Involvement of a ceramide activated protein phosphatase in the differentiation of neuroblastoma Neuro2a cells

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Abstract The possible involvement of protein phosphatase in ceramide-mediated neural cell differentiation was investigated. Neuroblastoma Neuro2a cell differentiation induced by retinoic acid, or conditions causing an increase in cellular ceramide, was significantly inhibited by the serine/threonine phosphatase inhibitor okadaic acid, at concentrations as low as 2.5 nM. A crude cytosolic preparation from Neuro2a cells was found to have a cation-independent protein phosphatase activity that was stimulated by ceramide in a dose-dependent manner. Short- and long-chain ceramides, but not sphingosine and related dihydro-derivatives, were active. Ceramide-activated protein phosphatase activity from Neuro2a cells was inhibited by 5 nM okadaic acid. The data indicate that a type 2A protein phosphatase is involved in ceramide-mediated differentiation of Neuro2a cells.

Key words: Neural cell differentiation; Ceramide; Protein phosphatases; Neuroblastoma cells

1. Introduction

Ceramide, the N-acylated derivative of sphingosine, has emerged as a widespread biomodulator, produced by cells when stimulated with different agents. It is involved in the regulation of fundamental cellular processes, like growth, differentiation and programmed cell death [1–5]. Also cells of neural origin share ceramide as an intracellular mediator of the effects of specific stimuli, especially with regard to differentiation [6–8]. In fact, in neuroblastoma and glioblastoma cell lines the administration of differentiating agents results in an increase in the cellular level of ceramide, which, in turn, appears to mediate their differentiation to, respectively, a neuron- [6,7] and an astrocyte-like phenotype [8]. In the same cells ceramide or ceramide analogs can mimick this effect. In particular in murine Neuro2a neuroblastoma cells retinoic acid and serum removal induce a neuron-like differentiation that is characterized by a rapid and persistent increase in the cellular levels of ceramide [6]. Moreover, removal of the differentiating agents results in a lowering of the ceramide levels and a reversal of differentiation. Also the experimental conditions that raise the cellular ceramide levels lead to stimulation of neurite outgrowth and growth inhibition in neuroblastoma cells [6,7]. Further support for the role of ceramide in neuronal differentiation comes from primary cultures of cerebellar neurons where the cellular content of ceramide markedly increases during differentiation in culture [7].

Present-day knowledge of molecular mechanisms at the basis of ceramide effects in cells are still limited. However one possibility appears to be the modulation of protein phosphorylation. In fact ceramide has been demonstrated to activate a proline-directed protein kinase, a protein kinase Cζ isotype and a serine/threonine type 2A protein phosphatase [9–15]. In particular, a ceramide-activated type 2A protein phosphatase has been reported to occur in rat brain and in glioblastoma cells [11,12].

Thus, prompted by the fact that protein phosphorylation appears to play a crucial role in neuroblastoma cell differentiation [16,17], we investigated the possible role of protein phosphorylation in the ceramide-mediated differentiation of Neuro2a cells. The data obtained in this work strongly suggest that a cytosolic type 2A protein phosphatase, specifically activated by ceramide, is involved in the regulation of Neuro2a cell differentiation.

2. Materials and methods

2.1. Chemicals

Reagents were of the highest purity available and solvents were redistilled before use. DMEM, FCS (heat-inactivated before use), bovine serum albumin, RA, PMSF, leupeptin, pepstatin A, bestatin, D-*erythro*-sphingosine, DL-*threo*-dihydrosphingosine, C18-Cer, ceramide from bovine brain sphingomyelin, C16-dihydrocer, ATP, histone type III-S, were from Sigma (St. Louis, MO, USA). C2-dihydrocer and protein kinase C from rat brain were from Calbiochem (La Jolla, CA, USA). C2-Cer was from Matreya Inc. (Pleasant Gap, PA, USA). Okadaic acid sodium salt was from LC Laboratories (Woburn, MA, USA). Phosphatidylserine was from BDH (Poole, UK). [γ-^{32}P]ATP (30 Ci/mmol) was from Amersham International (Amersham, Buckingamshire, UK).

