

# Activation of sphingosine kinase in pheochromocytoma PC12 neuronal cells in response to trophic factors

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**Abstract** Nerve growth factor (NGF), basic fibroblast growth factor (bFGF), dibutyryl cAMP and forskolin, known differentiating agents for pheochromocytoma PC12 cells, induced sustained activation of sphingosine kinase, the enzyme responsible for the formation of the sphingolipid second messenger, sphingosine-1-phosphate, which mediates the mitogenic effects of certain growth factors. In contrast, epidermal growth factor and insulin-like growth factor-1, which stimulate proliferation of PC12 cells, induced only small and transient increases in sphingosine kinase activity. Of the growth factors examined, NGF was the most potent activator of sphingosine kinase, inducing a 4-fold increase in  $V_{\max}$ . Sphingosine kinase activity induced by NGF, but not FGF, was blocked by the protein kinase inhibitor K252a when added simultaneously, with minimal effect when added after 60 min. Thus, activation of sphingosine kinase may have an important role in neural differentiation.

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**Key words:** Nerve growth factor; Neurotrophic factor; PC12 cell; Sphingosine kinase; trkA

## 1. Introduction

Given the complexity and extreme cellular diversity of the nervous system, the study of transformed neural crest-derived cell lines, such as rat pheochromocytoma PC12 cells which recapitulate many of growth and neurotrophic factor signaling events, has been instrumental in understanding the mechanisms of neuronal differentiation and proliferation [1–3]. Although binding of NGF and EGF to their respective cell surface receptors stimulates similar signal transduction cascades, NGF induces neurite outgrowth and neural markers, and cessation of cell growth, whereas EGF only stimulates proliferation [1,4]. It has been suggested that in neurons, discrimination between strong/prolonged and weak/transient activation of the extracellular signal regulated kinase (ERK) signaling pathway may be the cause of these opposite effects [4]. However, other studies indicate that this might be a more complex process, and other pathways are also probably involved [2,3].

Intermediates in biosynthesis and catabolism of sphingolipids, ceramide, sphingosine and SPP, have recently been implicated in intracellular signaling important for neuronal survival,

differentiation, development, and death [5–9]. Because increased levels of ceramide were observed during differentiation of neuroblastoma Neuro2a cells and SH-SY5Y cells [5,6], it has been suggested that ceramide plays a role in neuronal differentiation. Moreover, inhibitors of ceramide biosynthesis, such as fumonisins B1, interfere with differentiation of Neuro2a and cerebellar granule cells [6], and cultured hippocampal neurons [10]. However, increased ceramide levels within distal neurites inhibits neurite growth of cultured rat sympathetic neurons [7] and of NGF-differentiated PC12 cells [11]. Similarly, sphingosine, a metabolite of ceramide, suppresses NGF-directed neurite outgrowth in PC12 cells [12], likely by conversion to ceramide. The role of the further metabolite of sphingosine, SPP, produced by activation of sphingosine kinase, which generally provides positive signals for cell growth and survival [13–15], has not been established in neurons. Recently, SPP has been shown to play a distinct role in altering neuronal cell morphology [9,16] and stimulation of the ERK signaling pathway in diverse cell types [13,17,18], reminiscent of effects elicited upon growth factor stimulation. Furthermore, it has been suggested that the intracellular ratio of ceramide to SPP may be a critical factor in determining the fate of cells [13]. In this report, we examined the effects of diverse trophic agents on sphingosine kinase activity in PC12 cells. Our results suggest that sustained activation of sphingosine kinase may be important for neurotrophic responses.

## 2. Materials and methods

### 2.1. Materials

Mouse 2.5S nerve growth factor was obtained from Upstate Biotechnology; bFGF was purchased from Promega; EGF, IGF-1 and insulin were purchased from Collaborative Research; dibutyryl cAMP was from Boehringer Mannheim; sphingosine was purchased from Matreya; SPP was from Biomol.

### 2.2. Cell culture

Rat PC12 pheochromocytoma cells were kindly provided by Dr. Gordon Guroff (NICHD, NIH, Bethesda, MD). Cells were grown on polystyrene tissue culture dishes in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal calf serum (Gibco), plus 100 units/ml penicillin and 100 µg/ml streptomycin (Biofluids). Cells were seeded, except where indicated, at a density of  $5 \times 10^4/\text{cm}^2$  and allowed to grow for 2–4 days before experiments were performed. All experiments, unless otherwise described, were conducted after 3 day exposure to NGF. At this time, long processes and other morphological changes were clearly observed. Cells were maintained in a 37°C incubator in 5% CO<sub>2</sub>, water-saturated atmosphere.

