0026-895X/01/5905-1287–1297$3.00
MOLECULAR PHARMACOLOGY
Vol. 59, No. 5
299/899547
Mol Pharmacol 59:1287–1297, 2001
Printed in U.S.A.

Scabronine G-Methylester Enhances Secretion of Neurotrophic Factors Mediated by an Activation of Protein Kinase C-ζ

YUTARO OBARA, HIRONORI KOBAYASHI, TOMIHISA OHTA, YASUSHI OHIZUMI, and NORIMICHI NAKAHATA

Departments of Pharmaceutical Molecular Biology (Yu.O., Ya.O., N.N.) and Cellular Signaling (N.N.), Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan Department of Pharmacognosy and Natural Products Chemistry (H.K., T.O.), Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan

Received May 23, 2000; accepted January 23, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Glial cells release neurotrophic factors that maintain neurons functionally. Previously, we have shown that the scabronines isolated from *Sarcodon scabrosus* enhanced the secretion of neurotrophic factors from 1321N1 human astrocytoma cells. In the present study, we examined the mechanism of newly synthesized scabronine G-methylester (ME)-induced secretion of neurotrophic factors from 1321N1 cells. The dramatic neuronal differentiation of rat pheochromocytoma cells (PC-12) was observed by scabronine G-ME-conditioned medium of 1321N1 cells. Scabronine G-ME increased the secretion of nerve growth factor (NGF) and interleukin-6 (IL-6) from 1321N1 cells with the enhancement of their mRNA expressions. Scabronine G-ME concentration-dependently inhibited the carbachol-induced inositol phosphate accumulation in 1321N1 cells, which was reversed by GF109203X, an inhibitor of protein kinase C (PKC) isoforms. Furthermore, GF109203X inhibited the scabronine G-ME-induced mRNA expressions of both NGF and IL-6 and the differentiation of PC-12 cells, showing that scabronine G-ME activated PKC. Although scabronine G-ME enhanced activities of neither conventional nor novel types of PKCs, it translocated PKC-ζ to membranes in intact cells and cell-free condition. Furthermore, recombinant PKC-ζ activity was also increased by scabronine G-ME, suggesting the involvement of PKC-ζ in the effect of scabronine G-ME. Concerning the downstream effectors of the PKC-ζ, scabronine G-ME translocated nuclear factor-κB to nucleus, and enhanced its transcriptional activity. In addition, scabronine G-ME caused the degradation of inhibitor of nuclear factor-κB concentration-dependently, which was inhibited by GF109203X. These results suggest that scabronine G-ME potentially enhances the secretion of neurotrophic factors from 1321N1 cells mediated via the activation of PKC-ζ.

Neurotrophic factors are essential for neurons to maintain and organize themselves functionally. Glial cells support neurons by releasing neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor, neurotrophin 3 (Althaus and Richter-Landsberg, 2000), interleukin-6 (IL-6) (Schwaninger et al., 1997) and glia-derived neurotrophic factor (Lin et al., 1993). NGF has pleiotropic effects on the promotion of neuronal differentiation and survival (prevention of apoptosis) in various neurons (Levi-Montalcini, 1987). IL-6 promotes neuronal survival and differentiation of rat pheochromocytoma cells (PC-12) (Satoh et al., 1988), whereas it induces the proliferation of glial cells (Balasingam et al., 1994).

This work was partly supported by Grants-in-Aid 08672496 and 09470497 (N.N.) from Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan, and from Japan Society for the Promotion of Science Grant 01532 (Yu.O.).

It has been reported that C6 rat glioma cells synthesize NGF upon the stimulation by β-adrenergic agonists forskolin or cyclic AMP analogs (Fukumoto et al., 1994; Colangelo et al., 1996), indicating that the accumulated cyclic AMP increases NGF mRNA expression. On the other hand, NGF mRNA expression is dramatically enhanced by phorbol ester in astrocytes (Jehan et al., 1995), suggesting the involvement of protein kinase C (PKC). The phorbol ester-induced enhancement of NGF synthesis was assumed to be through the activation of activator protein-1 (AP-1) known as Fos/Jun homo- or heterodimer complex (Jehan et al., 1995; Colangelo et al., 1996). In fact, an AP-1 consensus sequence exists within downstream of the TATA box at the junction of the exon I/intron I region of rat and mouse NGF gene (D'Mello and Heinrich, 1991). It has been shown that nuclear factor κB (NF-κB) regulates gene expression in NGF synthesis induced by lipopolysaccharide, IL-1β, and tumor necrosis fac-

ABBREVIATIONS: NGF, nerve growth factor; IL-6, interleukin-6; PC-12, pheochromocytoma cells; PKC, protein kinase C; AP-1, activator protein-1; NF-κB, nuclear factor-κB; ME, methylester; EMEM, Eagle's minimal essential medium; I-κB, inhibitor of nuclear factor-κB; PMA, phorbol-12-myristate-13-acetate; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; PC-PLC, phosphatidylcholine-specific phospholipase C; RT, reverse transcription; PCR, polymerase chain reaction.

tor-α (Heese et al., 1998a,b). Galve-Roperh et al. (1997) showed that mitogen-activated protein kinase cascade was involved in NGF synthesis induced by sphingomyelinase and ceramide. In addition, we have shown that an increase in intracellular Ca^{2+} induced by maitotoxin stimulates the synthesis of NGF in C6-BU-1 glioma cells (Obara et al., 1999b). Thus, the expression of NGF mRNA is assumed to be regulated through multiple signaling pathways. However, the detailed mechanism of NGF synthesis remains unclear.

IL-6 gene transcription is also induced by various stimulations such as IL-1, tumor necrosis factor-α, platelet-derived growth factor, and interferons (Sehgal, 1992). Cyclic AMP, Ca^{2+}, and diacylglycerol are essential for IL-6 gene transcription (Sehgal, 1992). Furthermore, NF-κB binding site, located at the upstream of IL-6 gene, contributes to the regulation of IL-6 mRNA expression (Sehgal, 1992).

Molecular cloning has revealed the existence of 10 discrete isoforms of PKC, representing the products of separate genes (α, γ, δ, ϵ, ζ, η, θ, and λ/ι) and alternative splicing of one gene (β_I and β_{II}) (Zhou et al., 1994). With respect to structural features and diacylglycerol- or Ca^{2+} dependence, the members of the PKC family have been classified into three groups. The group that is dependent on both diacylglycerol and Ca^{2+} has been considered conventional/classic PKCs (α, β_I, β_{II}, and γ), whereas one that is dependent on diacylglycerol but not Ca^{2+} has been regarded as novel/nonclassic PKCs (δ, ϵ, η, and θ). Both conventional/classic PKC and novel/nonclassic PKC groups have been shown to be sensitive to phorbol esters. The third group of PKC isoform is considered as atypical PKCs (ζ and λ/ι), which require neither Ca^{2+} nor diacylglycerol. In addition, the atypical PKC family is neither activated nor down-regulated by phorbol esters.

PC-12 cells have been used as an in vitro model of neuronal differentiation. In response to NGF, these cells extend neurites and develop the characteristics of sympathetic neurons (Greene and Tischler, 1976). Human astrocytoma cells (1321N1) have been used as a model of glial cells to examine receptor-mediated events (Nakahata and Harden, 1987; Nakahata et al., 1989). We have previously reported that 1321N1 cells released a new neurotrophic factor that causes the differentiation of PC-12 cells (Obara et al., 1998).

Recently, we isolated new cyathane diterpenoids from *S. scabrosus,* termed scabronines (Kita et al., 1998; Ohta et al., 1998). Scabronine A and G have been shown to promote the secretion of neurotrophic factors, including NGF from 1321N1 cells, causing the enhancement of differentiation of PC-12 cells (Obara et al., 1999a).

The present study was undertaken to examine the effect of scabronine G-methylester (ME), the newly synthesized scabronine derivative, and its mechanism of the secretion of neurotrophic factors from 1321N1 cells.

Experimental Procedures

Materials. NGF was obtained from Sigma (St. Louis, MO). The NGF enzyme-linked immunosorbent assay (ELISA) kit was purchased from Roche Molecular Biochemicals (Mannheim, Germany). The IL-6 ELISA kit was purchased from Cosmobio (Tokyo, Japan). The total RNA extraction kit was from Amersham Pharmacia Biotech (Piscataway, NJ). The reverse transcription-polymerase chain reaction (RT-PCR) kit and LipoTAXI transfection kit were from Toyobo Co., Ltd (Osaka, Japan). Protein kinase C enzyme assay system and [γ-^{32}P]ATP were from Amersham Pharmacia Biotech (Buckinghamshire, England). Phorbol-12-myristate-13-acetate (PMA) and GF109203X were from Wako Pure Chemicals (Tokyo, Japan). Gö6976 was from Calbiochem (La Jolla, CA). [^{3}H]inositol (23.4 Ci/mmol) was from PerkinElmer Life Science Products (Boston, MA). Anion exchange column (AG1X8) was from Bio-Rad (Hercules, CA).

Purification of Scabronine G. Scabronine G was purified from the fruit bodies of the mushroom *S. scabrosus,* as described previously (Kita et al., 1998; Ohta et al., 1998). The melting point of scabronine G was 68.5–69.5°C. Its mass spectrometry analysis (high-resolution electron impact ionization-mass spectrometry) showed *m/z* 330.1829 (M^+, calcd 330.1831 for C20H2604).

Methyl Esterification of Scabronine G. Scabronine G-methylester was synthesized from scabronine G as follows: scabronine G was dissolved in acetic ester, and then diazomethane in ether was added into the solution until the color of the solution turned yellow. After the reaction, scabronine G-ME was isolated by column chromatography.

Cell Culture. 1321N1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (Cell Culture Laboratory, Cleveland, OH), penicillin (50 units/ml), and streptomycin (50 μg/ml) in an incubator containing 5% CO_2 at 37°C. PC-12 cells were grown in DMEM supplemented with 10% fetal calf serum, 5% horse serum (ICN Biochemicals, Costa Mesa, CA), and the above-mentioned antibiotics in an incubator containing 5% CO_2 at 37°C.

