N-Acylated Serinol Is a Novel Ceramide Mimic Inducing Apoptosis in Neuroblastoma Cells*

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A novel structural analog of ceramide was synthesized by N-acylation of serinol (2-amino-1,3-propanediol) and studied for its effects on glycolipid biosynthesis and cell differentiation of neuroblastoma cells. Incubation with N-palmitoylated serinol (C16-serinol) increased the concentration of endogenous ceramide by 50–80% and caused apoptosis in rapidly dividing low density cells but not in confluent cells. Cell death was not suppressed by simultaneous incubation with phorbol ester, known to antagonize ceramide-induced apoptosis by activation of protein kinase C (PKC). Purification of potential target proteins of C16-serinol was achieved by affinity chromatography of a protein preparation from rat brain on immobilized C16-serinol. A gel activity assay revealed that the eluate from C16-serinol-Sepharose contained three serine/threonine-specific protein kinases with molecular masses of 50, 70, and 95 kDa. The 70-kDa protein was immunostained on a Western blot using a PKCζ-specific antibody. The purified PKCζ could be activated directly by C16-serinol in an in vitro phosphorylation assay. Induction of apoptosis in neuroblastoma cells was suppressed by inhibition of PKCζ with Go 6983.

Our overall results indicate that apoptosis in neuroblastoma cells induced by C16-serinol was at least partially mediated by activation of PKCζ on condition of ongoing cell division. N-Acylated serinols may thus be useful for induction of apoptosis in mitotic cells and may be of therapeutic potential for treatment of cancer in the nervous system.

Sphingosine and its N-acylated derivative, ceramide, are important lipid second messengers for regulation of cell growth and apoptosis and entry substrates for the generation of phospho- and glycosphingolipids (1–11). In particular, apoptosis is known to be induced by elevation of endogenous ceramide or sphingosine (1–10). Structural analogs of these two compounds are expected to target sphingolipid-binding enzymes specifically, by acting as potential inhibitors or allosteric effectors. Commercially available ceramide analogs are either inhibitors of glycosyltransferase or ceramidase. The contribution of a second β-hydroxymethyl group in C16-serinol was evaluated by comparison with the effects of N-palmitoylthanolamine (C16-EA) on neuroblastoma cells. The activity of the new compounds was analyzed by their ability to affect sphingolipid metabolism and cellular differentiation, in particular apoptosis, and by their potential to bind directly to proteins prepared from neuronal cells or tissues. We will present an experimental approach for analyzing potential binding proteins of ceramide or its analogs by utilizing C16-serinol as an immobilized ligand for affinity chromatography purification of proteins from rat brain tissue.

EXPERIMENTAL PROCEDURES

MATERIALS—Murine neuroblastoma × rat glioma NG108-15 and murine neuroblastoma × rat dorsal root ganglion F-11 cells were kindly provided by Drs. Robert Ledeen (New Jersey School of Medicine, Newark, NJ) and Glyn Dawson (University of Chicago, Chicago, IL), respectively. PC12 (rat adrenal pheochromocytoma, ATCC CRL 1721) cells were purchased from the American Tissue Culture Collection (Rockville, MD). Culture dishes were from Falcon/Becton Dickinson Co. (Franklin Lakes, NJ). Serinol (2-amino-1,3-propanediol), octanoylcholride, palmitoylchlo-ride, and stearylochlide were purchased from Across/Fisher Scientific (Pittsburgh, PA). Ceramide, glycosylceramide, C16-EA, and NOE were from Matreya (Pleasant Gap, PA). Dulbecco’s modified Eagle’s medium was obtained from Life Technologies, Inc. Ceramide standards, phosphatidylcholine, phosphatidylserine, dioctanoylglycerol, N-palmitolysphingosine, β-phorbol 12-myristate 13-acetate (PMA), forskolin, rabbit polyclonal anti-PKCζ antibody, protein A-Sepharose, bovine brain myelin basic protein, and Hoechst dye 33258 were purchased from Sigma. Carrier-free 32P (9,000 Ci/mmol) and [γ-32P]ATP (6,000 Ci/mmol) were from NEN Life Science Products. Udp-[3H]glucoside (286 mCi/mmol) was from ICN Pharmaceuticals (Costa Mesa, CA). A polyclonal rabbit IgG anti-cyclin E antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and goat anti-rabbit IgG-rhodamine conjugate from Jackson ImmunoResearch (West Grove, PA). High performance TLC (HPTLC) plates were from Merck (Darmstadt, Germany). All other chemicals were of analytical grade or higher, and solvents were freshly redistilled before use.