### 2.3. Sphingosine kinase activity

Sphingosine kinase activity was measured as previously described [19]. Briefly, after treatment, cells were washed twice with PBS and harvested in kinase buffer (20 mM Tris buffer (pH 7.4) containing 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 15 mM NaF, 20 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 0.5 mM 4-deoxyypyridoxine,

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**Abbreviations:** SPP, sphingosine-1-phosphate; cAMP, dibutyryl cAMP; PBS, phosphate-buffered saline; TLC, thin layer chromatography; NGF, nerve growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1

1 mM PMSF, 10  $\mu$ g/ml leupeptin, and 10  $\mu$ g/ml aprotinin). After freeze-thawing three times, cytosolic extracts were obtained by centrifugation for 10 min at  $14000\times g$ . 20–50  $\mu$ g protein was used in each kinase assay. Reactions were started by addition of 250  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol, ICN) and 50  $\mu$ M sphingosine in a final volume of 100  $\mu$ l. Samples were incubated at 37°C for 10 min and reactions were terminated with 10  $\mu$ l of 1 N HCl followed by 400  $\mu$ l of chloroform/methanol/HCl (100:200:1, v/v). After vortexing, 125  $\mu$ l each of 2 M KCl and chloroform were added for phase separation. Lipids in the chloroform phase were resolved on silica gel 60 thin layer chromatography plates (EM Science) in butanol/ethanol/acetic acid/water (80:20:10:20, v/v). SPP was visualized by autoradiography, scraped from the plates and measured by liquid scintillation counting. For preparation of cytosolic and particulate fractions, cells were resuspended in kinase buffer, freeze-thawed three times, and centrifuged at  $100000\times g$  for 60 min.

#### 2.4. Measurement of SPP levels

After 2 days of NGF treatment, cells were labeled for the final 24 hours of NGF exposure with  $^{32}$ Pi (40 mCi/ml, ICN), in the presence of 0.5 mM 4-deoxypyridoxine to inhibit pyridoxal phosphate-dependent SPP aldolase. The media was removed and 1 ml of 0.1 N HCl and 4 ml of chloroform:methanol (2:1, v/v) were added to extract the lipids. The lipids in the organic phase were separated by two-dimensional TLC: chloroform/methanol/water (66:33:8, v/v) in the first dimension; and 1-butanol/methanol/acetic acid/ water (80:20:10:20, v/v) in the second dimension [15]. Standard SPP and sphingosine were applied with the samples and lipids were visualized with iodine vapors or by spraying with ninhydrin. The major cellular lipids and sphingolipids were excised from the plate and counted by liquid scintillation [15].

#### 2.5. Protein measurement

Protein measurements were performed following the procedure of Bradford with the Coomassie Plus Protein Assay Reagent (Pierce).

### 3. Results and discussion

#### 3.1. Activation of sphingosine kinase by prolonged treatment with NGF

Treatment of PC12 cells with NGF for 3 days, which causes differentiation and expression of neurofilament protein, markedly activated sphingosine kinase (Fig. 1A). Concomitantly, levels of SPP, the product of sphingosine kinase action, were increased 2.8-fold. After prolonged treatment of NGF for up to 2 weeks, sphingosine kinase activity remained markedly elevated (up to 5–6-fold). Activation of sphingosine kinase by NGF was dose-dependent, with maximal activation occurring at 100 ng/ml (Fig. 1B). Sphingosine kinase activity in extracts of untreated PC12 cells, as well as in cells treated with NGF, exhibited typical Michaelis-Menten kinetics, with a 4-fold increase in  $V_{max}$  induced by NGF without a significant change in the  $K_m$  for sphingosine (Fig. 1C). In a wide variety of cells and tissues, sphingosine kinase activity has been found to be present mainly in the cytosol (reviewed in