Evaluation of Neurite Outgrowth. After PC-12 cells were incubated with drugs, cells were fixed with 1% glutaraldehyde (Wako, Tokyo, Japan) in phosphate-buffered saline (PBS), and cell morphology was observed under a phase-contrast microscope. The neurite outgrowth of PC-12 cells observed under a phase contrast microscope was regarded as a sign of neuronal differentiation (Obara et al., 1998). The differentiation of PC-12 cells was scored as follows: cells without neurite outgrowth were scored 0, cells bearing neurites as long as one cell diameter were scored 1, cells bearing neurites 2 to 3 times longer length than their diameter were scored 2, and cells bearing neurites that were extremely long or forming a synapse were scored 3. The mean differentiation score was obtained for seventy PC-12 cells in each well. Data are expressed as means ± S.E.M. of the values of three to four wells.

Enzyme Immunoassay. Enzyme immunoassay of NGF was carried out as described in the previous report with slight modifications (Obara et al., 1999a). Briefly, 1321N1 cells were seeded onto six-well plates. The medium was substituted with DMEM supplemented with 0.5% fetal calf serum, and the cells were further cultivated overnight. Drugs in DMEM supplemented with 0.5% fetal calf serum were added to the well. The cells were cultivated for 24 h in the presence of the drugs, and 1 ml of the condition medium was collected. The NGF content in the medium was measured by using a sandwich ELISA according to the instructions of NGF ELISA kit. The same samples as NGF ELISA were used for IL-6 sandwich ELISA with horseradish peroxidase-conjugated anti-IL-6 monoclonal antibody according to the instructions of IL-6 ELISA kit.

Semiquantitative RT-PCR. Total RNA from 1321N1 cells was extracted by using a total RNA extraction kit, and semiquantitative RT-PCR was carried out by using a RT-PCR kit. NGF mRNA expression was examined as described previously (Obara et al., 1999a). In brief, the sense primer (5′-CTT CAG CAT TCC CTT GAC AC-3′, 316–335 of human NGF cDNA) and the antisense primer (5′-AGC CTT CCT GCT GAG CAC ACA-3′, 889–909) were complementary to conserved regions of the cDNA from both mouse and human NGF. The NGF cDNA of 594 base pairs was amplified 35 cycles (94°C for 60 s, 57°C for 30 s, and 72°C for 50 s). For analysis of IL-6 mRNA, the sense primer (5′-AAA TTC GGT ACA TCC TCG AC-3′ of human IL-6 cDNA) and the antisense primer (5′-CAG GAA CTG GAT CAG GAC TT-3′), which were complementary to conserved regions of cDNA from human IL-6, were used. The IL-6 cDNA of 295 base pairs was amplified 39 cycles (95°C for 60 s, 56°C for 60 s, and 74°C for 60 s).

Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) transcripts with 450- or 700-base pair length were used as a positive control. The number of cycles that yielded a quantitative amount of product was determined in a preliminary experiment.

Measurement of Inositol Phosphates. Accumulation of inositol phosphates was measured as follows: 1321N1 cells were cultivated in 12-well plates for 2 days at the density of 2×10^5 cells/ml/well, and were labeled with 2 μCi/ml [^{3}H]inositol overnight. After washing twice with Eagle's minimum essential medium (EMEM)-HEPES buffer (pH 7.4), the cells were preincubated in EMEM-HEPES containing 10 mM LiCl for 10 to 14 min, and they were incubated with drugs for an additional 10 to 14 min. Reaction was terminated by the addition of 1 ml of ice-cold 5% trichloroacetic acid after aspiration of the medium. The trichloroacetic acid extracts were washed three times with diethyl ether to remove trichloroacetic acid. Diethyl ether in the sample was removed by keeping at 47°C for 30 min. Total [^{3}H]inositol phosphates were separated by anion exchange column (AG-1X8, formate form, 100–200 mesh) as described previously (Nakahata et al., 1989).

PKC Kinase Assay. PKC kinase activity was examined using by protein kinase C enzyme assay system with slight modifications. Briefly, 1321N1 cells were cultivated on 35-mm dish for 2 days at a density of 4×10^5 cells/ml, the medium was replaced with DMEM supplemented with 0.5% fetal calf serum, and the cells were further cultivated overnight. The cells were incubated with drugs at 37°C for appropriate time in EMEM-HEPES. Then, after aspiration of the medium, the cells were scraped and homogenized by sonication in homogenization buffer (10 mM Tris-HCl, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.4 measured at 4°C). The cell homogenates were then incubated with the substrate and [γ-^{32}P]ATP (40 μCi/ml) for 15 min at 37°C in a final volume of 55 μl according to the instructions of protein kinase C enzyme assay system except that PMA in the buffer was removed. Synthetic peptide (Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-OH), which was a part of epidermal growth factor receptor peptide (amino acids 651–658), was used as the substrate for PKC. After terminating the reaction by addition of stop regent (10 μl), each sample (35 μl) was spotted onto phosphocellulose paper. The papers were washed twice for 2 min in 75 mM H_3PO_4, and then washed twice for 2 min in distilled water. The radioactivity on each binding paper was determined by scintillation counting. For PKC-ζ kinase assay in cell-free condition, human recombinant PKC-ζ (10 ng) (Upstate Biotechnology, Lake Placid, NY), PKC-α-derived pseudosubstrate peptide (2 μg) (Life Technologies, Rockville, MD) and phosphatidylserine (5 μg) (Avanti, Alabaster, AL) were dissolved in 40 μl of kinase buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 mM sodium orthovanadate). PKC-α-derived pseudosubstrate peptide {[Ser^{25}] PKC (19–31); Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val} was used as a substrate for PKC-ζ (Kochs et al., 1993). Drugs in 10 μl of the buffer were added to the tube and incubated at room temperature for 10 min. Then, the tube was incubated at 30°C for 15 min after addition of 5 μl of [^{32}P]ATP (0.2 μCi/tube) solution. The following procedure was the same as described above.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting for PKC and I-κBα. Samples for PKC detection were prepared

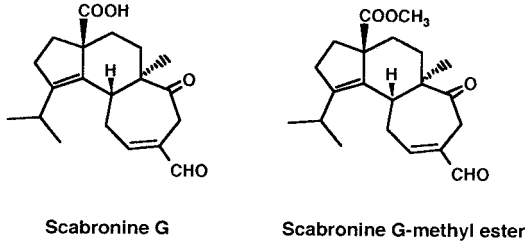

Fig. 1. Chemical structures of scabronine G and scabronine G-ME.

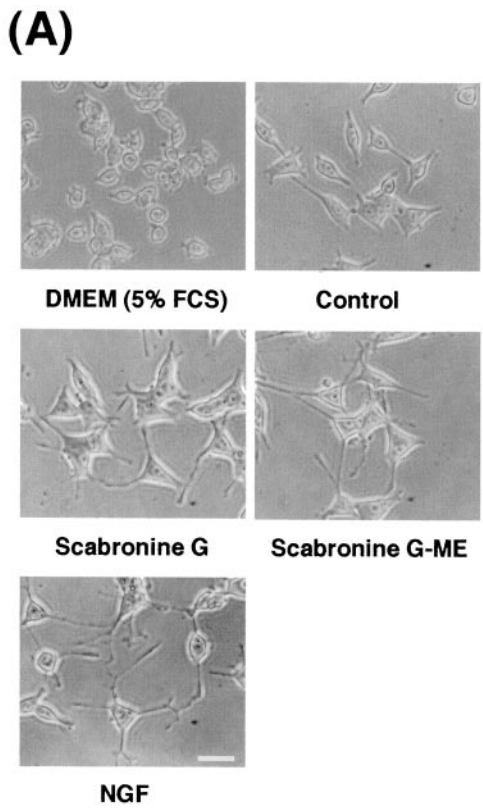

Fig. 2. Effects of scabronines on glial cell-mediated morphological changes in PC-12 cells. A, morphological changes of PC-12 cells by the 1321N1 cell culture medium conditioned by scabronines. 1321N1 cells (5×10^5 cells/ml) were incubated for 2 days in DMEM containing 5% fetal calf serum in the presence of scabronine G (100 μM) or scabronine G-ME (100 μM). Then PC-12 cells were cultivated for 2 days in the conditioned 1321N1 cell culture medium. Phase-contrast microscopy of PC-12 cells after addition of DMEM supplemented with 5% fetal calf serum, 1321N1 cell culture medium conditioned by 0.1% dimethyl sulfoxide as a vehicle of the scabronines (control), scabronine G (100 μM), scabronine G-ME (100 μM), and NGF (50 ng/ml). Scale bar, 50 μm. B, evaluation of differentiation scale of PC-12 cells. The differentiation of PC-12 cells was evaluated as described under *Experimental Procedures* and experiments were performed as shown in A. Values represent the means ± S.E.M. for three wells. Scabronine G and scabronine G-ME significantly accelerated differentiation compared with control (*$P < 0.05$). Scabronine G-ME also significantly promoted the differentiation of PC-12 cells compared with scabronine G (†$P < 0.05$).

as follows: 1321N1 cells were seeded onto a 35-mm dish at a density of 4×10^5 cells/ml. The medium was replaced with DMEM supplemented with 0.5% fetal calf serum, and the cells were cultivated overnight. Cells were incubated with drugs in serum-free EMEM-HEPES for various periods. After aspiration of the medium, the homogenization buffer (the same as described in the previous section) was added to terminate the reaction. The cells were homogenized and centrifuged at 100,000*g* for 60 min at 4°C. The supernatant was used as cytosolic fraction. The pellets were dissolved in the same buffer supplemented with 0.5% Triton X-100. After incubation for 30 min at 4°C, it was centrifuged at 100,000*g* for 30 min at 4°C. The supernatant was regarded as membrane fraction (Trilivas et al., 1991). Those fractions were dissolved in Laemmli sample buffer (final concentration, Tris-HCl 75 mM, SDS 2%, glycerol 15%, 2-mercaptoethanol 3%, pH 6.8), and boiled at 95°C for 5 min. For the preparation of samples for I-κBα detection, 1321N1 cells were seeded onto six-well plates at a density of 2×10^5 cells/ml. The medium was replaced with DMEM supplemented with 0.5% fetal calf serum and the cells were cultivated overnight. Cells were incubated with drugs in serum-free EMEM-HEPES for various periods. After aspiration of the medium, Laemmli sample buffer was added to terminate the reaction. The sample was boiled at 95°C for 5 min.

