Okadaic Acid Suppresses Neural Differentiation-dependent Expression of the Neurofilament-L Gene in P19 Embryonal Carcinoma Cells by Post-transcriptional Modification*

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Mouse P19 embryonal carcinoma cells in aggregation culture in the presence of 10^{-6} m retinoic acid followed by monolayer culture differentiate into nerve and glial cells. In this study, we demonstrated that the neurofilament-L (NF-L) mRNA and protein levels of these cells were enhanced in accordance with their retinoic acid-induced neural differentiation. Okadaic acid (OA) treatment of the cells markedly suppressed this differentiation-dependent NF-L gene expression increase and neurite outgrowth of the cells. Similar results were obtained when tautomycin was used instead of OA, suggesting that inhibition of protein phosphatase(s) is involved in the suppression of neural differentiation. OA treatment did not affect the NF-L gene transcription level, determined by the nuclear run-on transcription assay, but it did reduce the stability of both the 3.5- and 2.3-kilobase NF-L mRNAs. The expression and activity levels of protein phosphatase 2A (PP2A) and 2B (PP2B) but not protein phosphatase 1 (PP1) in P19 cells increased in accordance with the enhanced NF-L gene expression. The presence of OA in the culture medium during the course of the neural differentiation caused a reduced PP2A activity but not PP1 and PP2B activities of the cell extracts. On the other hand, both PP1 and PP2B activities but not PP2A activity of cell extracts were suppressed by the addition of cyclosporin A or FK506 in the culture medium. However, both cyclosporin A and FK506 treatments affected neither NF-L gene expression nor neurite outgrowth. These results demonstrate that the OA treatment inhibits the differentiation-dependent increase in NF-L gene expression by destabilizing its mRNAs and suggest that PP2A plays key roles in the differentiation-dependent enhanced expression of the NF-L gene and is the point of the action of OA.

Embryonal carcinoma cells have been used extensively as a model system for studying early embryonic development and differentiation. Because these pluripotent cells resemble the inner cell mass of early embryos, they can be differentiated in vitro, and the derivatives of all three germ layers (mesoderm, endoderm, and ectoderm) can be obtained depending on the culture conditions. P19 cells, a murine embryonal carcinoma cell line, in aggregation culture with a relatively high concentration of retinoic acid (RA) followed by monolayer culture differentiate into neural cells, and this system has been used as a model of neural differentiation. Expression of genes related to neural differentiation in vivo has also been observed during the process of RA-induced P19 cell differentiation. A study of signal transmission related to this differentiation revealed that receptor-type protein tyrosine phosphatase α (R-PTPα) participates in the early stage of differentiation. However, little is known about the involvement of phosphorylation and dephosphorylation of cellular protein serine and threonine residues in the regulation of such differentiation. We therefore were interested in studying the possible functions of major protein serine/threonine phosphatases during the course of P19 cell differentiation.

The major eukaryotic cellular protein serine/threonine phosphatases have been divided into four classes (PP1, PP2A, PP2B, and PP2C) on the basis of their sensitivities to two thermostable proteins (inhibitor-1 and -2) and their divalent cation requirements. Recent investigations revealed various physiological roles of each class of protein serine/threonine phosphatases in the regulation of cellular functions. Okadaic acid (OA) and tautomycin are known to be the inhibitors of PP1, PP2A, and PP2B and to be permeable through plasma membranes of cells. Cyclosporin A (CsA) and FK506, used clinically as immunosuppressants, have been found to form complexes with immunophilines in the cells and inhibit specifically PP2B activity. These specific inhibitors of protein phosphatases have provided new tools for exploring the roles of protein phosphatases in the cell culture system.

Neurofilaments (NFs) are intermediate filaments formed from three component proteins, termed NF-L (68 kDa), NF-M (150 kDa), and NF-H (200 kDa), which are encoded by three different genes and expressed specifically in neurons. PP2A but not PP1 was reported to be able to remove specifically the phosphate moieties from the NH2-terminal domain of the NF-L protein in vitro and OA treatment caused disruption of NF-L phosphorylation.

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of the NF network characterized by hyperphosphorylation of NF subunits (26), suggesting that PP2A participates in the regulation of NF-L network assembly.