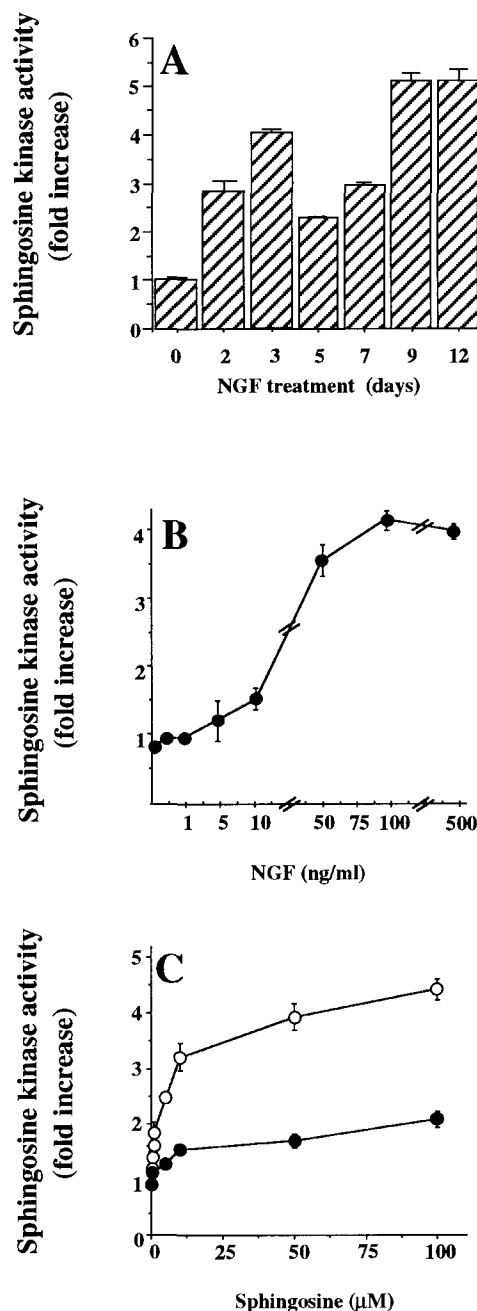


Fig. 1. Effect of NGF on sphingosine kinase activity. A: NGF activates sphingosine kinase. PC12 cells were treated with NGF (100 ng/ml) for the indicated times and sphingosine kinase activity was measured as described in Section 2. B: Dose response. PC12 cells were treated with the indicated concentrations of NGF for 3 days and sphingosine kinase activity was measured. C: Michaelis-Menten kinetics. Cells were treated in the absence (closed circles) or presence of NGF (open circles) for 3 days and cytosolic sphingosine kinase activity was determined in the presence of increasing concentrations of sphingosine. All data are expressed as fold increase in sphingosine kinase activity and are the mean  $\pm$  S.D. of triplicate cultures. Similar results were obtained in five independent experiments.

Table 1  
Distribution of sphingosine kinase in PC12 cells

Treatment	Sphingosine kinase activity		
	None	NGF	Fold increase
Cytosol	$9.6 \pm 0.3$	$25.0 \pm 0.7$	2.6
Particulate	$2.7 \pm 0.2$	$4.8 \pm 0.2$	1.8

Sphingosine kinase activity in cytosol and particulate fractions of PC12 cells was determined before and after treatment of PC12 with NGF (100 ng/ml) for 3 days. Sphingosine kinase activity is expressed as pmol/min/mg protein. Data are the means  $\pm$  S.D. of triplicate cell cultures. Similar results were obtained in two independent experiments.

[14]). However, membrane-bound forms of sphingosine kinase have also been detected [20,21]. In PC12 cells, the majority of sphingosine kinase activity is located in the cytoplasm and NGF treatment increased the activity to a similar extent in both cytoplasmic and particulate fractions (Table 1).