The electrophoresis was performed on 8% acrylamide gels. Proteins were transferred electrically from the gel onto polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA) by the semidry blotting method. The blots were blocked for 2 h with 1% bovine serum albumin in Tris-buffered saline at 25°C, and incubated with anti-PKC-α (Life Technologies, Gaithersbrug, MD) (1:2000 dilution), anti-PKC-ζ (Santa Cruz Biochemicals, Santa Cruz, CA) (1:2000 dilution), or anti-I-κBα (Santa Cruz Biochemicals) (1:1000 dilution) antibodies overnight at 4°C. The blots were washed several times and incubated with a 1:2000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (New England Biolabs, Beverly, MA) in Tris-buffered saline containing 1% bovine serum albumin at 25°C for 2 h. Blots were developed using a chemiluminescence assay kit, and visualized by the exposing the chemiluminescence from the membrane to the Hyperfilm enhanced chemiluminescence. The density of the bands corresponding to I-κBα was analyzed by densitometry (Advanced American Biotechnology, Fullerton, CA) and the data were expressed as percentage of control.

Immunostaining of NF-κB. 1321N1 cells were seeded onto 24-well plates at a density of 2×10^5 cells/ml. The medium was replaced with DMEM supplemented with 0.5% fetal calf serum and the cells

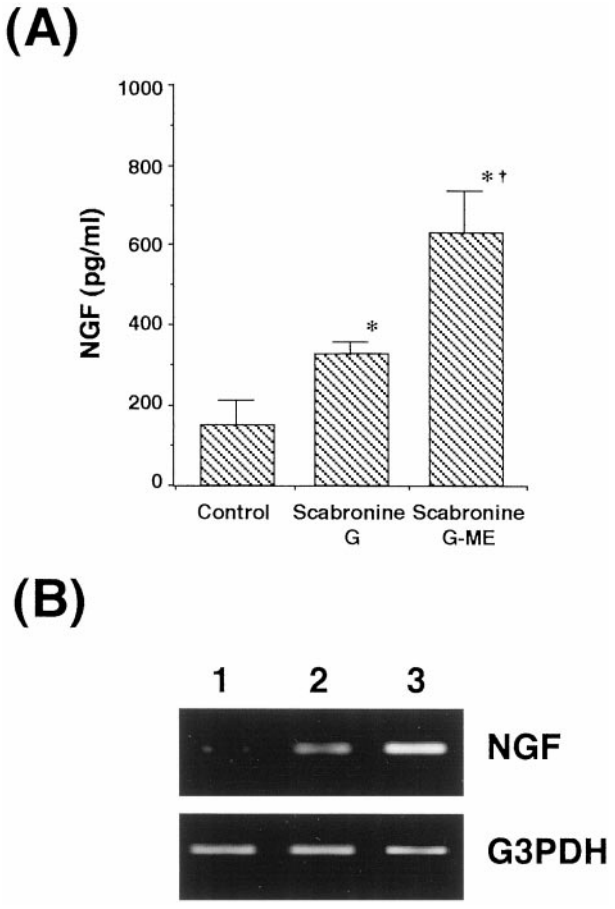

Fig. 3. Effects of scabronines on the synthesis and secretion of NGF from 1321N1 cells. A, effect of scabronines on NGF secretion from 1321N1 cells. After incubation with compounds (100 μM) for 24 h, NGF released from 1321N1 cells was measured using NGF ELISA kit. Values are the means ± S.E.M. of three determinations. Scabronine G and scabronine G-ME significantly increased NGF secretion, compared with control (*$P < 0.05$). Scabronine G-ME also significantly increased NGF secretion compared with scabronine G (†$P < 0.05$). B, effects of scabronines on NGF mRNA expression in 1321N1 cells. The cells were stimulated by the compounds for 4 h, and then total RNA from 1321N1 cells was reverse transcribed, followed by PCR as described under *Experimental Procedures*. The amount of G3PDH mRNA in each cell condition was also shown. Vehicle, lane 1; scabronine G (100 μM), lane 2; scabronine G-ME (100 μM), lane 3. Data are representative of three separate experiments.

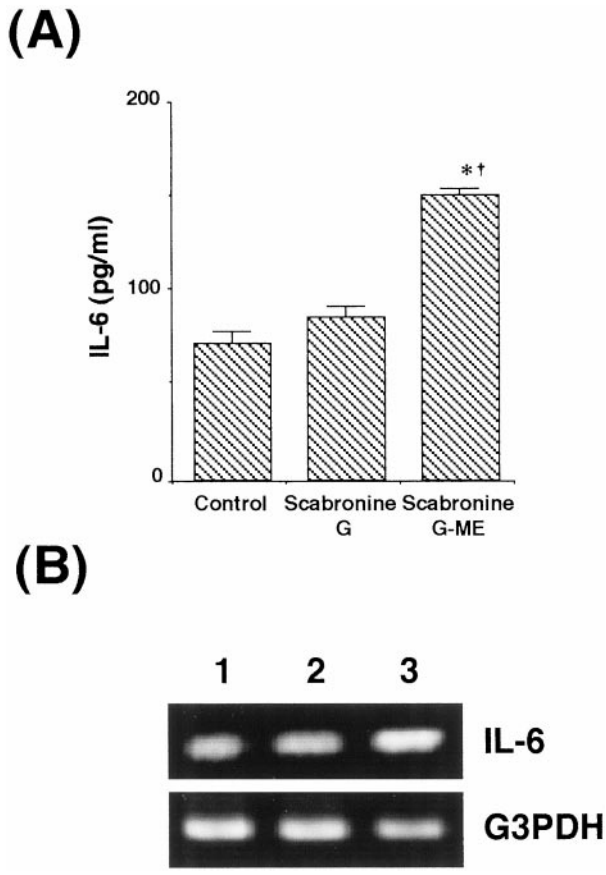

Fig. 4. Effects of scabronines on the synthesis and secretion of IL-6 from 1321N1 cells. A, effects of scabronines on IL-6 secretion from 1321N1 cells. After incubation with the indicated concentrations of the compounds for 24 h, IL-6 released from 1321N1 cells was measured using IL-6 ELISA kit. Values are the means ± S.E.M. of three determinations. Scabronine G-ME significantly increased IL-6 secretion, compared with control (*$P < 0.05$) and scabronine G (†$P < 0.05$). B, effects of scabronines on IL-6 mRNA expression in 1321N1 cells. The cells were stimulated by the compounds for 4 h, and then total RNA from 1321N1 cells was reverse transcribed, followed by PCR as described under *Experimental Procedures*. The G3PDH mRNA in each cell condition was also shown. Vehicle, lane 1; scabronine G (100 μM), lane 2; scabronine G-ME (100 μM), lane 3. Data are representative of two separate experiments.

were cultivated overnight. Cells were incubated with drugs in serum-free EMEM-HEPES for various periods. After the incubation, the cells were fixed with 1% glutaraldehyde/PBS for 15 min. Then, they were permeated with 0.5% Triton X-100/PBS for 5 min after washing with PBS. After blocking with skim milk (0.5%) for 1 h at 37°C, cells were incubated with a mouse anti-human NF-κB (p65) monoclonal antibody (2 μg/ml) (Roche Molecular Biochemicals) at 4°C overnight, followed by goat fluorescein-labeled anti-rabbit IgG antibody (6.25 μg/ml) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 37°C for 30 min. Then cells were visualized under a confocal laser microscope (DMRB/E, TCS NT; Leica, Wetzlar, Germany).

Transfection and Reporter Assay. The pNF-κB-SEAP reporter plasmid (CLONTECH, Palo Alto, CA) containing the four tandem copies of the NF-κB consensus sequence and secreted alkaline phosphatase was transfected into 1321N1 cells using LipoTAXI transfection kit. The pTAL-SEAP plasmid (CLONTECH), which lacks the NF-κB consensus sequence, was used as a negative control to measure the background signals. Briefly, 1321N1 cells were seeded onto 12-well plates at 3×10^5 cells/ml and cultivated for a day. The plasmid (0.7 μg/well) and transfection reagent (10 μl/well) were mixed gently in DMEM (90 μl) and incubated for 30 min at room temperature. After the addition of DMEM (200 μl), this entire mixture was transferred to the cell-cultured dish, and the cells were incubated for 5 h at 37°C. DMEM (300 μl) containing 10% fetal calf serum was added to the cell-cultured dish, and cells were incubated overnight. Next day, the medium was replaced with the normal fresh medium and incubated for 36 h. One night before the experiment, medium was substituted with DMEM containing 0.5% fetal calf serum. 1321N1 cells were incubated with drugs at 37°C for 6 h in Krebs-Ringer buffer-HEPES (130 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 4.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11.5 mM glucose, 20 mM HEPES, pH 7.4), and then medium was collected. After centrifugation to remove contaminating cells, 75 μl of sample was incubated in 20 μl of substrate solution (*p*-nitrophenyl phosphate disodium salt, 0.1 mg/ml of 1 M diethanolamine-HCl, pH 9.5) until the color of the solution turned light yellow. The absorbance at 415 nm subtracted with the value of background was regarded as the relative NF-κB activity.

Statistical Methods. Data were expressed as mean values with ± S.E.M., and the significant difference was determined using analysis of variance.