2.2. Cell culture

Murine neuroblastoma Neuro2a cells (CCL-131, American Cell Type Culture Collection) were cultured in plastic dishes or flasks in DMEM supplemented with 10% FCS, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin G and 100 μg/mL streptomycin sulphate at 37°C in a humidified atmosphere of 5% CO_2 and 5% air.

2.3. Cell differentiation and okadaic acid treatment in cultured cells

Neuro2a cells were plated at 3×10^3/cm^2 in 35-mm Falcon dishes and grown for 2 days in 10% FCS-DMEM; to induce neuronal differentiation, the cells were rinsed with DMEM containing 2% FCS and incubated in the same medium (1 mL/dish) with 20 μM RA [18] or 2–5 μM sphingoid molecules [6] for different times, up to 48 h. In some plates okadaic acid (OA), a cell-permeable serine/threonine protein phosphatase inhibitor, (2.5–10 nM in 0.05% DMSO, final concentration) was added to the culture medium [19–21], during treatment with inducers. Cell viability and neurite outgrowth evaluation were accomplished by phase-contrast microscopy as previously described [6].

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Abbreviations: Cer, ceramide; C18-Cer, *N*-stearoyl-D-*erythro*-sphingosine; C16-dihydrocer, *N*-palmitoyl-DL-dihydrosphingosine; C2-Cer, *N*-acetyl-D-*erythro*-sphingosine; C2-dihydrocer, *N*-acetyl-DL-dihydrosphingosine; DMEM, Dulbecco's modified Eagle's medium, DMSO, dimethyl sulfoxide; FCS, fetal calf serum; OA, okadaic acid; PMSF, phenylmethylsulphonylfluoride; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; RA, retinoic acid

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2.4. Cell fractionation

Neuro2a cells were cultured in 75 cm^2 flask, in supplemented DMEM. 2×10^7 cells from confluent cultures (approximately 10 mg of cellular protein) were rinsed with phosphate-buffered saline, resuspended in 2 mL of homogenization buffer (20 mM Tris-Cl pH 7.4, 1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.25 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 10 μg/mL bestatin) and disrupted by sonication. Cell homogenate was centrifuged at 700×g for 10 min at 4°C to remove nuclei and cell debris. The supernatant was centrifuged at 100 000×g for 1 h at 4°C and the resulting cytosolic fraction was used fresh or stored in aliquots at −80°C. Protein concentration was determined [22], using bovine serum albumin as standard.

2.5. Preparation of [^{32}P]phosphohistone

[^{32}P]phosphohistone was prepared as previously described [11], except that a commercially available rat brain protein kinase C preparation was used: 1 U/mL of the enzyme was incubated in the presence of 2 mg/mL histone type IIIS and 12.5 μM [γ-^{32}P]ATP (20 Ci/mmol). A typical preparation of [^{32}P]phosphohistone contained 4–6 nmol of Pi per mg protein.

2.6. Phosphatase assay

Dephosphorylation reactions were performed using 0.5 μM [^{32}P]phosphohistone as substrate and 10 μg/mL of crude cytosolic protein, as described by Dobrowsky and Hannun [11]. Incubations were performed at 37°C for 5 min. In the used conditions, protein phosphatase activity from Neuro2a cells was linear in the range 3–30 μg/ml up to 15 min incubation at 37°C.

