Nerve growth factor induces sphingomyelin accumulation in pheochromocytoma cells

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Abstract The pheochromocytoma cells are a well-known model for studying the nerve growth factor (NGF)-induced molecular changes during the differentiation process. The involvement of sphingomyelin (SM) was studied using the fluorescent analogue of ceramide, i.e. *N*-lissamine rhodaminyl-(12-aminododecanoyl) D-*erythro*-sphingosine (C12-LRh-Cer). This fluorescent analogue is metabolically active and can be used to follow the biosynthesis of SM in intact cells. NGF induces a 4-fold increase of fluorescent SM content in PC12 cells, when loaded with C12-LRh-Cer. Treatment of PC12 cells with actinomycin D or cycloheximide completely abolishes the NGF-induced elevation of SM. Inhibition of p140^{trkA} receptor by AG-879 prevents extracellular signal-regulated kinase 1/2 phosphorylation and suppresses the increase of SM. Inhibition of protein kinase C (PKC), protein kinase A (PKA) and phosphatidylinositol 3-kinase does not have any effect on NGF-induced C12-LRh-SM accumulation. On the other hand, activation of PKA or PKC with simultaneous treatment with NGF has a synergistic effect on increase of SM content. The NGF-induced SM increase in PC12 cells is an effect promoted by other differentiating agents like dibutyryl cyclic AMP or fibroblast growth factor-2 but not by a mitogenic agent like epidermal growth factor.

Key words: Ceramide; Sphingomyelin; Nerve growth factor; Rhodamine; Cell differentiation; PC12 cell

1. Introduction

The involvement of sphingolipids in the regulation of cellular proliferation, differentiation and programmed cell death [1] has clearly emerged during the last years [2].

Sphingomyelin (SM), an essential structural component of plasma membrane [3], is the precursor of molecules like ceramide and sphingosine-1-phosphate (S1P), which resulted to be second messengers. Formation of ceramide through the 'SM cycle' has been shown to have a specific role in cell cycle arrest [4], differentiation [5,6] and apoptosis [7–9]. S1P, in turn, plays an important role in neuronal growth regulation [10,11].

Nerve growth factor (NGF) modulates survival and differentiation of neuronal cells. The biological activity of NGF is mediated by the interaction with its high affinity p140^{trkA} and low affinity p75 receptors [12]. Pheochromocytoma PC12 cells, which co-express both p140^{trkA} and p75 receptors, are a widely used model system to study many biochemical and biological aspects of the differentiating process [13–16], inter alia, gangliosides biosynthesis [17,18].

Recent studies have shown that NGF reduces ceramide levels in PC12 cells through an increase of the synthesis of S1P [11] and that the developmental increase in SM levels is essential in neuronal maturation [19].

In the present paper, we have investigated the possibility that the neuronal differentiation process induced by NGF in PC12 cells could be associated to an increase in SM content. Using a fluorescent ceramide as SM precursor, we have observed that NGF induces an increase of SM content in PC12 cells and that this effect depends on the activation of p140^{trkA} receptor and extracellular signal-regulated kinase 1/2 ($ERK_{1/2}$).

2. Materials and methods

2.1. Materials

NGF 2.5S, epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), horse serum (HS), fetal bovine serum (FBS), RPMI 1640 medium, L-glutamine, penicillin and streptomycin were obtained from Gibco BRL (Life Technologies, Milan, Italy). Tetradecanoylphorbol-13-acetate (TPA), 3,5-di-*t*-butyl-4-hydroxybenzylidene cyanothioacetamide (AG-879), chelerythrine chloride, H-89, PD-98059, LY-294002 and N^6,2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate (dbcAMP) were purchased from Biomol (Plymouth Meeting, PA, USA). Essentially fatty acid-free human albumin, actinomycin D and poly-L-lysine were obtained from Sigma (Sigma-Aldrich, Milan, Italy). *N*-Lissamine rhodaminyl-(12-aminododecanoyl) D-*erythro*-sphingosine (C12-LRh-Cer) and *N*-lissamine rhodaminyl-(12-aminododecanoyl) sphingosyl phosphocholine (C12-LRh-SM) were synthesized as previously reported [20,21]. Solvents, thin layer chromatography (TLC) aluminum sheets silica gel 60 and LiChroprep RP-8 for reverse phase liquid chromatography were obtained from Merck (Merck, Darmstadt, Germany). Cycloheximide and miscellaneous reagents were from BDH (BDH Italia, Milan, Italy). Protein determination was carried out with Coomassie Protein Assay reagent from Pierce (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as internal standard.

