Mitogen-activated Protein Kinase-independent Pathways Mediate the Effects of Nerve Growth Factor and cAMP on Neuronal Survival*

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Nerve growth factor (NGF) is essential for the development and maintenance of sympathetic neurons and certain neural crest-derived sensory neurons (1, 2). During development, neurons that fail to obtain a sufficient amount of NGF from target or other tissues die. In the superior cervical ganglion (SCG), naturally occurring cell death results in a loss of 50% of the sympathetic neurons (3). If access to NGF is blocked during development, either by anti-NGF antibodies (4, 5) or by targeted disruption of the genes for NGF (6) or the NGF receptor, TrkA (7), virtually all of the SCG neurons die. Providing exogenous NGF rescues neurons that would otherwise die during development (8).

Despite the importance of NGF in the development and maintenance of the nervous system, the limitations of working with neurons has hindered our understanding of the mechanism of NGF signal transduction. Most of our knowledge of NGF signaling is based on experiments with PC12 cells, a cell line derived from a rat pheochromocytoma (9). PC12 cells treated with NGF exhibit characteristics of sympathetic neurons including extension of neurites (9, 10), electrical excitability, and expression of neuronal proteins (10, 11). Using neurite outgrowth in PC12 cells as a measure of differentiation, studies have implicated the MAP kinase pathway in NGF signaling. NGF binds to and activates the tyrosine kinase of TrkA, the high affinity NGF receptor (12–14). Phosphotyrosyl motifs on TrkA bind Shc proteins, which bind and activate Grb2/Sos, thereby activating Ras (15, 16). Ras activation results in activation of the protein kinases Raf-1, B-Raf, and MAP-ERK kinase (MEK) (17–19). Activation of one or more of these kinases leads to the phosphorylation and activation of MEK and the MAP kinase isozymes, ERK-1 and ERK-2 (19–22). Introducing activated forms of upstream regulators of MAP kinase, including Ras (23), Raf-1 (24), or MEK (25), mimic NGF by inducing neurite outgrowth in PC12 cells. The dominant negative forms of Ras (26, 27), Raf (28), and MEK (29) block the neurite outgrowth in PC12 cells stimulated by NGF.

One approach for identifying survival-promoting pathways in primary neurons has been to identify agents or conditions that support neuronal survival in the absence of NGF. The cAMP analog, chlorophenylthio-cAMP (CPTcA), supports the complete survival of SCG neurons in the absence of NGF (29–31). In PC12 cells, a direct link exists between the cAMP and NGF signaling pathways. Derivatives of cAMP induce neurite outgrowth in PC12 cells (32). Recently it was demonstrated that cAMP analogues increase MAP kinase activity in PC12 cells (35, 36) and potentiate NGF activation of MAP kinase (35). These findings are consistent with the hypothesis that cAMP potentiates NGF-induced neurite outgrowth in PC12 cells by activating MAP kinase.

The pathways mediating neuronal differentiation may differ from those involved in NGF-dependent survival. In a differentiated PC12 cell model, overexpression of MEK supports PC12 cell survival in the absence of NGF, suggesting that this pathway may be important for neuronal survival (37). Although the PC12 cell model offers distinct advantages for studying NGF signaling, PC12 cells are transformed and may not be representative of postmitotic neurons in all respects. Thus, primary sympathetic neurons represent a better model for investigating signaling events that are important for neuronal survival. The present experiments were performed to investigate the role of MAP kinase in the actions of CPTcA and NGF on the survival of primary sympathetic neurons derived from the superior cervical ganglion.

EXPERIMENTAL PROCEDURES

Neuronal Culture—Neuronal cultures were prepared from the SCG of embryonic day 21 or postnatal day 0 rats (38). The neurons were plated on collagen-coated dishes and maintained at 37 °C in a humid-
MAP Kinase-independent Neuronal Survival

Measurements of MAP Kinase Activities—MAP kinase activity was measured by using myelin basic protein (MBP) and [γ-32P]ATP as substrates essentially as described previously (40). Extract (10 μl) was combined with 20 μl of a reaction mixture (75 mM β-glycerophosphate, 45 mM NaF, 15 mM MgCl2, 2 mM dithiothreitol, 6.7 μM cAMP-dependent protein kinase inhibitory peptide (41), 0.5 mg/ml bovine serum albumin, 0.1% Triton X-100, pH 7.3), then incubated at 23°C for 60 min in buffer containing 0.1% Triton X-100. After incubation, the gels were rinsed and incubated for 12 h at 4°C in 5 mM β-mercaptoethanol, 0.4% Tween 20, and 1.5% normal goat serum to inhibit nonspecific binding, the cultures were incubated for 30 min in buffer containing 10 μM [γ-32P]ATP (900–1200 cpm/pmol). The gels were washed with 5% trichloroacetic acid and 1% sodium phosphate before the 32P incorporated into MBP was determined. For each condition tested, triplicate samples were homogenized and assayed.