C16-serinol, N-palmitoyl-2-amino-1,3-propanediol; C16-EA, N-palmitoylthanolamine; PMA, β-phorbol 12-myristate 13-acetate; PKC, protein kinase C; HPTLC, high performance thin layer chromatography; PAGE, polyacrylamide gel electrophoresis.

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‡ The abbreviations used are: PDMP, d-threo-1-phenyl-2-decanoylaminom-3-morpholinol-1-propanol; d-erythro-MAPP, d-erythro-2-(N-myristoylaminom)-1-phenyl-1-propanol; NOE, N-oleoylethanolamine; related derivatives are known to inhibit UDP-glucose:ceramide glucosyltransferase (hereafter glucosyltransferase), whereas d-erythro-MAPP and NOE are used for inhibition of ceramidase (12–17). It has been shown that several of these inhibitors may induce apoptosis by mechanisms other than elevation of endogenous ceramide (18). However, a direct binding to distinct proteins involved in regulation of apoptosis has not been demonstrated yet. The rational design of novel ceramide analogs was expected to facilitate the analysis of the structure/function relationship of ceramide analogs and the physiological activity of ceramide itself. Fig. 1 shows the structure of N-palmitoylated serinol (C16-serinol), which is derived from N-acylation of a β-hydroxymamide motif that serves as a common structural element in the sphingoid base and almost all effectors of glucosyltransferase or ceramidase. The contribution of a second β-hydroxymethyl group in C16-serinol was evaluated by comparison with the effects of N-palmitoylthanolamine (C16-EA) on neuroblastoma cells. The activity of the new compounds was analyzed by their ability to affect sphingolipid metabolism and cellular differentiation, in particular apoptosis, and by their potential to bind directly to proteins prepared from neuronal cells or tissues. We will present an experimental approach for analyzing potential binding proteins of ceramide or its analogs by utilizing C16-serinol as an immobilized ligand for affinity chromatography purification of proteins from rat brain tissue.
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Analysis of Lipid Composition and Metabolic Enzymes—NG108-15 and F-11 cells were incubated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a humidified atmosphere at 5% CO₂. Alternatively, cells were incubated for 12–72 h with different concentrations of N-acylated serinol, N-palmitoyl or N-oleoylthanolamine, or PMA as indicated under “Results.” For lipid extraction, protein determination, DNA laddering, or enzyme assays, cells were harvested by scraping and pelleted by centrifugation at 300 × g for 5 min. Lipids were prepared and analyzed by HPTLC as described elsewhere (19). HPTLC for ceramide was developed in CHCl₃/CH₃OH (9:1, v/v) as the running solvent. The yield of C₁₆-serinol was 75% (135 mg). The purity and structure were verified by NMR and mass spectrometry. C₁₆-serinol was found to be soluble at a concentration of 50 mg (549 μmol) of 2-amino-1,3-propanediol in 15 ml of pyridine supplemented with 1.65 mmol (457 μl) of palmitoyl chloride at −30 °C. The reaction mixture was stirred for 2 h at room temperature followed by the addition of 30 ml of CH₃OH. After stirring for another 2 h at room temperature the reaction product was concentrated by evaporation. For selective hydrolysis of the ester group, the concentrate was supplemented with 30 ml of CH₃OH and sodium methoxide (pH 11–12) and stirred for 2 h at room temperature. The mixture was neutralized and concentrated. The reaction product was purified by chromatography on a silica gel column (5 g) with CHCl₃/CH₃OH (5:1, v/v) as the running solvent. The yield of C₁₆-serinol was 75% (135 mg). The purity and structure were verified by NMR and mass spectrometry. C₁₆-serinol was found to be soluble at a concentration of up to 500 μM in aqueous solution. For optimal solubility sodium stearate was added to the solution at 20% of the concentration of C₁₆-serinol. The octanoyl and stearyl derivative of serinol (C₈-, C₁₈-acylated serinol, N-acetylated serinol) were synthesized following the same procedure used for the synthesis of C₁₆-serinol. C₁₆-serinol-Sepharose was synthesized as follows. Octyl Sepharose 4B, fast flow (Amersham Pharmacia Biotech), 2 ml, was washed three times with 10 ml of CH₃OH/0.1 M KCl (1:1, v/v) and then three times with 10 ml of solvent A (CHCl₃/CH₃OH/H₂O, 30:60:8, v/v/v). The Sepharose gel (2 ml) was supplemented with 2 ml of solvent A containing 4 mg of C₁₆-serinol and incubated for 30 min at room temperature. The gel was washed with 10 ml of CH₃OH/phosphate-buffered saline (1:1, v/v) and 10 ml of phosphate-buffered saline before use.