2.2. Cell culture

Rat pheochromocytoma PC12 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated HS, 5% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 4 mM L-glutamine at 37°C in a humid atmosphere containing 5% CO_2 and grown in 60 mm tissue dishes (Falcon, Becton Dickinson and Company, Plymouth, UK), after poly-L-lysine coating.

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2.3. Administration of sphingolipid fluorescent analogues

C12-LRh-Cer and C12-LRh-SM (5 μM) complexed with essentially fatty acid-free human albumin [22] were administered to PC12 cells plated in 60 mm tissue dishes, in a final volume of 2 ml RPMI 1640 medium supplemented with 1% HS and 0.5% FBS, L-glutamine and antibiotics.

2.4. Lipid extraction and quantification

After the incubation period, the medium was removed, the cells washed with phosphate-buffered saline (PBS) and collected. Cells were pelleted by centrifugation, resuspended in 500 μl Milli-Q water and 1/50 of the suspension was used for protein determination. Media and cell lipids were separated by reverse phase liquid column chromatography (LiChroprep RP-8), washing the column with water and eluting with methanol and chloroform/methanol (C/M) 6:4 (by volume). TLC separation of the eluates was carried out using the solvent system C/M/*n*-buthanol/ethyl acetate/0.25% KCl 25:16:25:25:9 (by volume). LRh fluorescent lipids were detected under UV-light and individual bands were scraped off the gel, extracted with C/M 6:4, or C/M/water 5:5:1 (by volume) in the case of SM and the fluorescence recorded in a spectrofluorimeter. Identification of C12-LRh-SM was carried out by comparing its R_f on TLC with a standard obtained by chemical synthesis.

SM was always quantified as total SM, that is, the sum of fluorescent SM recovered in the culture medium and SM associated to the cells. In pulse experiments, SM levels were expressed as nmol of total SM normalized to protein content and to ceramide uptake (i.e. divided by the ceramide uptake value), while in pulse/chase experiments, total SM levels were normalized only to protein content. Data were the mean ± S.D. values from at least three separate experiments.

Endogenous SM was determined, after perchloric acid digestion, as reported in [23,24].

2.5. Treatments

PC12 cells were treated with 50 ng/ml NGF for 1, 3 or 7 days, and successively incubated for 24 h with C12-LRh-Cer (5 μM) in the presence of NGF. Alternatively, PC12 cells were incubated for 8 h with C12-LRh-Cer (5 μM) in the presence of 50 ng/ml NGF or 10 ng/ml FGF-2 or 10 ng/ml EGF directly added into the medium at the start of the pulse. In the experiments with cycloheximide (2 μg/ml), actinomycin D (3 μg/ml), H-89 (10 μM), chelerythrine chloride (20 μM), LY-294002 (10 μM) and PD-98059 (50 μM) cells were pretreated with the inhibitors for 1 h and then incubated with 5 μM C12-LRh-Cer for 8 h in the presence or absence of NGF, maintaining the inhibitor into the culture medium. In experiments of protein kinase C (PKC) downregulation, cells were pretreated for 24 h with 1 μM TPA and then incubated with 5 μM C12-LRh-Cer for 8 h in the presence or absence of NGF, maintaining TPA into the culture medium. In experiments of PKC upregulation, 0.05 μM TPA was added into the medium at the start of the pulse period together with C12-LRh-Cer. In experiments with AG-879 (100 μM), PC12 cells were incubated with 5 μM C12-LRh-Cer for 16 h; at the last hour of pulse period, AG-879 was added to the medium and then cells were washed and chased for 8 h in the presence or absence of NGF, maintaining the inhibitor into the medium.