C2-Cer, sphingosine and their dihydroderivatives were prepared in absolute ethanol immediately before use and used at 0.1–25 μM (0.5% final ethanol concentration). C18-Cer, Cer and C16-dihydrocer were dissolved in ethanol/dodecane 98/2 (v/v) according to Ji et al. [23] and administered at 0.1–25 μM in 0.5% ethanol/0.01% dodecane (final concentrations) These conditions allowed a complete solubilization of ceramides in the dephosphorylation assay. OA, delivered from a stock solution in DMSO (final DMSO concentration never exceeding 0.05%), was added at 5 nM or 1 μM, final concentration, in order to separately determine protein phosphatase 1 (PP1) and 2A (PP2A) [19,24]. PP1 activity was calculated as the difference between protein phosphatase activity measured at 5 nM and 1 μM OA; PP2A activity as the difference between total protein phosphatase activity and that measured at 5 nM OA [19]. Ethanol, dodecane or DMSO at the utilized concentrations had no effect on enzyme activity.

3. Results

3.1. Effect of OA on neurite outgrowth in Neuro2a cells

In the first set of experiments we investigated the possible role of protein phosphatases in Neuro2a differentiation. For this purpose Neuro2a cell differentiation was induced by treatment with RA or Cer in the presence of the serine/threonine