ERK-1 and ERK-2 activities were measured after SDS-PAGE and renaturation essentially as described by Kameshita and Fujisawa (42). Briefly, extract samples (40 μl) were subjected to SDS-PAGE in 10% polyacrylamide gels that were polymerized with 0.3 mg/ml MBP. Where indicated, extract samples were prepared by homogenizing cells in sample buffer. After electrophoresis the gels were washed in 20% isopropanol (2 × 30 min); and 5 mM β-mercaptoethanol, 6 μm dithiothreitol, and 50 mM Tris-HCl, pH 8 (2 × 30 min). The gels were then rinsed and incubated for 12 h at 4°C in 5 mM β-mercaptoethanol. 0.4% Tween 20, and 50 mM Tris-HCl, pH 8. To assess kinase activities, the gels were rinsed for 30 min with buffer (50 mM β-glycerophosphate, 300 μM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 100 μM sodium vanadate, 2 mM benzamidine, 10 μM leupeptin, 10 μM aprotinin, 10 μM phenylmethylsulfonyl fluoride, pH 7.3) were added. The cells were immediately scraped off the dish and homogenized at 0°C in a glass Dounce homogenizer. Homogenates were centrifuged at 4°C for 10 min at 14,000 × g, and the supernatants were retained for analysis.

Results and Discussion

NGF increased MAP kinase activity in SCG neurons (Fig. 1). The maximum effect represented a 4-fold increase and was observed at 10 ng NGF. Significant activation of MAP kinase was observed at concentrations as low as 0.1 ng NGF. Neuronal survival decreases markedly below 0.05 ng NGF. Thus, the concentrations of NGF that increased MAP kinase activity were within the range of concentrations that support neuronal survival. The effects of NGF on activating MAP kinase were rapidly reversible. Within 30 min of removing NGF, MBP kinase activity fell to a stable plateau that was approximately 25% of maximal activity (Fig. 2). This fall in MBP kinase activity precedes many of the biochemical alterations that have been identified in NGF-deprived neurons (31). However, if the MAP kinase pathway is important for neuronal survival, then the residual activity observed after removing NGF is insufficient to support neuronal survival since all such neurons die via apoptosis within 48–72 h (31, 44, 45). The small number of nonneuronal cells in the cultures might account for some of this basal activity, although the number of such cells was minimized by adding the anti-mitotics, fluorodeoxyuridine and aphidicolin, to the culture medium. Based on cell counts, nonneuronal cells represented only 5–10% of the cultured cells. The NGF-stimulated activity was clearly associated with neurons, because NGF failed to increase MAP kinase activity in cultures in which the neurons had been eliminated by treatment with anti-NGF for 48 h.2

In contrast to NGF, CPTCa (500 μM) did not activate MAP kinase in SCG neurons, even after prolonged incubation (Fig. 3). By comparison, NGF (2 ng) increased activity by 4-fold within 5 min and activity was maintained for 24 h. Neurons deprived of NGF for up to 18 h can be rescued from cell death by adding either NGF or CPTCa; after 25 h of NGF deprivation, less than 40% of the neurons can be rescued by NGF or CPTCa treatment (30, 31). Thus, CPTCa rescues NGF-deprived SCG neurons with a time course very similar to that of NGF. To confirm that CPTCa promoted neuronal survival under conditions of the present experiments, 6-day neuronal cultures were deprived of NGF for 12 h and then treated with NGF or CPTCa for 5 days (Fig. 4). No neurons were found in the cultures

2 D. J. Creedon, E. M. Johnson, J. R., and J. C. Lawrence, J. R., unpublished observations.
Neurons that received either NGF (Fig. 4a) or CPTcA (Fig. 4c) exhibited phase-bright cell bodies and extensive neuritic networks characteristic of viable neurons.

That CPTcA increased survival but did not increase MBP kinase activity suggests that MAP kinase is not required for survival; however, only a small increase in MAP kinase may be necessary to promote neuronal survival. To address this point, neuronal cultures were treated with the phorbol ester, PMA, which activates the MAP kinase pathway in many cell types (19, 27, 35, 36). PMA (1 \( \mu \)M) transiently increased the MBP kinase activity in SCG neurons (Fig. 3). However, as observed previously (46), PMA did not maintain NGF-deprived neurons.