In this study, we demonstrated that the NF-L expression level was enhanced as neural differentiation of P19 cells progressed and that OA treatment of these cells inhibited this NF-L expression enhancement by reducing the stability of NF-L mRNA. Furthermore, the results suggest that PP2A participates in the mechanism(s) responsible for the differentiation-dependent increase in NF-L expression observed in these cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—
- $^{32}$P-ATP, $[^{32}]P$-CTP, and $[^{32}]P$-UTP were purchased from DuPont NEN, OA, tautomycin, all-trans retinoic acid (RA), and the human β-actin cDNA probe were purchased from Wako Pure Chemicals (Osaka, Japan). CsA was from Sandoz (Basel, Switzerland), and FK506 was from Fujisawa Pharmaceutical (Osaka, Japan). Inhibitor-2 was a gift from Dr. E. Y. C. Lee (University of Miami, FL), and protease inhibitors and calmodulin-agarose were purchased from Sigma. An anti-serum against the catalytic subunit of rat PP2A was raised by immunizing rabbits against C-terminal oligopeptides common to two distinct isoforms of the catalytic subunit of PP2A (Co and Cp) (27). The anti-rabbit PP1α catalytic subunit polyclonal antibody was purchased from UBI (New York, NY), and the monoclonal antibodies against the catalytic and regulatory subunits of PP2B were a gift from Dr. J. H. Wang (University of Manitoba, Canada). The anti-mouse neurofilament-L monoclonal antibody was purchased from Oncogene Science (New York, NY), the anti-rabbit immunoglobulin G (IgG)/alkaline phosphatase-conjugate antibody was from Promega (Madison, WI), the anti-mouse IgG/chorseredish peroxidase conjugate antibody was from BioRad, and pCR™III was purchased from Invitrogen (San Diego, CA). Myosin light chain and myosin light chain kinase was a gift from Dr. Masaaki Ito (Mie University, Japan).

**Cells and Culture**—
P19 embryonal carcinoma cells were obtained from ATCC (Rockville, MD) and cultured in bicarbonate-buffered α-modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum under a humidified 5% (v/v) CO$_2$ in air atmosphere at 37°C. In order to induce neural differentiation, the cells were cultured on bacterial grade dishes with 0.1% (v/v) OA or undifferentiated P19 cells (day 0) (A) were cultured on bacterial grade dishes with (C–G) or without (B) RA for 4 days and subsequently cultured on culture grade dishes without RA for 2 days. The photographs were taken on day 0 (A) and day 6 (B–G). For cell treatment, 10 nM OA (D), 50 nM tautomycin (E), 1 μg/ml CsA (F), or 100 ng/ml FK506 (G) was present in the medium from day 0 throughout the differentiation process, whereas no phosphatase inhibitors were present in B and C.

**Northern Blot Analysis**—The total RNA was isolated from the cells after incubation for the required times by the acid guanidium thiocyanate-phenol-chloroform method. 10-μg aliquots of denatured RNA were electrophoresed and transferred onto Hybond N+ membranes (Amersham Corp.), and Northern hybridization was carried out as described previously (28). The DNA probes for PPs used were as follows: the 259-base pair (bp) cDNA fragment unique to the α isoform of the catalytic subunit of rat PP1 prepared by the polymerase chain reaction using the upstream (5′-CTTGCGCGATTTGTGCAAGCGTGCTC-3′) and downstream (5′-TACATGTACCCATTGGGTCAACTGCGCTTCTCA-3′) primers (29), the 1.8-kilobase pair EcoRI fragment of the rat PP2A catalytic subunit (Co isoform) cDNA (30), and the 360-bp Psel fragment of the rat PP2B catalytic subunit (Ab isoform) cDNA (31). The DNA probe for mouse NF-L (720 bp) was prepared by the polymerase chain reaction using the upstream (5′-ACAAGCGGCGCTATGTGGAG-3′) and downstream (5′-TCGGCGTGGAGAGGAACGCTC-3′) primers (21). The 190-bp DNA probe for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was prepared by the polymerase chain reaction using the upstream (5′-TGCGATTGGAACGGCTCATGAC-3′) and downstream (5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′) primers (32). All these fragments were $^{32}$P-labeled by the random primer labeling method and used as probes. Radioactivity was measured by an auto-image analyzer (BAS 2000, Fuji, Japan) or autoradiography using Kodak x-ray films.