2.6. Fluorescence measurements

All fluorescence measurements were carried out using a Jasco FP770 spectrofluorometer. The fluorescence intensities of LRh derivatives were measured in C/M 6:4 (by volume) using excitation and emission wavelengths of 565 and 575 nm, respectively.

2.7. Detection of ERKs phosphorylation

PC12 cells were incubated in serum-free medium supplemented with 1 mg/ml BSA and 10 μg/ml transferrin for 1 h. Cells were then treated with 50 ng/ml NGF for 15 min alone or pretreated with 10 mM AG-879 for 30 min and incubated for an additional 15 min in the presence of both inhibitor and NGF. PC12 cells were washed once with PBS containing 100 μM sodium orthovanadate, collected with 80 μl of lysis buffer (50 mM β-glycerophosphate, 1.5 mM EGTA pH 8.5, 2 mM orthovanadate, 1 mM dithiothreitol, 5 μg/ml leupeptin, 1 μM benzamide, 1% Triton X-100, 2 mM sodium pyrophosphate, 100 U/ml Trasilol) and centrifuged (12 000 × *g*, 10 min, 4°C). Samples (30 μg of cell protein per lane) were run on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, and the proteins were transferred to a transfer membrane (NEF-1002 PolyScreen, Nen, Boston, MA, USA). The membrane was blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% powdered skimmed milk before being probed with an antibody directed against the dual phosphorylated form of ERK (Biolabs, Beverly, MA, USA) and further incubated with a horseradish peroxidase-conjugated secondary antibody (Sigma, Milan, Italy). Phospho-ERK was detected using chemioluminescence (ECL, Pierce, Rockford, IL, USA).

3. Results

3.1. NGF increases SM levels in PC12 cells

To investigate the effect of NGF on SM content in PC12 cells during neuronal differentiation, the capacity of PC12 cells to transform an exogenously added fluorescent analogue of ceramide (C12-LRh-Cer) into the corresponding fluorescent SM (C12-LRh-SM) was evaluated.

PC12 cells were cultured in the presence of 50 ng/ml NGF up to 7 days and pulsed with 5 μM C12-LRh-Cer for 24 h at different time points of NGF treatment. At the end of the 24 h pulse periods, incorporation of the fluorescent precursor into SM was evaluated. As shown in Table 1, NGF-treated cells showed a 2–4-fold increase in C12-LRh-SM content compared to untreated control cells at all the time points investigated. These data indicate that NGF caused a significant increase of the conversion of C12-LRh-Cer into SM during all the period of morphogenic differentiation induced by the growth factor. It must be pointed out that the pretreatment with NGF for different periods of time did not significantly modify the amount of C12-LRh-Cer cell-internalized during the 24 h pulse (data not shown).

To assess the kinetics of SM accumulation in NGF-treated versus control PC12 cells, cultures were incubated with the growth factor or vehicle in the presence of C12-LRh-Cer. At different time points from the beginning of the treatment, incorporation of the fluorescent precursor into SM was evaluated. As shown in Fig. 1, cumulative C12-LRh-SM content sharply increased 4–8 h after the beginning of NGF treatment, reaching a plateau at 24 h. In contrast, C12-LRh-SM accumulated at a much slower rate in control cells all throughout the experimental period.

The effect of NGF on C12-LRh-SM accumulation was dose-dependent, maximum stimulation being observed at 50–150 ng/ml of the growth factor (data not shown).

The above data indicate that NGF increases the conversion of exogenously added C12-LRh-Cer into SM.