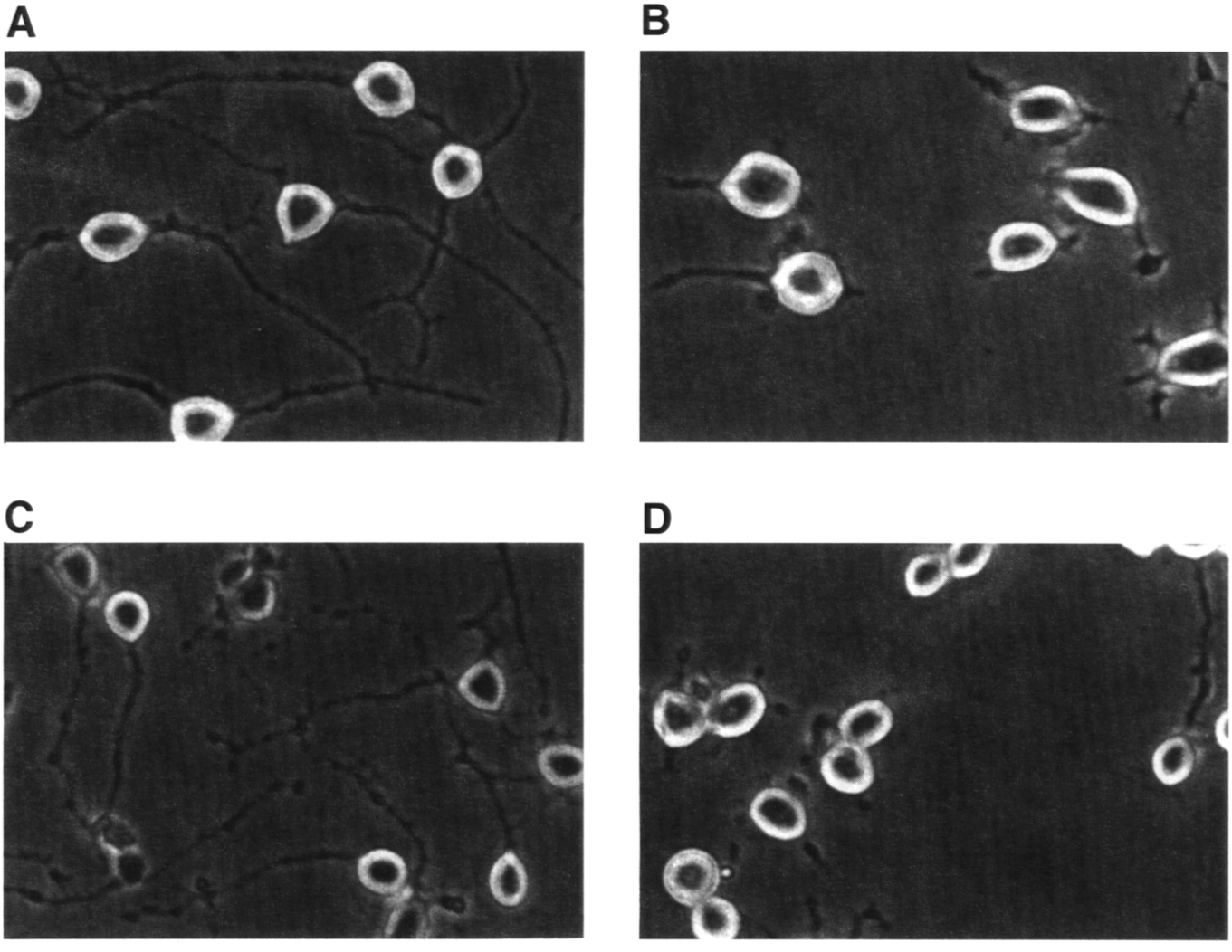

Fig. 1. Effect of okadaic acid on Neuro2a differentiation. Neuro2a cells were treated with 20 μM retinoic acid (A and B) or 5 μM ceramide (C and D) in the absence (A and C) or presence (B and D) of 5 nM okadaic acid for 24 h.

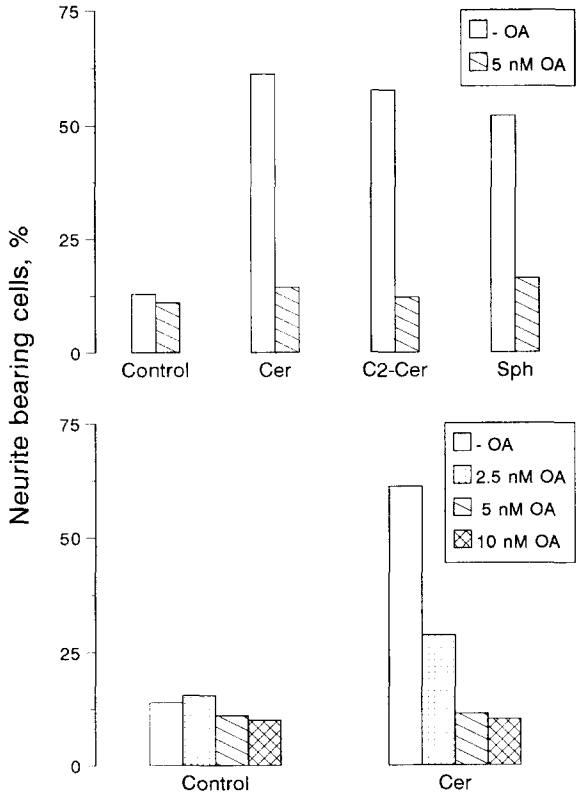

Fig. 2. Effect of okadaic acid on ceramide-induced Neuro2a differentiation. Neuro2a cells were treated with 5 nM (upper panel) or different concentrations (lower panel) of okadaic acid (OA) in the absence (Control), or presence of various sphingoids (2 μM) for 24 h. The extension of neurite-like processes was evaluated and quantified as described in Section 2. Data are the mean of three experiments in duplicate, S.D. never exceeding 15% of the mean. Cer, ceramide from bovine brain sphingomyelin; C2-Cer, C2-ceramide; Sph, sphingosine.

phosphatase inhibitor OA. In agreement with previous reports [6,18] Neuro2a cells extended neurites in response to RA (Fig. 1A) or ceramide (Fig. 1C). After 24 h treatment cells bearing neurites longer than twice the cell body diameter [6] accounted for 71.9 ± 8.3% (mean ± S.D.) in the presence of 20 μM RA and 61.2 ± 7.0% for 5 μM Cer. In the presence of 5 nM OA there was no neuritogenic effect due to RA (Fig. 1B) and Cer (Fig. 1D). As shown in Fig. 2 (upper part), the neuritogenic effect of C2-Cer or sphingosine was also impaired by 5 nM OA. The effect of different concentrations of OA on Cer-induced differentiation was then studied (Fig. 2, lower part). At 2.5 nM OA, neurite bearing cells were markedly reduced (46.7% of cells without OA) and at 5–10 nM, the neuritogenic effect of Cer was completely reversed. In cultured Neuro2a cells, 2.5–10 nM OA alone had little or no effect on cell viability and morphology for periods of up to 48 h.