**Preparation of Cell Extracts and Immunoblotting**—The cells were washed twice with isotonic saline and suspended in buffer C (10 mM Tris-HCl (pH 6.9), 50 mM NaCl, 5 mM β-mercaptoethanol, 0.1 mM EDTA, 1 mM EGTA, and 2% (v/v) glycerol) containing protease inhibitors (2 μg/ml pepstatin A, 2 μg/ml antipain, 2 μg/ml leupeptin, 2 μg/ml chymostatin, 0.1 mM Na$_3$-tosyl-l-phenylalanine chloromethyl ketone, 0.1 mM Na$_3$-tosyl-l-lysine chloromethyl ketone, 0.1 mM Nα-p-tosyl-l-lysine chloromethyl ketone, 0.1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride) and sonicated on ice. Each suspension was centrifuged at 10,000 g for 10 min, and the resulting supernatant was used as the cell extract. 10-μg aliquots of each extract were subjected to SDS-polyacrylamide gel electrophoresis as described previously (33), electrotransferred onto nitrocellulose membranes (Schleicher & Schuell), and immunostained with specific antibodies, as described previously (28), and immunoreactivity was detected using color development system (28). Protein concentrations were determined using the method described by Bradford (34) with bovine serum albumin as the standard.

**Assay of PP Activity**—PP activity was assayed by measuring the
amount of [32P]phosphate released from [32P]histone, [32P]casein, or [32P]myosin light chain, which were prepared with cyclic AMP-depend-ent protein kinase, casein kinase II, and myosin light chain kinase, respectively, as described previously (10, 35, 36). The PP1 activity assay was performed in a reaction mixture comprising 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM OA, 1 mM MnCl2, 60 μM (alkaline labile phosphate) [32P]casein, and the required cell extract in the presence or the absence of 0.2 mM inhibitor-2. The PP1 activity was defined as the inhibitor-2-sensitive PP activity (activity in the absence of inhibitor-2 minus that in its presence). The PP2A activity assay was performed in a reaction mixture comprising 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.2 μM inhibitor-2, 60 μM (alkaline labile phosphate) [32P]histone, and the required cell extract in the presence or the absence of 1 mM OA. The PP2A activity was defined as the OA-sensitive activity (activity in the absence of OA minus that in its presence). One unit of PP activity was defined as the amount of enzyme that catalyzed the release of 1 nmol phosphate/min at 30°C. The PP2B activity of cell extracts was assayed after purifying PP2B with calmodulin-agarose affinity column. The column chromatography of cell extracts was performed as described previously (37). The purified PP2B fraction was essentially free of PP1 and PP2A activities. The PP2B activity assay was performed in a reaction mixture comprising 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 0.2 μM inhibitor-2, 0.5 μM OA, 1 mM MnCl2, 2 mM CaCl2, 3 μM calmodulin, 60 μM (alkaline labile phosphate) [32P]myosin light chain, and purified PP2B fractions.

Nuclear Run-on Transcription Assay—Nuclear preparation was performed as described previously (38), except that 10 mM OA was present in the Nonidet P-40 lysis buffer. 10-μg aliquots of mouse NF-L cDNA fragment, prepared by the polymerase chain reaction as described above, were subcloned into pcDNAIII. pcDNAIII with or without the insert was linearized by BamHI digestion, denatured, and fixed on Hybond N membranes with a Dot-blot apparatus (Advantec, Japan). 10-μg aliquots of the 440-bp fragment of human β-actin cDNA and the 1.2-kilobase pair fragment of chicken GAPDH cDNA (39) were also fixed on the membranes. The run-on products from the same number of nuclei (1.0 × 107 nuclei) labeled with [3H]UTP were hybridized with the cDNA probes on the membranes at 45°C for 3 days. Then the membranes were washed until no detectable nonspecific radioactivity of the spot of pCR<sup>™</sup>II without an insert remained (see Fig. 4).

Analysis of mRNA Stability—The stability of NF-L mRNA was analy-ized as described previously (24).