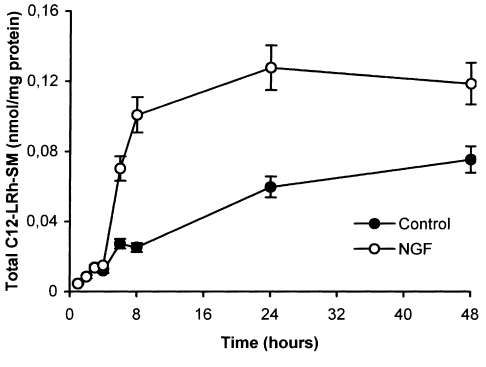

Fig. 1. Kinetics of C12-LRh-SM accumulation in PC12 cells. PC12 cells were pulsed with C12-LRh-Cer (5 μM) for 1–48 h, in the presence (open circles) or absence (closed circles) of 50 ng/ml NGF. Total C12-LRh-SM is expressed as described in Section 2.

Table 1
Effects of NGF on SM levels

Treatment	C12-LRh-SM level	
	nmol/mg/24 h uptake ± S.D.	Fold increase
Control	0.0324 ± 0.007	1.00
NGF pretreatment (days)		
0	0.0682 ± 0.010	2.10
1	0.1100 ± 0.020	3.40
3	0.1340 ± 0.022	4.14
7	0.0929 ± 0.025	2.90

PC12 cells were pretreated with NGF for 0, 1, 3 or 7 days and than incubated with 5 μM C12-LRh-Cer for a further 24 h in the presence of the growth factor. Control cultures were treated for an additional 24 h with C12-LRh-Cer only. Total C12-LRh-SM values are expressed as described in Section 2. In the last column, C12-LRh-SM levels are expressed as fold increase over control values.

In order to be sure that the observed increases were not due to an artifact arising from the use of a fluorescent analogue of ceramide as precursor for SM, we investigated the effect of NGF on endogenous SM content in PC12 cells. Therefore, PC12 cells were incubated in the presence of NGF for different times and the SM detected. As shown in Fig. 2, the endogenous SM content significantly increased over time.

3.2. Upregulation of SM content by NGF depends on transcriptional/translational events

PC12 cells pretreated for 1 h with the transcriptional inhibitor actinomycin D (3 μg/ml) or the translational inhibitor cycloheximide (2 μg/ml) were incubated for 8 h with 5 μM C12-LRh-Cer in the presence or absence of NGF. The addition of actinomycin D or cycloheximide resulted in a complete abolishment of the NGF effect on C12-LRh-SM accumulation (Fig. 3), indicating that the growth factor increases SM levels via mRNA and protein synthesis. It must be pointed out that actinomycin D and cycloheximide pretreatment did not affect the capacity of NGF to cause activation of $ERK_{1/2}$ in PC12 cells (data not shown), thus indicating that the inhibition of mRNA or protein synthesis did not interfere with the early intracellular signaling activated by the growth factor.

3.3. Degradation of SM is slowed down by NGF

Degradation of SM to the corresponding ceramide was assessed in PC12 cells in the presence or absence of NGF. Cells were pulsed for 3 h in the presence of 5 μM C12-LRh-SM and then chased for 4 h in fresh medium with or without 50 ng/ml NGF. Cells treated with the growth factor showed a fluorescent ceramide content (0.017 nmol/mg protein) of which was 50% lower than the control (0.034 nmol/mg protein).

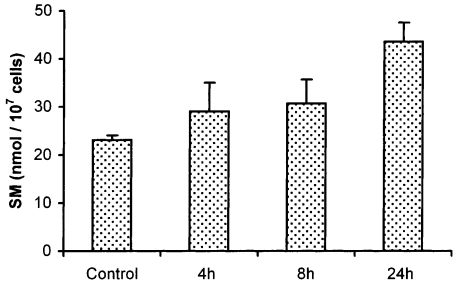

Fig. 2. Effect of NGF on endogenous SM levels. PC12 cells were treated for 4, 8 and 24 h with 50 ng/ml NGF or cultured for the same period in the absence of the growth factor (control value), and the SM content was determined as reported in Section 2 and expressed as nmol per 10 million cells.