Results

Effects of Scabronine G and Scabronine G-ME on the Differentiation of PC-12 Cells Mediated by the Secre-

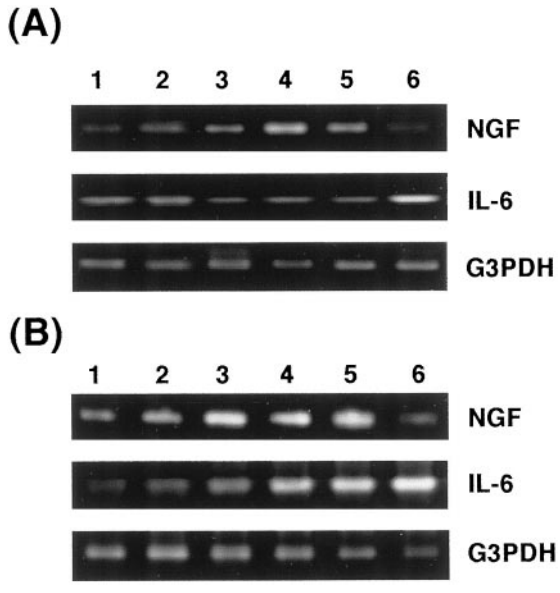

Fig. 5. Time dependence in the mRNA expressions of NGF and IL-6 by scabronines in 1321N1 cells. A, effect of scabronine G on the NGF and IL-6 mRNA expressions in 1321N1 cells. The cells were stimulated by the compounds for the indicated time, and then total RNA from 1321N1 cells was reverse transcribed, followed by PCR as described under *Experimental Procedures*. 1321N1 cells were stimulated with 100 μM scabronine G for 0 h (lane 1), 2 h (lane 2), 3 h (lane 3), 4 h (lane 4), 5 h (lane 5), or 6 h (lane 6). Data are representative of two separate experiments. B, effect of scabronine G-ME on the NGF and IL-6 mRNA expressions in 1321N1 cells. 1321N1 cells were stimulated with 100 μM scabronine G-ME for 0 h (lane 1), 2 h (lane 2), 3 h (lane 3), 4 h (lane 4), 5 h (lane 5), or 6 h (lane 6). Data are representative of two separate experiments.

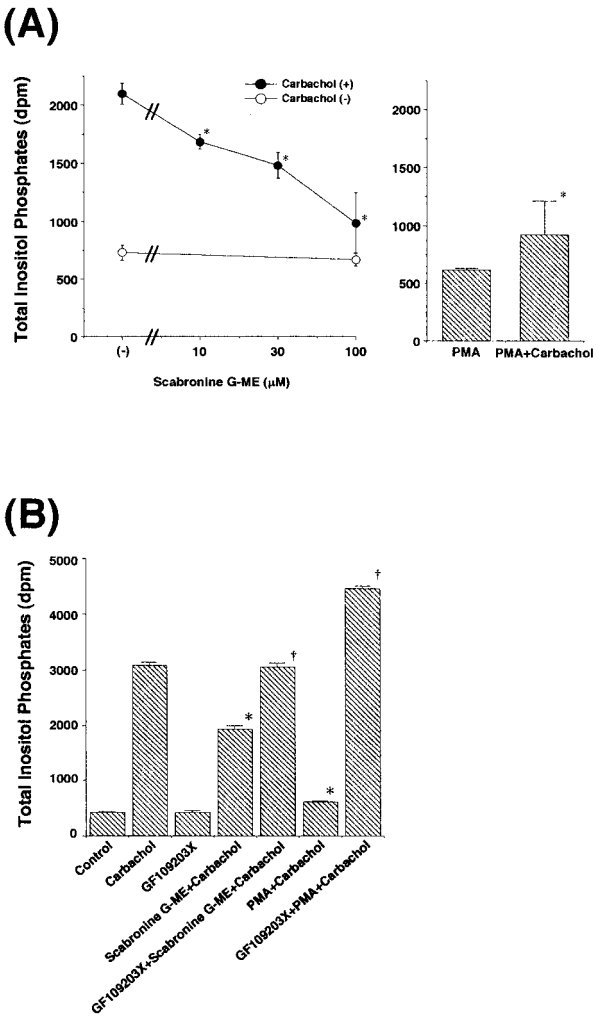

Fig. 6. Inhibition of carbachol-induced phosphoinositide hydrolysis by scabronine G-ME through an activation of PKC. A, inhibitory effect of scabronine G-ME on the accumulation of inositol phosphates. 1321N1 cells were incubated with scabronine G-ME (10–100 μM) or PMA (100 nM) for 10 min, and further incubated with carbachol (100 μM) for an additional 10 min. Total inositol phosphates were measured as described under *Experimental Procedures*. Vehicle (○), carbachol (100 μM) (●). Values are the means ± S.E.M. of three determinations. Scabronine G-ME and PMA significantly inhibited the carbachol-induced accumulation of inositol phosphates (*$P < 0.05$). B, inhibitory effect of GF109203X on scabronine G-ME-induced inhibition of phosphoinositide hydrolysis. 1321N1 cells were incubated with GF109203X (10 μM) for 14 min, and further incubated with PMA (30 nM) or scabronine G-ME (100 μM) for an additional 14 min. Then, the cells were incubated with carbachol (100 μM) for 14 min. Values are the means ± S.E.M. of three determinations. Scabronine G-ME and PMA significantly inhibited the carbachol-induced accumulation of inositol phosphates (*$P < 0.05$), and GF109203X significantly recovered the inhibition by scabronine G-ME and PMA (†$P < 0.05$).

tion of Neurotrophic Factors from 1321N1 Cells. To investigate effects of scabronine G and scabronine G-ME (Fig. 1), 1321N1 cells were incubated for 2 days in DMEM containing 5% fetal calf serum supplemented with 100 μM scabronine G or scabronine G-ME, and then PC-12 cells were cultivated for 2 days in the conditioned 1321N1 cell culture medium. The culture medium conditioned by 100 μM scabronine G or scabronine G-ME promoted neurite outgrowth of PC-12 cells (Fig. 2A). When the differentiation of PC-12 cells was evaluated by scoring as described under *Experimental Procedures*, scabronine G-ME had a greater potency than scabronine G, which was almost the same degree as that of 50 ng/ml NGF (Fig. 2B). Neither scabronine G nor scabronine G-ME directly caused neurite outgrowth of PC-12 cells by themselves in this condition (data not shown).

Effects of Scabronines G and Scabronine G-ME on Neurotrophic Factor Synthesis and Secretion from 1321N1 Cells. NGF and IL-6 are major neurotrophic factors released from astrocytes (Schwaninger et al., 1997; Althaus and Richter-Landsberg, 2000). Therefore, we measured NGF and IL-6 in the culture medium after 1321N1 cells were cultivated in the presence of 100 μM scabronine G or scabronine G-ME for 24 h. Scabronine G and scabronine G-ME significantly augmented NGF secretion. Scabronine G-ME had a greater potency than scabronine G (Fig. 3A). Scabronine-G ME also significantly increased IL-6 secretion from the cells (Fig. 4A). In contrast to NGF, scabronine G had a weak stimulation of IL-6 secretion. In addition, the mRNA expressions of NGF and IL-6 in 1321N1 cells were examined by a semiquantitative RT-PCR method. 1321N1 cells were incubated with 100 μM scabronine G or scabronine G-ME for 4 h. The mRNA expressions for both NGF and IL-6 were enhanced by scabronine G and scabronine G-ME (Figs. 3B and 4B). Scabronine G-ME had a greater potency than scabronine G in the mRNA expressions for both neurotrophic factors, consistent with the results of their protein secretion level. Scabronine G increased NGF mRNA expression in a time-dependent manner (Fig. 5A). Its expression began to increase 3 h after the addition of scabronine G, attained to a maximum at 4 h, and returned to control level at 6 h. On the other hand, scabronine G-ME enhanced the expression of NGF mRNA with the peak at 3 h, which was 1 h before the peak of scabronine G (Fig. 5B). Scabronine G-ME induced the IL-6 mRNA expression in a time-dependent manner with a delay compared with the expression of NGF mRNA (Fig. 5B). It is suggested that scabronine G-ME enhances the secretion of both neurotrophic factors by initiating the increase of mRNA level in 1321N1 cells.

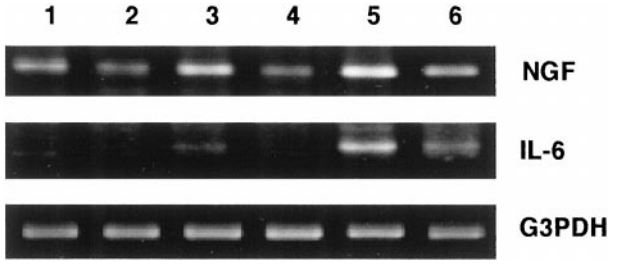

Fig. 7. Inhibitory effect of PKC inhibitor GF109203X on the mRNA expressions of NGF and IL-6 induced by scabronine G-ME in 1321N1 cells. 1321N1 cells were incubated with PMA (100 nM) or scabronine G-ME (100 μM) for 4 h in the presence or absence of GF109203X (1 μM), and then semiquantitative RT-PCR analysis was carried out as described before. Control, lane 1; GF109203X, lane 2; PMA, lane 3; PMA+GF109203X, lane 4; scabronine G-ME, lane 5; and scabronine G-ME + GF109203X, lane 6. Data are representative of three separate experiments.

Involvement of PKC Activation in the Action of Scabronine G-ME. To investigate the mechanism of action of scabronine G-ME, phosphoinositide hydrolysis was examined by monitoring inositol phosphates (Fig. 6). Scabronine G-ME (100 μM) and PMA (100 nM) did not affect the accumulation of inositol phosphates by themselves. However, scabronine G-ME and PMA inhibited the carbachol (100 μM)-induced accumulation of inositol phosphates in a concentration-dependent manner (Fig. 6A). The inhibitory effects were recovered by pretreatment with GF109203X (10 μM), a specific inhibitor of PKC isoforms (Fig. 6B).