RESULTS

Expression of Neurofilament-L during the Course of P19 Cell Neural Differentiation—First we determined whether the expression level of neurofilament-L (NF-L), a marker of neural differentiation, altered during the course of RA-induced differ-entiation of P19 cells into neurons and glia. The morphological changes during the course of neural differentiation are shown in Fig. 1. When the P19 cells were cultured on bacterial grade dishes, cell aggregates termed embryoid bodies were formed about 24 h after seeding both in the presence and the absence of 10−6 M RA. Neurite outgrowth was observed about 48 h after replating onto tissue culture grade dishes (monolayer culture) in the absence of RA only when the preceding aggregation culture had been performed in the presence of RA, essentially confirming the previous report (Fig. 1, B and C) (4). The amount of cellular NF-L protein in the aggregation culture increased markedly only when RA was present, and this elevated level was maintained in the subsequent monolayer cul-ture (Fig. 2D). The NF-L mRNA level was found to increase in accordance with the enhanced NF-L protein level (Fig. 2D).

Effect of Protein Phosphatase Inhibitors on Neurite Out-growth and NF-L Expression—In order to determine whether...
protein serine/threonine phosphatases participate in the regulation of P19 cell differentiation, the effects of OA and tautomycin, inhibitors of PP1, PP2A, and PP2B, and CsA and FK 506, specific inhibitors of PP2B, were studied. When 10 nM OA was present in the culture medium throughout the differentiation process, neurite outgrowth and the mRNA (both 2.3- and 3.5-kilobase mRNAs) and protein levels of NF-L were suppressed markedly on day 6 (Figs. 1D, 2B, and 2D). OA at 10 nM had no effect on the viability of P19 cells (Fig. 3). Treatment with 5 nM OA had a considerably less marked inhibitory effect on neurite outgrowth and NF-L expression (data not shown). The presence of 50 nM tautomycin in the culture medium during the course of differentiation partially suppressed the neurite outgrowth and NF-L expression (Figs. 1E, 2C, and 2E), but this concentration of tautomycin did not affect the viability of the cells. The presence of 10 nM OA during the last 2 days (days 4–6) only of the course of differentiation was enough to suppress NF-L expression (Fig. 2, B and D). However, no decrease in the NF-L level was observed when the cells were treated with OA for the first (day 0–2) or second (day 2–4) 48 h only of the 6-day course of P19 cell neural differentiation. In accordance with the effect of OA treatment on NF-L expression, neurite outgrowth was partially inhibited by treating the cells with OA during days 4–6 but not during days 0–2 or 2–4 (data not shown).

The addition of 1 μg/ml CsA to the culture medium throughout the differentiation process affected neither neurite outgrowth nor the NF-L expression level (Figs. 1F and 2F). The addition of 100 ng/ml FK506 did not affect the neurite outgrowth, but it caused an enhanced expression of NF-L (Figs. 1G and 2F).

Effect of OA Treatment on NF-L Gene Transcription and NF-L mRNA Stability—In order to determine the mechanism by which OA treatment reduced the NF-L mRNA levels, we performed the nuclear run-on transcription assay using nuclei isolated from neurally differentiated P19 cells (day 6) treated with or without 10 nM OA (Fig. 4). The results showed that there were no significant differences between the NF-L gene transcriptional levels of cells treated with and without OA.

Next, the effect of OA treatment on NF-L mRNA stability was studied. The cytoplasmic NF-L mRNA level of differentiated cells treated with OA was compared with that of untreated cells after incubating both sets of cells with actinomycin D for 0, 2, 4, and 6 h (Fig. 5). OA treatment enhanced degradation of both 3.5- and 2.3-kilobase NF-L mRNAs, whereas GAPDH mRNA stability was affected little by OA treatment.


![Fig. 3. Effect of OA treatment on the growth rate of undifferentiated P19 cells.](http://www.bac.org/)

![Fig. 4. Effect of OA treatment on NF-L gene transcription in P19 cells.](http://www.bac.org/)

![Fig. 5. Effect of OA treatment on NF-L mRNA stability.](http://www.bac.org/)
showed that the alteration in the pattern of the protein level of the PP2A catalytic subunits (mixture of PP2A-Cα and PP2A-Cβ proteins) during the course of differentiation was similar to that of the mRNA levels (Fig. 7B). In parallel with the mRNA and PP2A catalytic subunit protein levels, the total PP2A activity increased (1.5-fold) in the aggregation culture in the presence of RA and increased further (2.2-fold) in the subsequent monolayer culture (Fig. 7C), whereas no alterations of PP2A expression were observed when the cells were cultured in the absence of RA.