3.2. *Effect of ceramide on cytosolic protein phosphatase activity in Neuro2a cells*

Further experiments were performed to evaluate the possible presence of a ceramide-sensitive protein phosphatase in Neuro2a cells. For this purpose the cation-independent protein phosphatase activity was measured in a crude cytosolic preparation from Neuro2a cells using [^{32}P]phosphohistone as substrate. The early experiments were performed with the short-chain analogue C2-Cer. As shown in Fig. 3 (upper part), C2-Cer increased the cytosolic protein phosphatase activity of the Neuro2a cells in a dose-dependent fashion, stimulation being detectable at 1–25 μM. At the same concentrations, also C18-Cer and Cer from bovine brain sphingomyelin (containing primarily stearic and nervonic acids) were effective (Fig. 3, middle and lower part).

3.3. *Specificity of ceramide effect on Neuro2a cytosolic protein phosphatase activity*

To evaluate the specificity of Cer on protein phosphatase stimulation the effect of different molecules structurally closely

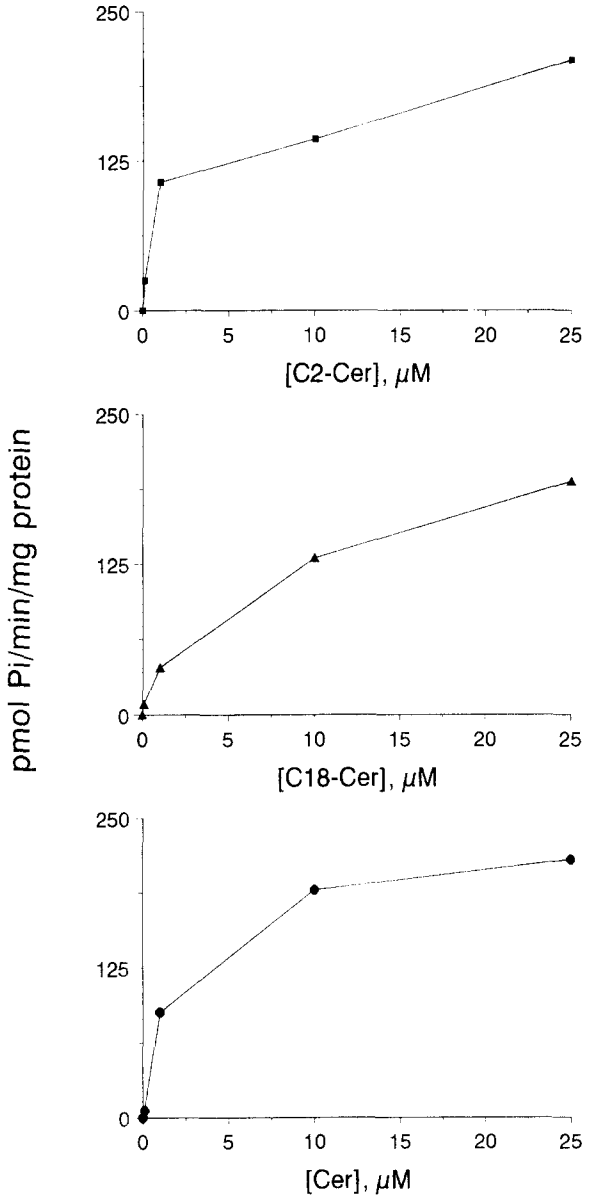

Fig. 3. Effect of ceramide on cytosolic protein phosphatase activity in Neuro2a cells. Phosphatase activity was determined toward [^{32}P]phosphohistone III-S in a cytosolic fraction from Neuro2a cells in the absence or presence of different concentrations of C2-ceramide (C2-Cer, upper part) or C18-ceramide (C18-Cer, middle part) or ceramide from bovine brain sphingomyelin (Cer, lower part). Data are obtained after subtraction of the activity measured in the absence of ceramide. Basal activity was 706 ± 51 pmol Pi/min per mg protein. Data are the mean values of two experiments in triplicate, S.D. never exceeding 12% of the mean.