Because a possibility arose that scabronine G-ME stimulated PKC, we examined whether an activation of PKC was involved in the expressions of NGF and IL-6 induced by scabronine G-ME. GF109203X (1 μM) was added 15 min before an addition of scabronine G-ME (100 μM), and the cells were incubated for 4 h. The mRNA expressions of NGF and IL-6 were analyzed by RT-PCR method. GF109203X inhibited the mRNA expressions of both NGF and IL-6 induced by scabronine G-ME and PMA (Fig. 7), indicating that the mRNA expressions of NGF and IL-6 by scabronine G-ME were mediated by the activation of PKC. In addition, we also determined the involvement of PKC in the secretion of neurotrophic factors, which promoted the differentiation of PC-12 cells, using GF109203X and Gö6976 (Fig. 8). As expected, the medium of 1321N1 cells conditioned with scabronine G-ME (50 μM) induced the remarkable neurite outgrowth in PC-12 cells, and this effect was diminished by GF109203X (10 μM). The effect of PMA (100 nM) was also significantly inhibited by GF109203X (10 μM) (Fig. 8B). On the other hand, Gö6976 (1 μM), a specific inhibitor of conventional PKCs, did not affect on the scabronine G-ME (50 μM)-induced secretion of neurotrophic factors, whereas it clearly inhibited PMA-induced one (Fig. 8C). These results indicated that the effect of scabronine G-ME was mediated through the activation of PKC isoforms except the conventional one in 1321N1 cells.

To establish the involvement of PKC in the action of scabronine G-ME, we determined PKC activity using a synthetic peptide of epidermal growth factor receptor (amino acids 651–658) as an exogenous substrate for PKC (Fig. 9A). PMA (100 nM) increased PKC activity linearly up to 30 min, whereas scabronine G and scabronine G-ME (100 μM) did not (Fig. 9A). It has been reported that this substrate is not phosphorylated by human PKC-ζ (Kochs et al., 1993). Therefore, the PKC translocation was investigated by Western blotting to examine the participation of an atypical PKC, PKC-ζ (Fig. 9B). Scabronine G and scabronine G-ME (100 μM) did not change PKC-α localization, whereas PMA (100 nM) translocated it to membrane fraction from cytosolic fraction. Interestingly, the translocation of PKC-ζ was observed in response to scabronine G-ME (100 μM) as well as PMA (100 nM). Anti-PKC-ζ antibody detected the doublet bands in our experiments. According to the report of Allen et al. (1994), the upper band was assumed to be PKC-α, consistent with the present result of its molecular weight and the responsiveness to PMA. To examine whether scabronine G-ME activates PKC-ζ directly, PKC-ζ translocation to membranes

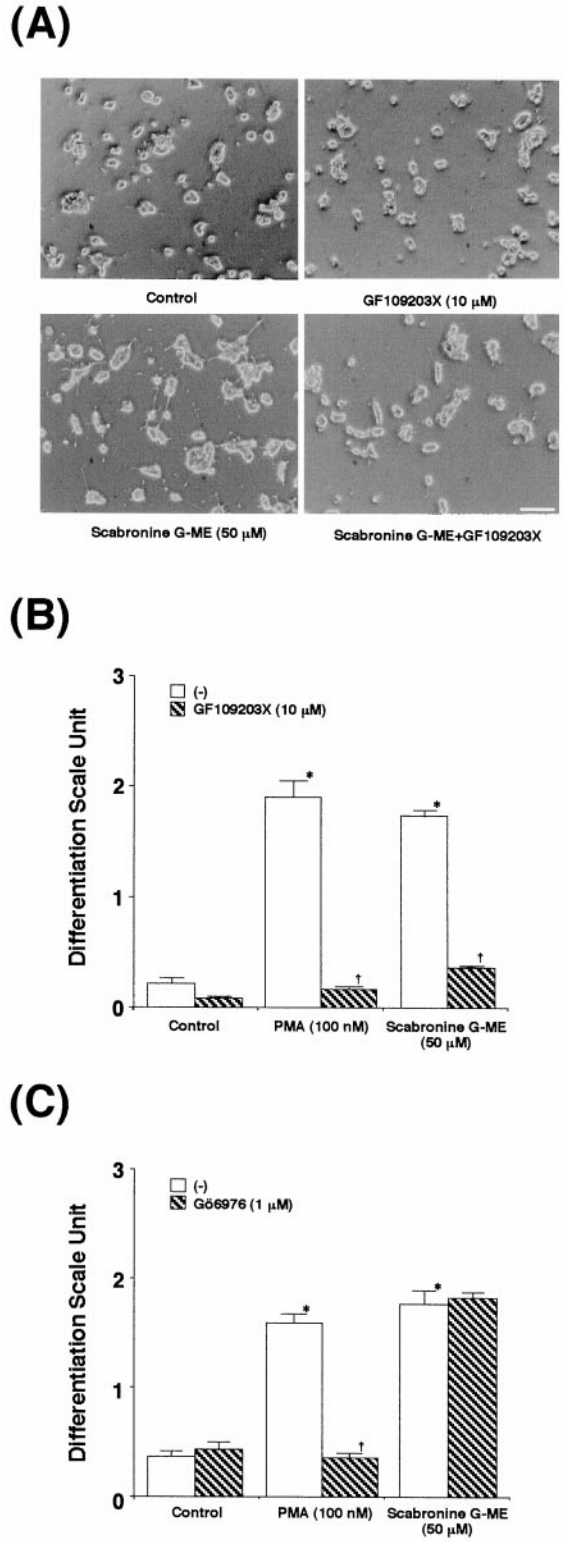

Fig. 8. Inhibitory effects of GF109203X and Gö6976 on the scabronine-induced differentiation of PC-12 cells mediated through the secretion of neurotrophic factors from 1321N1 cells. A, inhibitory effect of GF109203X on the morphological changes of PC-12 cells. 1321 N1 cells (5×10^5 cells/ml) were cultivated for 2 days in DMEM supplemented with 5% fetal calf serum in the presence of scabronine G-ME (50 μM) after the preincubation with GF109203X (10 μM) for 15 min. Then, PC-12 cells were cultivated in the conditioned medium for 24 h. Scale bar, 50 μm. B, effect of GF109203X on the neurotrophic factor secretion from 1321N1 cells determined by the differentiation of PC-12 cells. The differentiation of PC-12 cells was evaluated as described under *Experimental Procedures*. Values represent the means ± S.E.M. for three wells. PMA (100 nM) and scabronine G-ME (50 μM) significantly accelerated differentiation as compared with control (*$P < 0.05$). GF109203X (10 μM) significantly inhibited effects of PMA and scabronine G-ME (†$P < 0.05$). C, effect of Gö6976 on the neurotrophic factor secretion from 1321N1 cells determined by the differentiation of PC-12 cells. The differentiation of PC-12 cells was evaluated as described above, except that Gö6976 was used as a specific inhibitor of conventional PKC instead of GF109203X. PMA (100 nM) and scabronine G-ME (50 μM) significantly accelerated differentiation compared with control (*$P < 0.05$). Gö6976 (1 μM) did not cause the inhibitory effect on scabronine G-ME-induced neurotrophic factor secretion, whereas it significantly inhibited the effect of PMA (†$P < 0.05$).

by scabronine G-ME was investigated in cell-free condition. 1321N1 cells were homogenized before the drug treatment, and then scabronine G-ME (10 and 100 μM) or PMA (100 nM) was added to the homogenates (Fig. 10A). PMA caused PKC-α translocation whereas scabronine G-ME did not. Conversely, scabronine G-ME even at a concentration of 10 μM still translocated PKC-ζ to membranes, whereas PMA did not. In this condition, PMA-induced translocation of PKC-ζ as observed in intact cells disappeared. In addition, PKC-ζ activity in cell-free condition was examined using human recombinant PKC-ζ to confirm the interaction between scabronines and PKC-ζ. Scabronine G-ME (10 and 100 μM) significantly increased the kinase activity, whereas PMA did not (Fig. 10B). These results strongly suggested that scabronine G-ME directly activated PKC-ζ.

Involvement of NF-κB Activation at the Downstream of PKC-ζ Activated by Scabronine G-ME. 1321N1 cells were stimulated with scabronine G-ME (100 μM) for 60 min, and NF-κB translocation to nucleus was examined by immunostaining. NF-κB translocated to nucleus in response to scabronine G-ME (100 μM) (Fig. 11A). In addition, the transcriptional activity of NF-κB was measured by the reporter assay. Scabronine G-ME (100 μM) enhanced the synthesis and secretion of alkaline phosphatase from 1321N1 cells transfected with reporter gene (Fig. 11B). Furthermore, I-κBα degradation accompanied by the activation of NF-κB was investigated by immunoblotting. The I-κBα degradation by scabronine G-ME was observed from 5 min up to 15 min, and then I-κBα contents started to increase up to 60 min by de novo synthesis (data not shown). Scabronine G-ME caused I-κBα degradation in a concentration-dependent manner (Fig. 12). The I-κBα degradation by scabronine G-ME (100 μM) was concentration-dependently inhibited by pretreatment of GF109203X (Fig. 13). These results indicate that NF-κB is activated by scabronine G-ME through PKC-ζ.

Discussion

In the present study, we synthesized a new scabronine derivative to examine the mechanism of its action and clearly demonstrated that the methylester form of scabronine G caused the enhancement of neurotrophic factor secretion from 1321N1 cells with a greater potency than the nonmethylester form. PC-12 cells were differentiated by the culture medium of 1321N1 cells conditioned by scabronine G-ME with a similar degree to NGF. The increased potency of methyl esterification of scabronine was supposed to be due to the decrease of its hydrophilicity, because hydrophobic compounds are easily permeable through plasma membranes.