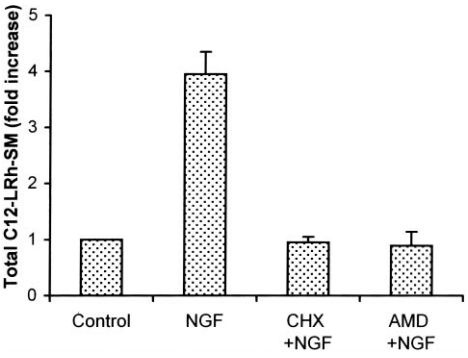

Fig. 3. Effect of transcriptional and translational inhibitors on C12-LRh-SM levels increased by NGF. 1 h preincubation with 2 μg/ml cycloheximide or 3 μg/ml actinomycin D completely abolished the increase of SM levels induced by 8 h treatment with 50 ng/ml NGF. Total SM values (see Section 2) are expressed as fold increase over the control. 1 (control value) corresponds to 0.038 ± 0.006 nmol/mg/8 h uptake.

3.4. Increase of SM accumulation by NGF depends on the activity of $p140^{trkA}$ receptor

Since NGF is known to exert most of its activities in PC12 cells by binding to the $p140^{trkA}$ receptor [12], we decided to evaluate the effect of the $p140^{trkA}$ inhibitor AG-879 [25] on NGF-mediated C12-LRh-SM accumulation. As shown in Fig. 4, AG-879 completely abolished $ERK_{1/2}$ phosphorylation and the increase of C12-LRh-SM levels induced by the growth factor in PC12 cells, indicating that the tyrosine kinase activity of $p140^{trkA}$ receptor is essential for this effect.

3.5. Increase of SM accumulation by NGF requires $ERK_{1/2}$ activation

Activation of $p140^{trkA}$ receptor by NGF triggers various intracellular signaling pathways, including PKC-, phosphatidylinositol 3-kinase (PI3-K)- and $ERK_{1/2}$-dependent pathways [26]. In order to identify the signal transduction pathway involved in NGF-mediated upregulation of SM content, PC12 cells were pretreated for 1 h with the protein kinase A (PKA) inhibitor H-89 [27], the PKC inhibitor chelerythrine chloride [28], the PI3-K inhibitor LY-294002 [29] or the mitogen-activated protein kinase kinase 1 (MEK1) inhibitor PD-98059 [30]. Then, cells were pulsed for 8 h with 5 μM C12-LRh-

Table 2
Synergistic effect on SM accumulation between NGF and PKA or PKC activation

Treatment	NGF	C12-LRh-SM (fold increase ± S.D.)
−	−	1.00
	+	3.91 ± 0.45
10 ng/ml dbcAMP	−	1.73 ± 0.17
	+	9.75 ± 0.51
0.05 μM TPA for 5 h	−	1.02 ± 0.11
	+	8.75 ± 0.62

PC12 cells were incubated with 5 μM C12-LRh-Cer for 5 h in the absence or presence of 50 ng/ml NGF and in the presence of 10 ng/ml dbcAMP or 0.05 μM TPA, respectively. Total SM values (see Section 2) are expressed as fold increase over the control. 1.00 (control value) corresponds to 0.038 ± 0.006 nmol/mg/8 h uptake.