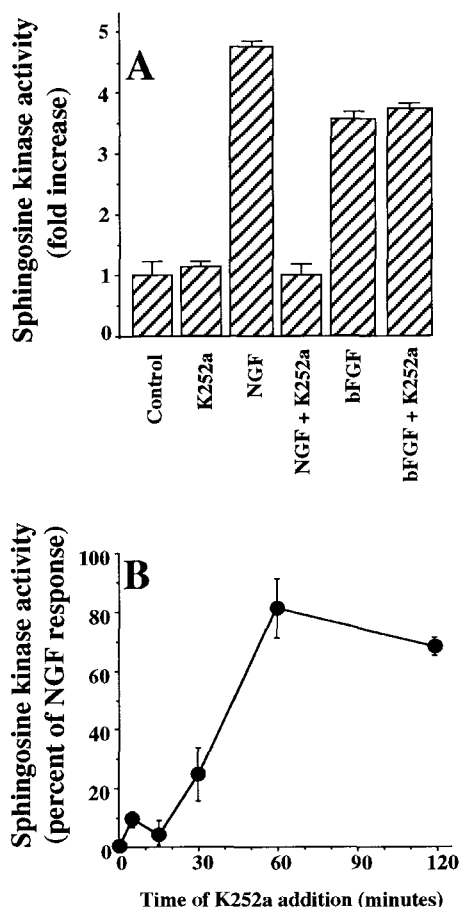


Fig. 2. Involvement of *trkA* in NGF activation of sphingosine kinase. **A**: K252a inhibits NGF induced sphingosine kinase. Sphingosine kinase activity in PC12 cells stimulated with bFGF (20 ng/ml) or NGF (100 ng/ml) for 3 days was determined in the absence or presence of K252a (200 nM). Data are the mean  $\pm$  S.D. of fold increases in kinase activity. Similar results were obtained in two independent experiments. **B**: Time course of K252a inhibition of NGF-induced increase in sphingosine kinase activity. PC12 cells were treated with NGF (100 ng/ml) at 0 time and K252a (200 nM) was added at the indicated time points after addition of NGF. Sphingosine kinase activity was measured after 3 days and is expressed as percent of the activity in untreated cells. Data are the means  $\pm$  S.D. of triplicate cultures, and similar results were obtained in two independent experiments.

### 3.2. Activation of sphingosine kinase by NGF occurs through the *trkA* receptor

Most of the actions of NGF are mediated by binding to the *trkA* receptor resulting in the dimerization of the receptor and autophosphorylation of key tyrosine residues [1–4,22]. Due to the potent activation of sphingosine kinase in response to NGF treatment, we investigated whether *trkA* mediated activation of sphingosine kinase. K252a, an inhibitor of *trkA* [23], completely blocked the activation of sphingosine kinase induced by NGF after 3 days of treatment (Fig. 2A), indicating that the *trkA* receptor is essential for NGF-induced sphingosine kinase activation. In contrast, K252a had no effect on activation of sphingosine kinase induced by bFGF or on basal sphingosine kinase activity. *TrkA* activation and subsequent autophosphorylation of key tyrosine residues has been shown to occur within minutes of neurotrophin binding and quickly

attenuates thereafter [22]. Inhibition of the activation of sphingosine kinase by NGF was observed only when K252a was added within the first 30 min of exposure to NGF, with a minimal effect resulting when added after 60 min (Fig. 2B).

### 3.3. Long-term activation of sphingosine kinase by NGF

*requires protein synthesis and is dependent on cell density*

Sustained elevation of sphingosine kinase activity induced by chronic treatment with NGF suggested that NGF is likely stimulating synthesis of sphingosine kinase protein. Treatment of PC12 cells with the protein synthesis inhibitor cycloheximide dramatically reduced the extent of NGF-stimulated sphingosine kinase activity, indicating that the observed increase in  $V_{\max}$  was due to an increase in enzyme concentration (Fig. 3A). The effects of NGF on sphingosine kinase activity were also dependent on cell density, since increasing the cell density produced a corresponding increase in NGF-induced sphingosine kinase activation (Fig. 3B). Thus, cell-cell contact may be important for some of the trophic effects of NGF in PC12 cells.

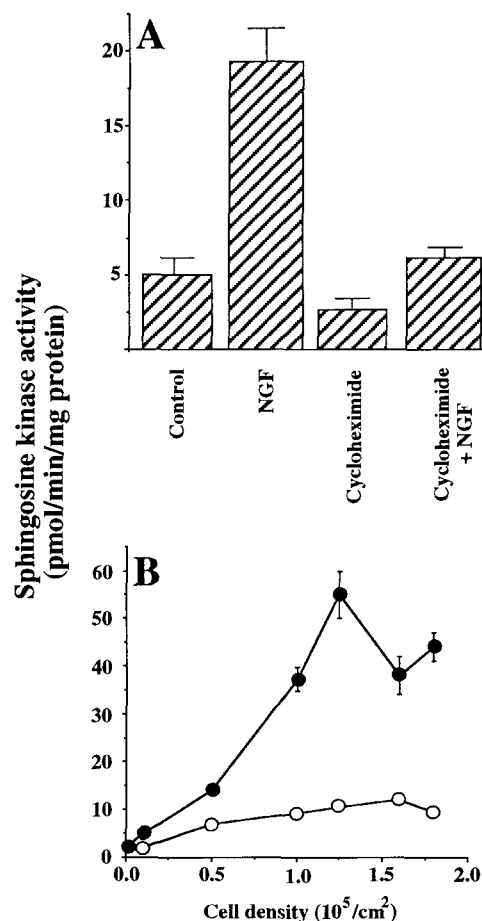


Fig. 3. Protein synthesis is required for NGF-induced sustained activation of sphingosine kinase. **A**: Effect of cycloheximide. PC12 cells were treated with cycloheximide (10  $\mu\text{g}/\text{ml}$ ) with and without the presence of NGF (100 ng/ml) and sphingosine kinase activity was measured. **B**: Effect of cell density. PC12 cells were plated at the indicated densities, in the absence (open circles) or presence of NGF (closed circles). Sphingosine kinase activity is expressed as pmol/min/mg protein and all values are the mean  $\pm$  S.D. of triplicate cultures.