We previously showed that scabronine G promoted the differentiation of PC-12 cells through the enhanced secretion

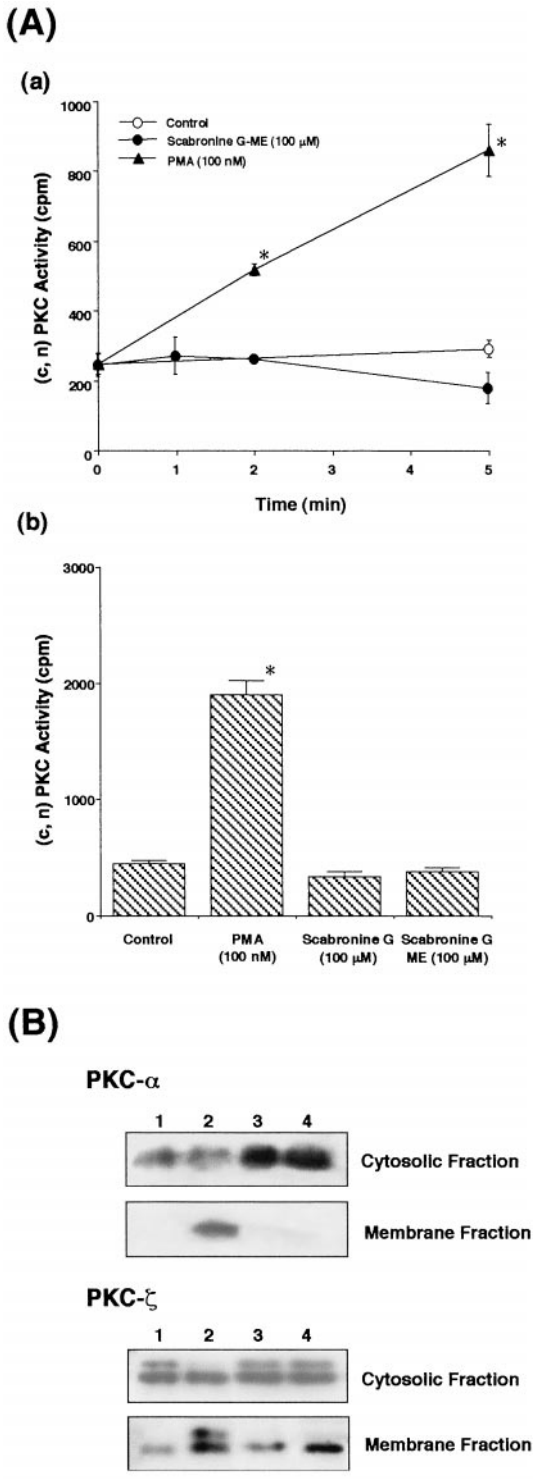

Fig. 9. Effect of scabronines on the activation of atypical PKC. A, effects of scabronines on the activity of conventional and novel PKCs in 1321N1 cells. 1321N1 cell homogenates were used as sample for PKC kinase assay as described under *Experimental Procedures*. a, time course within 5 min. Control (○), Scabronine G-ME (100 μM) (●), PMA (100 nM) (▲). b, 1321N1 cells were incubated with PMA (100 nM), scabronine G (100 μM), or scabronine G-ME (100 μM) for 30 min. Values are the means ± S.E.M. of three determinations. Significant difference from control ($*P < 0.05$). B, effect of scabronines on the translocation of PKC-α and -ζ. 1321N1 cells were incubated with drugs for 30 min, and the cell homogenates were separated to cytosolic and membrane fractions. PKC-α and -ζ in both fractions were investigated by Western blotting as described under *Experimental Procedures*. Control, lane 1; PMA (100 nM), lane 2; scabronine G (100 μM), lane 3; and scabronine G-ME (100 μM), lane 4.

of neurotrophic factors, including NGF from 1321N1 cells (Obara et al., 1999a). In the study, a NGF neutralizing antibody only partially (less than 10%) inhibited the differentiation of PC-12 cells by the culture medium conditioned with scabronine G. The neutralizing antibody also inhibited scabronine G-ME-induced differentiation of PC-12 cells by 32% (Y. Obara and N. Nakahata, unpublished observations). Therefore, NGF secreted by scabronine G-ME was not enough to induce the differentiation of PC-12 cells. Thus, it is expected that scabronines also cause the enhancement of secretion of neurotrophic factors other than NGF from 1321N1 cells. The present study demonstrated that one of other neurotrophic factors secreted by scabronine G-ME was IL-6. Because IL-6 is known to cause the differentiation of

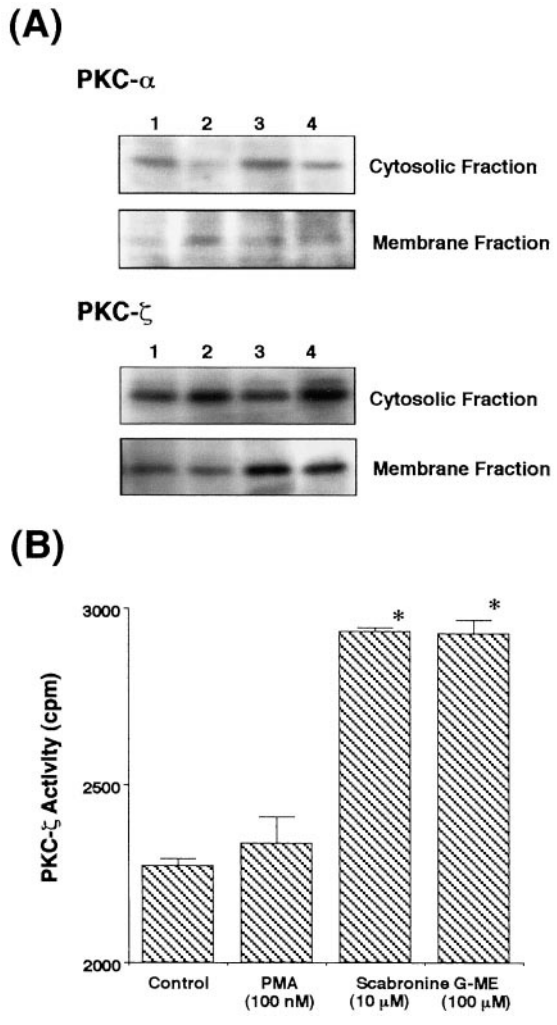

Fig. 10. Effect of scabronine G-ME on the direct activation of PKC-ζ. A, effect of scabronine G-ME on the translocation of PKC-α and -ζ in cell-free condition. Scabronine G-ME (10 and 100 μM) or PMA (100 nM) were added to 1321N1 cell homogenate in 250 μl of homogenization buffer and incubated for 37°C for 30 min. Then, the homogenate was separated to cytosolic fraction and membrane fraction. PKC-α and -ζ in both fractions were determined by Western blotting as described under *Experimental Procedures*. Control, lane 1; PMA (100 nM), lane 2; scabronine G-ME (10 μM), lane 3; and scabronine G-ME (100 μM), lane 4. B, effect of Scabronine G-ME on the PKC-ζ activity in an enzymatic assay. The human recombinant PKC-ζ was incubated with PMA (100 nM) or scabronine G-ME (10 and 100 μM) for 10 min at room temperature, and then PKC-ζ activity was determined as described under *Experimental Procedures*. Values are the means ± S.E.M. of three to four determinations. Significant difference from control ($*P < 0.05$).

PC-12 cells (Satoh et al., 1988), NGF and IL-6 secreted from 1321N1 cells by scabronine G-ME may cause the neuronal differentiation cooperatively.

1321N1 cells expressed at least PKC-α, -δ, -ε, and -ζ, but not PKC-β or -γ (Trilivas et al., 1991; Y. Obara and N. Nakahata, unpublished observations). In the present study, we showed that scabronine G-ME caused the enhancement of mRNA expressions for neurotrophic factors, the differentiation of PC-12 cells by consequent neurotrophic factor secretion, and the inhibition of phosphatidylinositol turnover, all of which were inhibited by GF109203X, a specific inhibitor of PKC isoforms (Figs. 6B, 7, and 8B). Although scabronine G-ME neither activated conventional nor novel types of PKC, it could activate PKC-ζ, an atypical PKC, based on the results of 1) scabronine G-ME translocated PKC-ζ to membranes, and 2) scabronine G-ME caused PKC-ζ-catalyzed phosphorylation of the specific peptide in vitro. In the experiment of

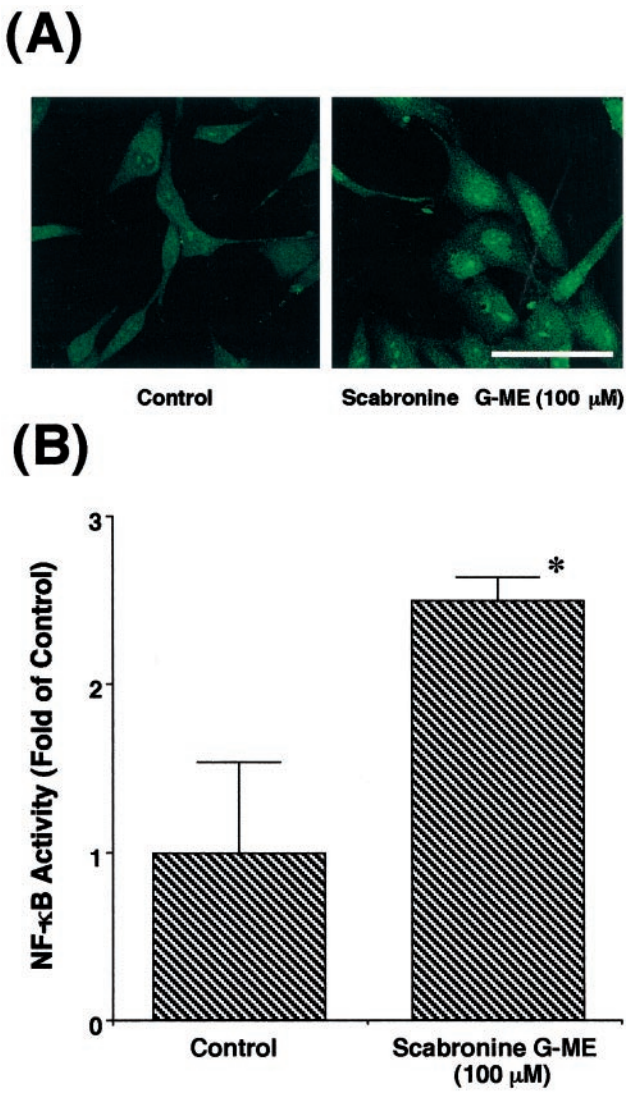

Fig. 11. Effect of scabronine G-ME on the activation of NF-κB in 1321N1 cells. A, effect of scabronine G-ME on the translocation of NF-κB to nucleus in 1321N1 cells. 1321N1 cells were incubated with scabronine G-ME (100 μM) for 60 min and immunostained with anti-NF-κB antibody as described under *Experimental Procedures*. Scale bar, 100 μm. B, effect of scabronine G-ME on the transcriptional activity of NF-κB in 1321N1 cells. The pNF-κB-SEAP reporter plasmid was transfected into 1321N1 cells as described under *Experimental Procedures*. 1321N1 cells were incubated with scabronine G-ME (100 μM) for 6 h. The collected medium containing newly secreted alkaline phosphatase was incubated with *p*-nitrophenyl phosphate, and then the enzyme activity is regarded as the NF-κB activity. Values are the means ± S.E.M. of four determinations. Significant difference from corresponding to control (*$P < 0.05$).