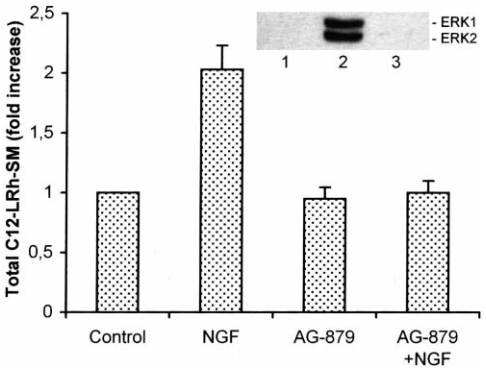

Fig. 4. Effect of tyrosine kinase inhibitor AG-879 on increase of C12-LRh-SM levels induced by NGF. PC12 cells were pulsed for 16 h with 5 μM C12-LRh-Cer, washed and treated for 8 h in the presence of AG-879 and/or NGF. AG-879 completely abolished the effect of NGF on SM levels. Total SM values are expressed as fold increase over the control (see Section 2). 1 (control value) corresponds to 0.063 ± 0.005 nmol/mg. Inset: blocking of NGF-activated ERKs phosphorylation in PC12 cells by AG-879 inhibitor. PC12 cells were incubated in serum-free medium supplemented with 1 mg/ml BSA and 10 μg/ml transferrin for 1 h (lane 1). Cells were then treated with 50 ng/ml NGF for 15 min alone (lane 2) or pretreated with 10 mM AG-879 for 30 min and incubated for an additional 15 min in the presence of both inhibitor and NGF (lane 3). ERKs phosphorylation was detected as described in Section 2.

Cer in the absence or presence of NGF, being each specific inhibitor added. As shown in Fig. 5, only PD-98059 caused a significant reduction of the synthesis of C12-LRh-SM induced by NGF, whereas all the other inhibitors were ineffective. The lack of a role of PKC in NGF-induced C12-LRh-SM accumulation was confirmed in PC12 cells in which PKC has been downregulated by 1 μM TPA pretreatment (Fig. 5). Taken together, these data indicate that activation of the $ERK_{1/2}$ signaling pathway by $p140^{trkA}$ is responsible for the increase of C12-LRh-SM synthesis induced by NGF in PC12 cells.

3.6. Activation of PKA or PKC with simultaneous treatment with NGF further enhances SM content in PC12 cells

Activation of PKA with dbcAMP, a permeable analogue of cAMP, caused a significant increase of SM levels in PC12 cells (Table 2). However, the concomitant presence of NGF had a synergistic effect on SM content which was more than 2-fold greater than in cells treated with NGF alone (Table 2).

Table 3
Effect of differentiating and mitogenic agents on increasing of SM content

Treatment (8 h pulse)	C12-LRh-SM levels (fold increase ± S.D.)
Control	1.00
NGF	3.91 ± 0.45
FGF-2	1.85 ± 0.19
dbcAMP	1.73 ± 0.17
EGF	1.08 ± 0.08

PC12 cells were incubated with 5 μM C12-LRh-Cer for 8 h in the absence (control) or presence of 50 ng/ml NGF, 10 ng/ml FGF-2, 1 mM dbcAMP and 10 ng/ml EGF, respectively. Total SM values (see Section 2) are expressed as fold increase over the control. 1.00 (control value) corresponds to 0.038 ± 0.006 nmol/mg/8 h uptake.

Moreover, even though 0.05 μM TPA over 5 h did not affect the basal levels of SM, the phorbol ester caused a 2-fold increase of the SM accumulation induced by NGF alone (Table 2). Thus, activation of PKA or PKC strongly potentiates the effect of NGF on SM accumulation in PC12 cells.

3.7. Other differentiation factors cause SM accumulation in PC12 cells

The ability of NGF and dbcAMP [31] of increasing SM content in PC12 cells was compared with that of the FGF-2, another differentiating agent [32], and that of the EGF, a mitogenic agent in PC12 cells [33].

As shown in Table 3, administration of either the differentiation factors NGF, FGF-2 and dbcAMP but not the mitogenic growth factor EGF induced a significant increase in SM content in PC12 cells.

4. Discussion

The present study provides evidence that NGF stimulates the accumulation of SM in PC12 cells. This result derives both from evidences obtained following the incorporation into SM of a fluorescent analogue of ceramide (i.e. C12-LRh-Cer) and from determination of endogenous SM, as well.