The mRNA and protein levels of the PP2B catalytic subunit also increased when aggregation culture was performed in the presence but not the absence of RA, and the expression level increased further in the subsequent monolayer culture (Fig. 8, A and B). In contrast with PP2B catalytic subunit, the presence of substantial amount of PP2B regulatory subunit protein was observed in the control P19 cell extracts, but the expression level of the regulatory subunit was also enhanced during the course of the differentiation process (Fig. 8B). In parallel with the enhanced expression of the catalytic and regulatory subunits of PP2B, the PP2B activity of the cell extract increased in the aggregation culture in the presence of RA, and the activity
level reached 4.3-fold above control in the subsequent monolayer culture. However, no increase in the activity level was observed when the cells were not treated with RA (Fig. 8C).

**Effects of OA, CsA, and FK506 Addition during the Course of Differentiation on the PP1, PP2A, and PP2B Activities**—It has been reported that endogenous PP2B activity of cell extracts was suppressed when the enzyme assay was performed following the incubation of mammalian cells in the presence of CsA (40). Therefore, we tested whether the treatment of P19 cells with the protein phosphatase inhibitors in the course of neural differentiation affected the protein phosphatase activities of the cell extract. The PP1, PP2A, and PP2B activities of cell extracts were determined after culturing the cells in the presence of OA, CsA, or FK506 during the 6-day course of the neural differentiation (Fig. 9). The PP1 activity decreased 29 and 38% when the cells were treated with CsA and FK506, respectively, compared with that of the control cells without treatments, but the activity was little affected by the OA treatment (Fig. 9A). The PP2A activity decreased 68% by the OA treatment of the cells, whereas the CsA or FK506 treatment affected the enzyme activity very little (Fig. 9B). On the other hand, the PP2B activity decreased 90 and 77% by the CsA and FK506 treatments, respectively, whereas the 10 nM OA treatment did not significantly affect the PP2B activity in the cell extracts (Fig. 9C).

**DISCUSSION**

In this study we demonstrated that adding OA or tautomycin to the culture medium morphologically suppressed RA-induced neural differentiation of P19 cells. We demonstrated also that NF-L gene expression increased in accordance with P19 cell neural differentiation, and both OA and tautomycin showed similar inhibitory effects on the neural differentiation of P19 cells suggests that the inhibition was operated through the suppression of protein phosphatase activities by these agents.

The inhibition by OA treatment was due primarily to reduced NF-L mRNA stability. As the GAPDH mRNA level was hardly affected by OA treatment, the reduced NF-L mRNA stability did not appear to be a nonspecific effect of OA (Fig. 2D). Regulation of NF-L gene expression in rat PC12 cells by post-transcriptional modification has been reported (41), and in this cell system, nerve growth factor increased NF-L mRNA levels by stabilizing the mRNAs.

We found that expression of PP2A and PP2B but not PP1 increased in parallel with the NF-L expression level of P19 cells (Figs. 6–8). Because adding CsA or FK506, specific inhibitors of PP2B, to the culture medium did not inhibit NF-L expression, PP2B was unlikely to be responsible for the differentiation-dependent increase in NF-L expression (Figs. 1 and 2). In this context, FK506 treatment was reported to rather stimulate nerve growth factor-induced neural differentiation of PC12 cells (42). OA inhibited NF-L expression only when high levels of PP2A activity were maintained, and a relatively low concentration of OA (10 nM) was required for this inhibition (Fig. 2B and D). OA addition in the culture medium inhibited the PP2A activity but not the PP1 activity of the cell extracts harvested at day 6 of the cell differentiation process. In addition, both CsA and FK506 treatments, which did not suppress the NF-L expression, were found to inhibit not only the PP2B activity but also the PP1 activity of the cell extracts (Fig. 9). These lines of evidence suggest strongly that of the three major OA-sensitive protein phosphatases PP2A but not PP1 or PP2B is responsible for the differentiation-dependent increase in NF-L expression and is the point of the action of OA. The inhibition of PP1 activity by CsA or FK506 treatment was presumably through inhibition of PP2B by these agents, because it has been reported that a CsA- or FK506-induced decrease in the PP2B activity in nerve cells contributed to maintenance of the high phosphorylation level of protein phosphatase inhibitor-1, which as a result caused decrease in the PP1 activity of the cells (13).