Measurements of MBP phosphorylation in extracts reflect the activities of the ERK-1 and ERK-2 isoforms of MAP kinase (and possibly other kinases). To investigate the possibility that CPTcA selectively activated one of the MAP kinase isoforms, ERK-1 and ERK-2 activities were assessed by using an in-gel assay. Two major MBP kinases were resolved by SDS-PAGE (Fig. 5a). The apparent molecular weights (44,000 and 42,000) corresponded to the expected sizes of ERK-1 and ERK-2, respectively (47). Immunoprecipitation experiments with specific antibodies confirmed the assignment of these bands as ERK-1 and ERK-2. NGF markedly increased the activities of both ERK-1 (Fig. 5b) and ERK-2 (Fig. 5c). In contrast, CPTcA increased the activity of neither isoform. CPTcA appeared to enhance the effect of NGF slightly on increasing ERK-1 activity, but it had no effect on the stimulation of ERK-2 by NGF. CPTcA was also without effect on the activation of ERK-1 and ERK-2 by PMA.

In PC12 cells, CPTcA promotes a 12-100-fold increase in MAP kinase activity (35, 36) and potentiates the effects of NGF and PMA on increasing MAP kinase activity (35). In SCG neurons, CPTcA alone activated neither ERK-1 nor ERK-2 and caused only a slight potentiation of ERK-1 activity in neurons treated with both cAMP and NGF (Fig. 5, b and c). Although the reason the response to CPTcA differs in sympathetic neurons and PC12 cells is not clear, different responses may even occur in different subclones of PC12 cells. Vaillancourt et al. (22) find that increasing cAMP with forskolin has no effect on MAP kinase activity in PC12 cells. In other cells, such as...
adipocytes (48), fibroblasts (49, 50), and vascular smooth muscle cells (51), increasing cAMP actually decreases the activation of MAP kinase activity. Activated cAMP-dependent protein kinase phosphorylates Raf-1 and B-Raf, inhibiting both Ras-Raf interactions and Raf kinase activity (52, 53). Regardless of the mechanisms involved, the differences between the effect of cAMP on MAP kinase in PC12 cells and SCG neurons underscore the importance of not relying solely on the PC12 model to define the mechanisms of action of neurotrophins.

To test the possibility that neuronal survival is enhanced through activation of a subcellular pool of MAP kinase that is not detected in assays of cell extracts, control neurons and cells that had been treated with CPTcA or NGF were immunostained with an antibody that recognizes only the active phosphorylated form of MAP kinase. Six-day neuronal cultures were deprived of NGF for 12 h, then treated with no additions, 2 nm NGF, or 500 μM CPTcA. Neurons were fixed 3 h after treatment and stained with a phospho-specific MAP kinase antibody. Phase-contrast (a, c, and e) and corresponding immunofluorescent (b, d, and f) images reveal that control (a and b), and CPTcA-treated (c and d) neurons contain no detectable phospho-MAP kinase, whereas in NGF-treated (e and f) cultures, the neuronal cell bodies and processes are stained. A similar staining pattern was seen in cultures fixed 1 h after treatment.

The failure of CPTcA to increase MAP kinase activity in SCG neurons does not mean that the MAP kinase pathway was not necessary for NGF-mediated neuronal survival. To investigate this possibility, we assessed the effect of the MEK inhibitor, PD 098059 (55, 56), on dissociated SCG neurons. PD 098059 caused a concentration-dependent inhibition of the activation of both ERK-1 and ERK-2 by NGF (Fig. 7a). The amount of activity that remained after treatment with 100 μM PD 098059 (lane 5) was comparable to that seen in either control (NGF-deprived) cultures (lane 1) or cultures treated with 500 nm

**FIG. 6.** Phospho-MAP kinase antibodies stain NGF, but not control or CPTcA-treated SCG neurons. Six-day neuronal cultures were deprived of NGF for 12 h, then treated with no additions, 2 nm NGF, or 500 μM CPTcA. Neurons were fixed 12 h after treatment and stained with phospho-specific MAP kinase antibody. Phase-contrast (a, c, and e) and corresponding immunofluorescent (b, d, and f) images reveal that control (a and b), and CPTcA-treated (c and d) neurons contain no detectable phospho-MAP kinase, whereas in NGF-treated (e and f) cultures, the neuronal cell bodies and processes are stained. A similar staining pattern was seen in cultures fixed 1 h after treatment.
K252a (lane 6), a Trk inhibitor that causes neuronal cell death indistinguishable from that produced by NGF deprivation (57–59). PD 098059 also blocked the increase in MAP kinase staining after NGF treatment.