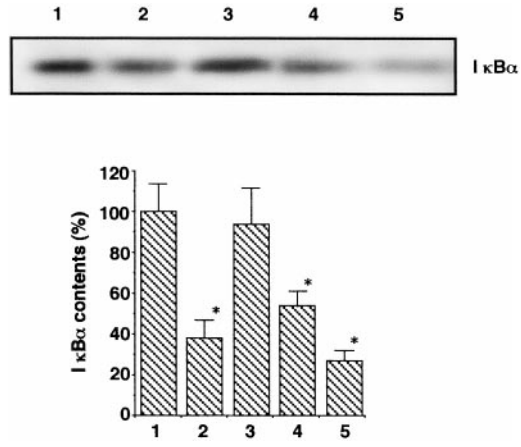

Fig. 12. Concentration-dependent effect of scabronine G-ME on I-κBα degradation in 1321N1 cells. 1321N1 cells were incubated with PMA (100 nM) or scabronine G (10–100 μM) for 5 min, and the I-κBα was detected by Western blotting as described under *Experimental Procedures*. Control, lane 1; PMA (100 nM), lane 2; scabronine G-ME (10 μM), lane 3; scabronine G-ME (30 μM), lane 4; and scabronine G-ME (100 μM), lane 5. Data are representative of three experiments. The density of the bands corresponding to I-κBα was analyzed by densitometry after normalization to protein content to correct for loading. Values are the means ± S.E.M. of three determinations. Scabronine G-ME and PMA significantly caused the degradation of I-κBα (*$P < 0.05$).

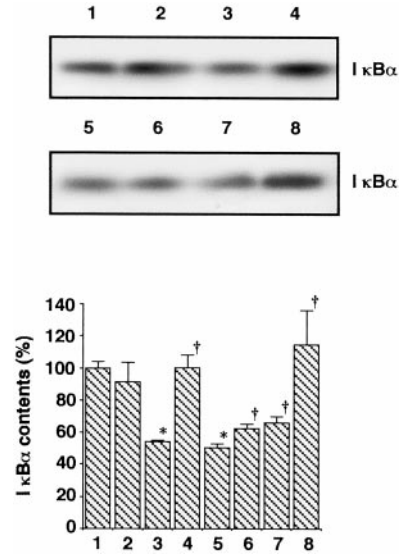

Fig. 13. Inhibitory effect of GF109203X on scabronine G-ME-induced I-κBα degradation in 1321N1 cells. The cells were preincubated with GF109203X (0.1–10 μM) for 15 min, and then they were incubated with PMA (100 nM) or scabronine G-ME (100 μM). Control, lane 1; GF109203X (10 μM), lane 2; PMA (100 nM), lane 3; PMA (100 nM) + GF109293X (10 μM), lane 4; scabronine G-ME (100 μM), lane 5; scabronine G-ME (100 μM) + GF109203X (0.1 μM), lane 6; scabronine G-ME (100 μM) + GF109203X (1 μM), lane 7; and scabronine G-ME (100 μM) + GF109203X (10 μM), lane 8. The I-κBα was detected by Western blotting as described under *Experimental Procedures*. Data are representative of three experiments. The density of the bands corresponding to I-κBα was analyzed by densitometry after normalization to protein content to correct for loading. Values are the means ± S.E.M. of three determinations. Scabronine G-ME and PMA significantly caused the degradation of I-κBα (*$P < 0.05$), and GF109203X significantly inhibited these effects of scabronine G-ME and PMA (†$P < 0.05$).

PKC-ζ translocation, scabronine G-ME translocated PKC-ζ clearly without detectable loss of PKC-ζ in cytosolic fraction. Thus, it seems likely that a small part of PKC-ζ in cytosol is translocated to membranes in response to scabronine G-ME, and the translocated PKC-ζ in membranes is enough to activate the following signal pathway. As described before, atypical PKCs are not generally activated by either diacylglycerol or calcium. Nevertheless, the present study demonstrated that PKC-ζ was also translocated by PMA in intact cells (Fig. 9B). The insensitivity of PKC-ζ to PMA is generally believed to be due to the existence of only one cysteine-rich, zinc-finger-like motif in its C_1 domain, and the loss of one zinc finger decreased binding of phorbol-12,13-dibutyrate by approximately 10- to 20-fold (Zhou et al., 1994). Nakanishi and Exton (1992) suggested that PKC-ζ was specifically activated by phosphatidic acid. It is known that PMA is an activator of phospholipase D, which generates phosphatidic acid in 1321N1 cells (Mizuno et al., 1995). Therefore, the interpretation of the PMA-induced activation of PKC-ζ in the present study is that PKC-ζ is activated by phosphatidic acid generated by phospholipase D, which is activated by PMA-sensitive PKC isoforms. This idea is consistent with the results that PMA did not translocate PKC-ζ to membranes in cell-free conditions (Fig. 10A) and that PMA did not increase PKC-ζ activity directly in an enzymatic assay (Fig. 10B).

With respect to contribution of atypical PKCs to NGF synthesis, a few studies have been provided. It has been shown that phosphatidylcholine-specific phospholipase C (PC-PLC) mediates the NGF synthesis in cultured glial cells (Laviada et al., 1995). The activation of PC-PLC results in ceramide generation mediated by the activation of acidic sphingomyelinase (Schutze et al., 1992). Galve-Roperh et al. (1996) showed that treatment of C6 glioma cells with PC-PLC led to translocation and phosphorylation of PKC-ζ, which was one of the molecular targets of ceramide (Lozano et al., 1994). These reports indicate that PKC-ζ in addition to conventional or novel PKCs contributes to the promotion of NGF synthesis in glial cells, although the signaling pathway in NGF production through atypical PKCs remains unclear.

To date, phorbol esters have been used as a PKC activator. Recently, some PKC isoform-selective natural products have been reported (Way et al., 2000). For example, thymeleatoxin stimulates specifically PKC-α, -βI, and -γ. Sapintoxin D, mezerein, indolactam V, and resiniferatoxin activate PKC-α with a mild selectivity. Bistratene A is known as a PKC-δ-selective activator. However, no natural product has been reported yet that activates atypical PKCs selectively, although intracellular lipid mediators such as phosphatidic acid (Nakanishi and Exton, 1992), phosphatidylinositol 3,4,5-trisphosphate (cooperating with 3-phosphoinositide-dependent protein kinase-1) (Nakanishi and Exton, 1992; Balendran et al., 2000; Rust et al., 2000), and ceramide (Lozano et al., 1994) are known as PKC-ζ activators. In the present study, scabronine G-ME translocated PKC-ζ to membranes directly without affecting conventional or novel type of PKC (Figs. 9 and 10). This observation indicates that scabronines activate atypical PKC selectively, whereas PMA activates conventional and novel PKCs except atypical PKCs. Although further study is necessary to examine how the compounds activate PKC-ζ in detail, scabronines will be the first natural products as a PKC-ζ-selective activator, and can be used as a promising pharmacological tool.

Among the downstream effectors of PKC-ζ, it is suggested that mitogen-activated protein kinase cascade (Berra et al., 1995; Mizukami et al., 2000), AP-1 (Huang et al., 1997, 2000), and NF-κB (Diaz-Meco et al., 1994; Lozano et al., 1994) are associated with NGF synthesis. We examined the involvement of NF-κB as a target of PKC-ζ, because NF-κB plays a key role in the regulation of genes involved in immune and inflammatory responses in brain (O'Neill and Kaltschmidt, 1997). In the present study, we demonstrated that scabronine G-ME potently activated NF-κB via PKC-ζ. The NF-κB activated by PKC-ζ was assumed to be through the phosphorylation of I-κB kinase, which in turn inactivates I-κB (Diaz-Meco et al., 1994; Lozano et al., 1994). It is known that lipopolysaccharide (Heese et al., 1998a) and inflammatory cytokines such as IL-1β and tumor necrosis factor-α (Heese et al., 1998b) caused the expression of NGF mRNA through an activation of NF-κB. Furthermore, a potential NF-κB binding site has been identified in the promoter region of the NGF gene (Jehan et al., 1993). Thus, it is strongly suggested that NF-κB may be involved in scabronine-induced NGF synthesis mediated by PKC-ξ.

The present study demonstrated that the mRNA expression of IL-6 in addition to NGF was also increased by scabronine G-ME. It is known that the AP-1 binding site (12-*O*-tetradecanoylphorbol-13-acetate-responsive element) and NF-κB binding site exist in the upstream of IL-6 gene (Sehgal, 1992). Although it has been shown that IL-6 induces the promotion of NGF mRNA expression in astrocytes (Kossmann et al., 1996), the time course experiment revealed that the IL-6 mRNA expression was delayed from NGF mRNA expression (Fig. 5). Thus, it is suggested that NGF mRNA expression is independent of IL-6 mRNA expression in this cell line. Alternatively, the delayed expression of IL-6 mRNA may be due to other newly expressed gene products, which lead to the expression of IL-6 mRNA.