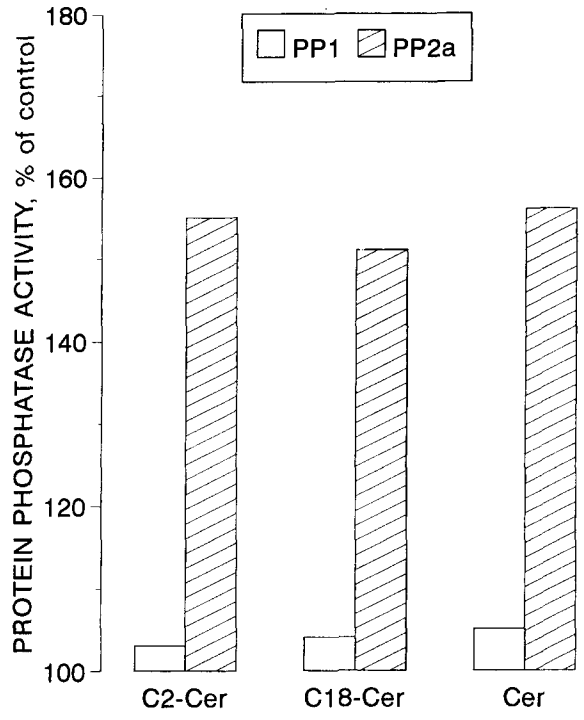

Fig. 4. Effect of ceramides on protein phosphatase type 1 (PP1) and type 2A (PP2A) from Neuro2a cells. Protein phosphatase activity was evaluated in a cytosolic fraction from Neuro2a cells in the presence of 25 μM of different ceramides as described in Section 2. PP1 activity was calculated by the difference between 5 nM OA-residual activity and 1 μM OA residual activity and PP2A by the difference between total cytosolic protein phosphatase activity and the residual activity in the presence of 5 nM OA. Data are expressed as percent of activity in the absence of ceramides and are the mean of three experiments in triplicate. Control activity was 0.394 and 0.410 nmol Pi/min per mg protein for PP1 and PP2A, respectively.

related to ceramide was investigated. Under experimental conditions where C2-Cer or long-chain ceramides were active, C2- or C16-dihydroceramide, sphingosine and racemic *threo*-dihydrosphingosine had no effect on the Neuro2a cytosolic protein phosphatase activity (Table 1).

3.4. *Effect of OA on protein phosphatase activity in Neuro2a cells*

To evaluate whether a specific phosphatase type could be responsible for ceramide-stimulated activity, protein phosphatase activity was measured in the presence of different OA concentrations. It is known that OA, a complex fatty acid polyether, completely inhibits PP2A at 1–5 nM, while higher concentrations are needed to inhibit PP1 [19]. In a cytosolic fraction from Neuro2a cells, protein phosphatase activity (0.815 pmol Pi/min per mg protein) was reduced to approximately one-half the total by 5 nM OA (0.405 pmol Pi/min per mg protein) and was almost completely abolished by 1 μM OA (0.011 pmol Pi/min per mg protein). Thus, in the experimental conditions used, the PP1 and PP2A activities each contributed about 50% of the total cytosolic protein phosphatase.

The effect of 5 nM and 1 μM OA on Cer-activated protein phosphatase allowed the identification of the type of activated phosphatase. As shown in Fig. 4, 25 μM C2-Cer and C18-Cer, as well as Cer from bovine brain sphingomyelin, significantly increase PP2A activity in the cytosol of Neuro2a cells but had no effect on PP1.