Neurons deprived of NGF can be rescued if NGF is added after 12 h. If NGF is added after 18 h, approximately 80% of the neurons can be rescued; however, if NGF is added after 36 h, essentially none of the neurons survive (30, 31). Based on this time course, a three-stage paradigm was used to investigate the effect of MEK inhibition on neuronal survival. In stage 1, SCG cultures were deprived of NGF for 12 h to initiate the cell death program. In stage 2, cells were incubated without or with 100 μM PD 098059 for 30 min. NGF was then added to half of the cultures, and the cells were incubated for 24 h. To ensure that MEK inhibition was maintained during this period, the medium was replaced every 6 h with medium containing fresh inhibitor. Control experiments indicated that a single addition of 100 μM PD 098059 inhibited MAP kinase activation for at least 8 h. In stage 3, NGF was added to all the cultures to rescue neurons that were not committed to die. The numbers of surviving neurons were counted after an additional 2 days in culture. Essentially all the neurons that did not receive NGF in stage 2 died (Fig. 7b). In contrast, neurons that received NGF in stage 2 survived, even those incubated with PD 098059 (Fig. 7b).

The results with PD 098059 indicate that MAP kinase activation was not required for neuronal survival stimulated by NGF. CPTcA also appeared to promote survival independently of the MAP kinase signaling pathway as the cyclic nucleotide derivative increased neither ERK-1 nor ERK-2 activity (Fig. 5). Interestingly, several lines of evidence indicate that Ras, an upstream element of the MAP kinase signaling pathway, is part of the survival pathway for both these agents. Chicken sensory neurons survive and extend neurites in the absence of NGF if isolated by mechanical dissociation in the presence of activated T-24 Ras (60). Microinjection of Y13–259, a neutralizing Ras antibody, or dissociation in the presence of Y13–259 Fab fragments blocks the survival of chicken sensory neurons (61) or rat SCG neurons (62) maintained in NGF. Dissociation of rat sympathetic neurons in the presence of Ras antibodies also blocks CPTcA-mediated neuronal survival (62). This issue is further complicated, however, by the observation that a dominant negative Ras prevents apoptosis of PC12 cells (63). Considered in the context of these previous findings, our results suggest that the neuronal survival pathway branches from the MAP kinase pathway at the level of Ras.

Sustained activation of MAP kinase may be sufficient for survival, although not necessary for NGF-mediated survival. Overexpression of activated MEK in NGF-differentiated PC12 cells blocks the apoptosis triggered by NGF withdrawal, possibly by inhibiting the JNK and/or p38 signaling pathways (37). Both JNK and p38 phosphorylate the transcription factor Jun, which has been implicated in neuronal cell death. As SCG neurons undergo apoptosis, the cells increase the transcription (64) and translation (65) of jun. These increases appear to be required for the cell death process since microinjecting Jun antibodies (64) or a dominant negative Jun construct (65) blocks apoptosis. In PC12 cells, NGF withdrawal activates both JNK and p38; overexpression of either kinase promotes apoptosis even in the presence of NGF (37). Greenberg and colleagues (37) have further proposed that the fate of a cell depends on the balance between growth factor-activated MAP kinase activity (life) and stress-activated JNK and p38 activity (death). Thus, inhibition of MEK might render cells more susceptible to stress-induced apoptosis. However, our results demonstrate that inhibition of MEK was not sufficient to activate programmed cell death.

Results from PC12 cells indicate that sustained activation of the MAP kinase pathway is important for neurite extension (56, 66–68). NGF caused sustained activation of MAP kinase in SCG neurons (Fig. 3), but we were unable to evaluate the effect on neurite outgrowth as the primary neurons had already extended neurites. The observation that neurons maintained in cAMP analogs have smaller somas and less extensive neurite formations than those maintained in NGF (Ref. 29; see also Fig. 4) is correlative evidence that MAP kinase activity may be important for neurite extension in neurons. Although the neurons do not die in the presence of CPTcA, the rate of growth is less than with NGF. Thus, our findings are consistent with the hypothesis that MAP kinase activity is sufficient for promoting neuronal growth and/or differentiation. Additional investigation is needed to define the MAP kinase-independent pathway(s) mediating the effects of NGF on neuronal survival.

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