentially inhibiting Jun activity (40), which is consistent with our observations. However, NGF does not appear to activate PKDs in sympathetic neurons, in contrast to PMA treatment of these neurons. How does PKC promote the survival of NGF-dependent sympathetic neurons, and these two pathways accounted for the majority of the survival-promoting effects of NGF. The simultaneous inhibition of the PI3K and PKC pathways caused the phosphorylation and induction of the proapoptotic transcription factor c-Jun, suggesting that these signaling pathways functioned to inhibit programmed cell death at the level of proapoptotic gene expression. In summary, the PKC pathway had a critical function in the survival of sympathetic neurons maintained by NGF, regardless of the culture conditions used.

Given the size and diversity of the PKC family, an important question is which PKC family member(s) functions in trophic factor-dependent survival. The ability of PMA and GF109203X to inhibit the survival of sympathetic neurons suggests that the relevant PKC was either a conventional or novel PKC, which comprise 10 different kinases. As a first step toward identifying which PKC was involved, we examined sympathetic neurons from PKCγ mutant mice and compared them with neurons from wild-type littermates. There were no significant differences in the kinetics of death after NGF withdrawal or in their sensitivity to PI3K inhibition (data not shown). This experiment, however, suggested to us that identification of the relevant PKC by using either genetic or dominant-negative strategies may well be unmanageable, given the number of possible combinations of PKCs that could be involved and the technical difficulties associated with the ectopic expression genes in sympathetic neurons. Another approach that was taken to determine which PKC is involved in survival was a pharmacologic one, using several inhibitors reported to inhibit specific subsets of PKCs. Unfortunately, many of the compounds tested, such as rottlerin, chelerythrine, and G66976, were toxic to sympathetic neurons, even at concentrations below those described in the literature to inhibit PKC activity, thus limiting their use in cell death assays. Of note, the toxicity exhibited by these compounds did not have the time course or characteristics of apoptosis, suggesting that they inhibit other essential enzymatic processes. Although these attempts to determine which PKC contributes to trophic factor-dependent survival signaling were unsuccessful, it remains an important future direction.

The results reported here represent a dramatic example of how variations in the in vitro conditions in which the neurons are maintained affects their intracellular signaling processes. Importantly, irrespective of whether serum-containing medium or a defined medium is used, both the PKC and PI3K pathways had pivotal roles in neuronal survival initiated by NGF. The more general issue that these experiments raise is which culture condition better represents the in vivo environment for sympathetic neurons. Sympathetic neurons in vivo would not normally be exposed to serum nor would they be exposed to the high levels of many of the constituents of defined medium, such as putrescine, transferrin, and insulin, thus making this question difficult to answer. Perhaps the most direct method for discerning the relative contribution of both the PI3K and the PKC pathways in neuronal survival will be with genetic or molecular genetic approaches. However, the redundancy inherent to the PI3K and PKC families may make this a difficult task.
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neurons, based on an antibody that specifically recognizes PKDs when phosphorylated on activation loop residues necessary for catalytic activity (P-744/748-PKD; data not shown). PKCα phosphorylates mitochondrial Bel-2 on Ser70, which enhances its antiapoptotic activity (41, 42). Although a possibility, our observations place the probable function of PKCs proximal to Jun activation, well upstream of the mitochondrial events regulated by Bel-2. In an effort to understand how PKCs promote survival in neurons, we made several attempts to identify some of the PKC substrates phosphorylated after NGF treatment in a biased manner but were unsuccessful. For example, the 32-kDa protein that is tightly regulated by NGF (Fig. 2A) was not BAG-1, cyclin D1, or CDK5, all of which have similar molecular weights and isoelectric points (data not shown). These conclusions were obtained by performing biochemical experiments with antibodies specific to BAG-1, cyclin D1, and CDK5 and by analyzing neurons from CDK5 knock-out mice. Thus, the mechanism by which PKCs function to inhibit apoptosis in neurons remains unclear.

The observation that combined PI3K and PKC inhibition did not fully induce the proapoptotic BH3 domain-containing proteins such as BIM that are necessary for programmed cell death suggests that additional signaling pathways inhibited the apoptotic pathway in sympathetic neurons, either at a transcriptional or post-transcriptional level. One such mechanism is the MAPK-dependent phosphorylation and destabilization of BIM (43), which could explain the inability of PI3K and PKC inhibition to induce BIM fully. Additional survival signaling pathways, such as those that regulate the prosurvival transcription factors cAMP-response element-binding protein and NF-κB (44–46) and those that repress proapoptotic genes (47), may also account for the somewhat slower kinetics of cell death induced by PI3K and PKC inhibition. The impressive array of signaling mechanisms used by neurons to maintain survival highlights the importance of protecting postmitotic populations of cells from inappropriate or premature apoptosis. The PKC pathway is one such pathway that, in conjunction with PI3K, inhibits the expression of genes responsible for neuronal programmed cell death.

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