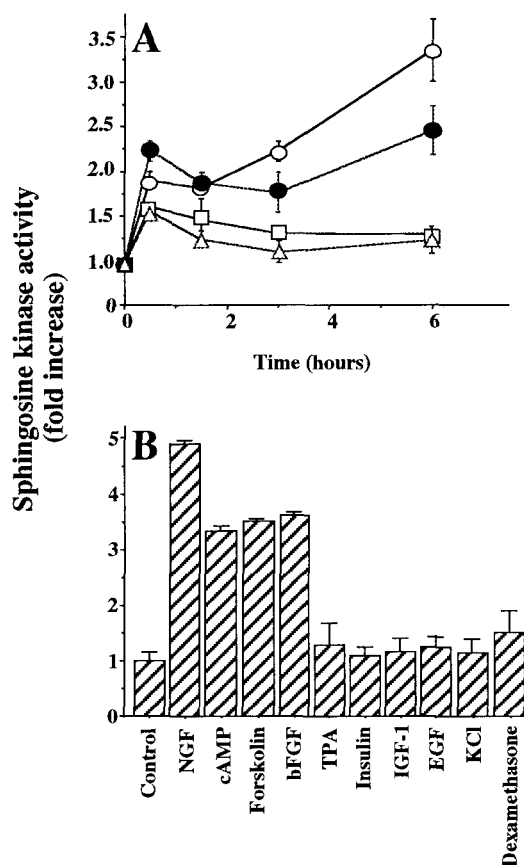


Fig. 4. Effect of various growth factors on sphingosine kinase activity in PC12 cells. A: Cells were treated for the indicated times with NGF (100 ng/ml; open circles), EGF (100 ng/ml; open squares), IGF-1 (100 nM; open triangles), bFGF (20 ng/ml, closed circles) and sphingosine kinase activity was measured. B: Cells were treated with NGF (100 ng/ml), dibutyryl cAMP (1 mM), forskolin (10  $\mu$ M), TPA (100 nM), insulin (100 nM), IGF-1 (100 nM), EGF (100 ng/ml), KCl (50 mM) and dexamethasone (100 nM) and sphingosine kinase activity was measured after 3 days. All data are the mean  $\pm$  S.D. of fold increases in kinase activity. Similar results were obtained in three independent experiments.

### 3.4. Effects of neurotrophic factors on sphingosine kinase activity in PC12 cells

It was of interest to examine whether other growth factors and trophic agents besides NGF could also affect sphingosine kinase in PC12 cells. Similar to NGF, bFGF also induced a biphasic increase in sphingosine kinase activity, although the magnitude of the response was smaller (Fig. 4A). In contrast, other mitogens, including EGF and IGF-1, induced only small and transient increases. Although treatment of PC12 cells for 3 days with TPA, insulin, IGF-1, or EGF did not have any effect on sphingosine kinase activity, dibutyryl cAMP and forskolin, which promote survival [24] and differentiation of neuronal cells [25], significantly activated sphingosine kinase to similar levels observed after exposure to bFGF, but all were less effective than NGF (Fig. 4B). Thus, agents that promote both survival and differentiation of PC12 cells were the most effective activators of sphingosine kinase.

Studies of events mediating neuronal cell homeostasis indicate that a variety of neurotrophin-induced, receptor-mediated events can influence the type of biological responses produced. Our findings suggest that activation of sphingosine kinase, the enzyme responsible for the production of the second messenger SPP, correlates with the neurotrophic effects of NGF and bFGF on PC12 cells. Subtle differences in signaling pathways mediated by distinct production of second messengers, such as SPP, may modulate intrinsic cellular homeostasis. Thus, activation of sphingosine kinase may help fine-tune responses to the many signals a cell receives and aid in mediating cellular events leading to proliferation, differentiation, or apoptosis.

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