The highest fold increase obtained for fluorescent SM probably reflects the fact that the C12-LRh-SM does not accumulate into the cell because the polarity of the fluorochrome determines its release into the medium [22], and PC12 cells

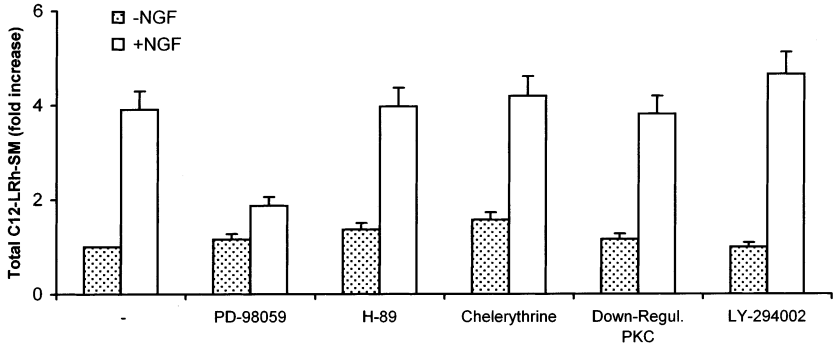

Fig. 5. Identification of the signal transduction pathway involved in NGF-mediated upregulation of SM content. PC12 cells were incubated for 8 h in the absence or in presence of 50 ng/ml NGF and in the presence of 50 μM PD-98059 (MEK1 inhibitor), 10 μM H-89 (PKA inhibitor), 20 μM chelerythrine chloride (PKC inhibitor), 1 μM TPA (for downregulating PKC activity) and 10 μM LY-294002 (PI3-K inhibitor), respectively. Total SM values (see Section 2) are expressed as fold increase over the control. 1 (control value) corresponds to 0.038 ± 0.006 nmol/mg/8 h uptake.

are continuously stimulated to synthesize SM. The use of the fluorescent tool has revealed to be more practical and has been preferred to the classical method for SM determination.

Inhibition of transcription or inhibition of protein synthesis completely abolished the effect of NGF on SM accumulation. Thus, a process of protein neosynthesis is essential for NGF modulation of SM levels. Therefore, an increase of the enzyme SM synthase and/or the synthesis of an activator protein can be hypothesized. Moreover, the prolonged half life of SM in the presence of NGF, as resulted from our experiments in which PC12 cells were loaded with C12-LRh-SM, can partially contribute to the observed increase of SM content during differentiation in PC12 cells.

The role of the activation of kinases ERK1 and ERK2 via $p140^{trkA}$ receptor in the differentiation process induced by NGF has been clearly established [34]. We have demonstrated that AG-879, an inhibitor of NGF $p140^{trkA}$ receptor, and PD-98059, a specific inhibitor of MEK1 [30], prevented accumulation of SM by NGF. This indicates that activation of the Ras cascade by $p140^{trkA}$ receptor engagement is required for the observed biological effect of NGF.

The use of PKA, PKC or PI3-K by H-89, chelerythrine chloride or LY-294002, respectively, did not influence the SM elevation promoted by NGF. This means that NGF does not influence the SM elevation promoted by NGF. This means that NGF does not primarily use these signaling components to induce SM synthesis. The activation of PKC by TPA did not induce any increase in SM levels in PC12 cells; however, if the cells are treated with NGF, concomitant stimulation of PKC by TPA results in a striking synergistic effect. A similar situation was obtained when PKA was activated by dbcAMP. NGF and dbcAMP are known to induce differentiation in PC12 cells and have additive and synergistic effects on neuritogenesis [32]. As shown in Table 2, the concurrent presence of dbcAMP and NGF induced a greater increase in SM synthesis than did the individual agents. DbcAMP added alone to control undifferentiated cells had a limited but clear effect on increasing SM content. Thus, the cellular mechanisms involved in the SM increase by the two differentiating agents does not appear to be the same, as reported in the case of dopamine increase in PC12 cells [35].

Besides NGF, other differentiating factors in PC12 cells, like FGF-2 and dbcAMP, but not the mitogenic factor EGF, induce an accumulation of SM. These data are in keeping with the hypothesis that increase in SM could play an important role in neuronal differentiation [19].

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