4. Discussion

Previous studies in our laboratory have demonstrated that Cer plays a mediator role in Neuro2a differentiation [6,7] and this prompted us to carry out further investigations. The data from the present work demonstrate the involvement of an OA-sensitive pathway in the Cer-mediated differentiation of Neuro2a cells. In fact, the neuritogenic effects of RA (which induces Cer increase), as well as of exogenous Cer, are strongly reduced by treating cells with OA. OA concentrations as low as 2.5 nM were effective and in the presence of 5 nM OA, neurite extension induced by either RA or ceramide or sphingosine was completely inhibited. Thus, the activity of a type 1 and/or 2A serine/threonine protein phosphatase appears to be involved in ceramide-mediated Neuro2a cell differentiation. Despite the difficulty in achieving selective PP1 or PP2A inhibition in intact cells [19–21], the efficacy of low nanomolar concentrations of OA in the inhibition of Neuro2a cell differentiation suggests that PP2A rather than PP1 is involved in this phenomenon. These data are in agreement with other studies that demonstrate an OA inhibitory effect in other models of neuronal differentiation [21,25]. Direct proof of the existence of a type 2A ceramide-activated protein phosphatase in Neuro2a cells was found using a cell-free system, i.e. a cytosolic extract from these cells. Even in the micromolar range ceramide activates a cytosolic protein phosphatase in a dose-dependent manner. Furthermore both C2-Cer and natural occurring long-chain ceramides have similar potency. The ceramide-activated protein phosphatase activity was sensitive to OA inhibition and was completely inhibited in the presence of 5 nM OA. These results support the notion that the involved protein phosphatase belongs to the type 2A.

Stimulation of cytosolic protein phosphatase by Cer was specific. In fact molecules structurally related to Cer but lacking the double bond between C4 and C5 of the sphingoid base (C2-, C16-dihydrocer and DL-*threo*-dihydrosphingosine) were

Table 1
Effect of different sphingoid molecules on cytosolic protein phosphatase activity in Neuro2a cells

Sphingoid molecule	Protein phosphatase activity (nmol/min per mg protein)	%
None	0.815 ± 0.030	100
Ceramide	1.056 ± 0.063**	130
C2-dihydroceramide	0.798 ± 0.180	98
C18-ceramide	1.055 ± 0.047**	130
C16-dihydroceramide	0.805 ± 0.034	99
Sphingosine	0.802 ± 0.005	98
Threo-dihydrosphingosine	0.851 ± 0.015	104

Neuro2a cytosolic protein phosphatase activity was evaluated (see Section 2 in the absence (control) or presence of 25 μM sphingoid molecule. Data are expressed as nmol Pi/min per mg protein and are the mean ± S.D. of 3 experiments in triplicate. $^{**}P < 0.01$.

ineffective. It is noteworthy that the same specificity was observed in the effect on Neuro2a cell differentiation [6]. On the other hand sphingosine, which is able to induce neuritogenesis when administered to Neuro2a cells, had no effect on the activity of protein phosphatases in vitro. This apparent discrepancy can be explained by the fact that in Neuro2a cells sphingosine, when administered to cells at neuritogenic doses, is rapidly and efficiently converted to ceramide [6] which is the true effector.

Taken together, these results provide evidence of intracellular targets that mediate Cer-induced neuronal differentiation, and indicate a specific activation of a cytosolic type 2A protein phosphatase. This is in agreement with the evidence that the phosphorylation control of key cellular proteins is a crucial event in neural differentiation. In fact compounds activating cyclic AMP-dependent PKA (such as cAMP-analogues or phosphodiesterase inhibitors) [17,26], or inhibiting/down-regulating PKC [16,27,28] are able to induce neurite extension an the arrest of cell proliferation in Neuro2a cells. The present work demonstrates that a signal-mediated dephosphorylation pathway involving type 2A protein phosphatase also contributes to this regulation. Future research will be aimed at identifying the protein target(s) of type 2A protein-phosphatase that is(are) instrumental to Neuro2a cell differentiation.

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