It is generally accepted that PP2A is present in various
oligomeric forms in mammalian cells. The activity of the free catalytic subunit changes considerably when it recombines with the regulatory subunits (43), and PP2A activity can be regulated by post-translational modifications (44, 45). Therefore, it is unlikely that the differentiation-induced PP2A activity increase was caused merely by enhanced expression of the catalytic subunit of PP2A. The possibility that some post-translational modification of PP2A induced by P19 cell differentiation contributed to the increased PP2A activity should be considered.

Various information regarding the involvement of protein phosphorylation in the regulation of mRNA stabilization has been obtained. In LLC-PK1 cells, down-regulation of protein kinase C was found to stabilize urokinase-type plasminogen activator mRNA (46), and CsA treatment of mast cell tumor lines was reported to destabilize interleukin-3 mRNA through the mechanism involved the 3′-untranslated region (3′-UTR) (47), suggesting that PP2B enhances mRNA stability. However, in contrast to its destabilizing effect on NF-L mRNA observed in this study (Fig. 5), OA treatment was found to increase the stability of nerve growth factor mRNAs in primary cultures of cortical astrocytes (48). Such conflicting evidence suggests that the stability of mRNAs is controlled by various signal transfer systems and phosphorylation of cellular proteins results in distinct effects that depend on the particular mRNA species and/or the signal transfer system employed. Schwartz et al. (49) reported recently that 3′-UTR of NF-L mRNA may contain determinants that regulate the stability of NF-L mRNA because studies of transfected P19 cells showed 3′-UTR deletion led to a severalfold stabilization of NF-L mRNA, providing the possibility that OA-sensitive dephosphorylation of cellular proteins is involved in the inhibition of destabilizing function of 3′-UTR of NF-L mRNA.

OA treatment of rat dorsal root ganglion neurons was reported to cause NF network disruption characterized by hyperphosphorylation of NF subunits (26), and the amino-terminal domain of NF-L was preferentially dephosphorylated by the catalytic subunit of PP2A but not that of PP1 in vitro (25). Therefore, the differentiation-dependent increase in PP2A expression in P19 cells may contribute not only to enhanced NF-L expression but also to NF-L dephosphorylation, thereby inducing assembly of the NF network.

It is not known what type(s) of signal is/are required to enhance the expression levels of the PP2A and PP2B catalytic subunits in P19 cells. In this context, den Hertog et al. (9) reported that R-PTPα participated in signal transmission related to P19 cell aggregation. They found that the R-PTPα expression level was enhanced when P19 cells in monolayer culture were transferred to aggregation culture and that P19 cells harboring the R-PTPα expression vector could be differentiated into neurons in a RA-dependent manner, even in the monolayer culture. Their results also demonstrated that endogenous pp60c-src is activated by a high R-PTPα expression level. Therefore, in order to clarify the mechanism(s) responsible for the differentiation-dependent increases in PP2A and PP2B expression, it is important to determine whether high expression levels of these enzyme molecules are related to R-PTPα/pp60c-src-dependent signal transmission.

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![Suppression of NF-L Gene Expression by Okadaic Acid](http://www.jbc.org/)

**Fig. 9. Effects of OA, CsA, and FK506 treatments on PP activities.** PP1 (A), PP2A (B), and PP2B (C) activities of cell extracts were determined as described under “Experimental Procedures” after culturing the cells in the absence or the presence of 10 nM OA, 1 μg/ml CsA, or 100 ng/ml FK506 during the 6-day course of the neural differentiation. Each activity is expressed as the percentage of the activity of the control cells that were not treated with protein phosphatase inhibitors. The results represent the mean of two experiments ± standard error of the mean.
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