In conclusion, scabronine G-ME potently promoted the secretion of neurotrophic factors, including NGF and IL-6, mediated via an activation of PKC-ζ. Scabronine G-ME is a promising lead compound of neurotrophic factor inducers with low molecular weight. In addition, the drug is useful to clarify the mechanisms underlying the synthesis and secretion of neurotrophic factors.

References

Allen BG, Andrea JE and Walsh MP (1994) Identification and characterization of protein kinase C ζ-immunoreactive proteins. *J Biol Chem* **269:**29288–29298.

Althaus HH and Richter-Landsberg C (2000) Glial cells as targets and producers of neurotrophins. *Int Rev Cytol* **197:**203–277.

Balasingam V, Tejada-Berges T, Wright E, Bouckova R and Yong VW (1994) Reactive astrogliosis in the neonatal mouse brain and its modulation by cytokines. *J Neurosci* **14:**846–856.

Balendran A, Biondi RM, Cheung PCF, Casamayor A, Deak M and Alessi DR (2000) A 3-phosphoinositide-dependent protein kinase-1 (PDK) docking site is required for the phosphorylation of protein kinase cζ (PKCζ) and PKC-related kinase 2 by PDK1. *J Biol Chem* **275:**20806–20813.

Berra E, Diaz-Meco MT, Lozano J, Frutos S, Municio MM, Sanchez P, Sanz L and Moscat J (1995) Evidence for a role of MEK and MAPK during signal transduction by protein kinase C ζ. *EMBO J* **14:**6157–6163.

Colangelo AM, Pani L and Mocchetti I (1996) Correlation between increased AP-1_{NGF} binding activity and induction of nerve growth factor transcription by multiple signal transduction pathways in C6–2B glioma cells. *Mol Brain Res* **35:**1–10.

Diaz-Meco MT, Dominguez I, Sanz L, Dent P, Lozano J, Municio MM, Berra E, Hay RT, Sturgill TW and Moscat J (1994) ζPKC induces phosphorylation and inactivation of IκB-α in vitro. *EMBO J* **13:**2842–2848.

D'Mello SR and Heinrich G (1991) Structural and functional identification of regulatory regions and cis elements surrounding the nerve growth factor gene promoter. *Mol Brain Res* **11:**255–264.

Fukumoto H, Kakihana M and Suno M (1994) Characterization of C6–10A glioma cells highly responsive to β-adrenergic receptor agonist-induced NGF synthesis/secretion. *Glia* **12:**151–160.

Galve-Roperh I, Haro A and Diaz-Laviada I (1997) Induction of nerve growth factor synthesis by sphingomyelinase and ceramide in primary astrocyte cultures. *Mol Brain Res* **52:**90–97.
Galve-Roperh I, Malpartida JM, Haro A and Diaz-Laviada I (1996) Addition of phosphatidylcholine-phospholipase C induces cellular redistribution and phosphorylation of protein kinase C zeta in C 6 glial cells. *Neurosci Lett* **219:**68–70.
Greene LA and Tischler AS (1976) Establishment of noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci USA* **73:**2424–2428.
Heese K, Fiebich BL, Bauer J and Otten U (1998a) NF-kappaB modulates lipopolysaccharide-induced microglial nerve growth factor expression. *Glia* **22:**401–407.
Heese K, Hock C and Otten U (1998b) Inflammatory signals induces neurotrophin expression in human microglial cells. *J Neurochem* **70:**699–707.
Huang C, Li J, Chen N, Ma W, Bowden GT and Dong Z (2000) Inhibition of atypical PKC blocks ultraviolet-induced AP-1 activation by specifically inhibiting ERKs activation. *Mol Carcinog* **27:**65–67.
Huang C, Ma W and Dong Z (1997) Signal transduction through atypical PKCs, but not the EGF receptor, is necessary for UV-induced AP-1 activation in immortal murine cells. *Oncogene* **14:**1945–1954.
Jehan F, Neveu I, Naveilhan P, Brachet P and Wion D (1993) Complex interactions among second messenger pathways, steroid hormones, and protooncogenes of the Fos and Jun families converge in the regulation of the nerve growth factor gene. *J Neurochem* **60:**1843–1853.
Jehan F, Neveu I, Philippe N, Naveihan P, Wion D and Brachet P (1995) Interactions between second messenger pathways influence NGF synthesis in mouse primary astrocytes. *Brain Res* **672:**128–136.
Kita T, Takaya Y, Oshima Y, Ohta T, Aizawa K, Hirano T and Inakuma T (1998) Scabronines B, C, D, E and F, novel diterpenopids showing stimulating activity of nerve growth factor-synthesis, from the mushroom *Sarcodon scabrosus. Tetrahedron* **54:**11877–11886.
Kochs G, Hummel R, Meyer D, Hug H, Marme D and Sarre TF (1993) Activation and substrate specificity of the human protein kinase C alpha and zeta isoenzymes. *Eur J Biochem* **216:**597–606.
Kossmann T, Hans V, Imhof HG, Trentz O and Morganti-Kossmann MC (1996) Interleukin-6 released in human cerebrospinal fluid following traumatic brain injury may trigger nerve growth factor production in astrocytes. *Brain Res* **713:** 143–152.
Laviada ID, Baudet C, Galve-Roperh I, Naveilhan P and Brachet P (1995) Phosphatidylcholine-phospholipase C mediates the induction of nerve growth factor in cultured glial cells. *FEBS Lett* **364:**301–304.
Levi-Montalcini R (1987) The nerve growth factor 35 years later. *Science (Wash DC)* **237:**1154–1162.
Lin LH, Doherty DH, Lile JD, Bektesh S and Collins F (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science (Wash DC)* **260:**1130–1132.
Lozano J, Berra E, Municio MM, Diaz-Meco MT, Dominguez I, Sanz L and Moscat J (1994) Protein kinase C zeta isoform is critical for kappa B-dependent promoter activation by sphingomyelinase. *J Biol Chem* **269:**19200–19202.
Mizukami Y, Kobayashi S, Uberall F, Hellbert K, Kobayashi N and Yoshida K (2000) Nuclear mitogen-activated protein kinase activation by protein kinase czeta during reoxygenation after ischemic hypoxia. *J Biol Chem* **275:**19921–19927.
Mizuno K, Nakahata N and Ohizumi Y (1995) Mastoparan-induced phosphatidylcholin hydrolysis by phospholipase D activation in human astrocytoma cells. *Br J Pharmacol* **116:**2090–2096.
Nakahata N and Harden TK (1987) Regulation of inositol trisphosphate accumulation by muscarinic cholinergic and H_1-histamine receptors on human astrocytoma cells. Differential induction of desensitization by agonists. *Biochem J* **24:**337–344.
Nakahata N, Matsuoka I, Ono T and Nakanishi H (1989) Thromboxane A_2 activates phospholipase C in astrocytoma cells via pertussis toxin-insensitive G-protein. *Eur J Pharmacol* **162:**407–417.
Nakanishi H and Exton JH (1992) Purification and characterization of the zeta isoform of protein kinase C from bovine kidney. *J Biol Chem* **267:**16347–16354.
Obara Y, Nakahata N, Kita T, Takaya Y, Kobayashi H, Hosoi S, Kiuchi F, Ohta T, Oshima Y and Ohizumi Y (1999a) Stimulation of neurotrophic factor secretion from 1321N1 human astrocytoma cells by novel diterpenoids, scabronines A and G. *Eur J Pharmacol* **370:**79–84.
Obara Y, Nakahata N and Ohizumi Y (1998) A new factor derived from 1321N1 human astrocytoma cells causes the differentiation of PC-12 cells mediated through mitogen-activated protein kinase cascade. *Brain Res* **806:**79–88.
Obara Y, Takahashi M, Nakahata N and Ohizumi Y (1999b) Maitotoxin-induced nerve growth factor production accompanied by the activation of a voltage-insensitive Ca^{2+} channel in C6-BU-1 glioma cells. *Br J Pharmacol* **127:**1577–1582.
Ohta T, Kita T, Kobayashi N, Obara Y, Nakahata N, Ohizumi Y, Takaya Y and Oshima Y (1998) Scabronine A, a novel diterpenoid having a potent inductive activity of nerve growth factor synthesis isolated from the mushroom *Sarcodon scabrosus. Tetrahedron Lett* **39:**6229–6232.
O'Neill LAJ and Kaltschmidt K (1997) NF-κB: a crucial transcription factor for glial and neuronal cell function. *Trends Neurosci* **20:**252–258
Rust C, Karnitz LM, Paya CV, Moscat J, Simari RD and Gores GJ (2000) The bile acid taurochenodeoxycholate activates a phosphatidylinositol 3-kinase-dependent survival signaling cascade. *J Biol Chem* **275:**20210–20216.
Satoh T, Nakamura S, Taga T, Hirano T, Kishimoto T and Kaziro Y (1988) Induction of neuronal differentiation in PC-12 cells by B-cell stimulatory factor 2/interleukin 6. *Mol Cell Biol* **8:**3546–3549.
Schutze S, Potthoff K, Machleidt T, Berkovic D, Wiegmann K and Kronke M (1992) TNF activates NF-kappa B by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown. *Cell* **71:**765–776.
Schwaninger M, Neher M, Viegas E, Schneider A and Spranger M (1997) Stimulation of interleukin-6 secretion and gene transcription in primary astrocytes by adenosine. *J Neurochem* **69:**1145–1150.
Sehgal PB (1992) Regulation of IL6 gene expression. *Res Immunol* **143:**724–734.
Trilivas I, McDonough PM and Brown JH (1991) Dissociation of protein kinase C redistribution from the phosphorylation of its substrates. *J Biol Chem* **266:**8431–8438.
Way KJ, Chou E and King GL (2000) Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol Sci* **21:**181–187.
Zhou G, Wooten MW and Coleman ES (1994) Regulation of atypical zeta-protein kinase C in cellular signaling. *Exp Cell Res* **214:**1–11.

Send reprint requests to: Norimichi Nakahata, Ph.D, Professor, Department of Cellular Signaling, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan. E-mail: nakahata@mail.pharm.tohoku.ac.jp