Affinity Purification of Rat Brain Protein on C₁₆-Serinol-Sepharose—2 g of rat brain was solubilized with 10 ml of 50 mM Hepes buffer, pH 7.0, supplemented with 0.5 mM NaCl, 0.5% Triton X-100, 1 mM β-mercaptoethanol, 250 μM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin, pepstatin, aprotinin by five strokes with a Teflon-glass homogenizer at 2,500 rpm. The solubilized protein was first incubated for 1 h at 4 °C and then any insoluble material removed by centrifugation at 60,000 × g for 1 h using a Beckman SW50.1 rotor. The supernatant was diluted 1:30 with Hepes buffer omitting Triton X-100 and centrifuged again for 60 min at 60,000 × g. The resulting supernatant was then incubated with 1.0 ml of C₁₆-serinol-Sepharose and incubated for 2 h (or overnight) at 4 °C. The gel was washed in a column with 100 volumes of Hepes buffer without Triton X-100 and then eluted with 1 ml of buffer or 0.5 mM C₁₆-serinol in buffer for 2 h at 4 °C. The protein in the elution fractions was precipitated and analyzed by SDS-PAGE and immunoblotting or renatured in a polyacrylamide gel and used for a gel activity assay.

Analysis of Protein Kinases and in Vitro Phosphorylation of PKCγ—Protein eluted from C₁₆-serinol-Sepharose was precipitated following the Wessel-Flügge method and the precipitated protein resolubilized by boiling with SDS-sample buffer. An amount of 30 μg of protein affinity-purified from 1 g of rat brain was separated by SDS-PAGE. Renaturation and assay of serine/threonine protein kinases were performed according to the method of Kamishita and Fujisawa (24) as modified by Mangoura and Dawson (27).

The in vitro phosphorylation assay was initiated by adding 10 ng of human recombinant PKCγ to 100 μl of a reaction mixture consisting of 25 mM Tris-HCl, pH 7.5, supplemented with 0.5 mM MgCl₂, 0.5 mM EGTA, 1 mM diethiothreitol, 0.1 mg/ml PKCε substrate peptide, 0.1 mM ATP, and 10 μCi of [γ-³²P]ATP. Alternatively, the reaction mixture was supplemented with 0.1 mg/ml phosphatidyserine for full activation of the enzyme and various concentrations of C₁₆-serinol. After incubation for 15 min at 37 °C, the reaction was stopped by the addition of 10 μl of 5% phosphoric acid and incubated on ice for 5 min. The transferred radioactivity was determined by binding of the substrate peptide to phosphocellulose membranes according to the manufacturer’s procedure for the use of microcentrifuge columns (Pierce).

Analysis of Apoptosis and General Methods—Apoptosis was analyzed by DNA fragmentation, in situ terminal nucleotidyl transferase assay, and staining of condensed chromatin with Hoechst dye 33258 as described elsewhere (9, 18). The degree of cell death was monitored by detection of the number of floating cells and cells stained with 0.4% trypan blue (28). The amount of protein was determined according to a modification of the Folin phenol reagent assay as described elsewhere (29). Protein precipitation was performed according to Wessel and Flügge (30). SDS-PAGE was performed using the Laemmli method (31), and immunoblotting followed the procedure described by Gershoni and Palade (32).

RESULTS

Analysis of Sphingolipid Metabolism upon Incubation with C₁₆-Serinol—Murine neuroblastoma NG108-15 or F-11 cells were incubated with C₁₆-serinol and the sphingolipid composition analyzed by HPTLC. In particular, the levels of ceramide, sphingomyelin, and neutral glycosphingolipids were determined by densitometric analysis and comparison with various amounts of standard lipids. There was no detectable alteration of the glycosphingolipid composition or the level of sphingomyelin upon incubation with 100 μM C₁₆-serinol. As shown in Fig. 2, incubation with C₁₆-serinol, however, elevated the concentration of ceramide in F-11 and NG108-15 cells with 20% confluence (lane 3, NG108-15; lane 7, F-11) by about 50–80%.

Incubation of 100% confluent cells showed no significant alteration of endogenous ceramide (lane 4, NG108-15; lane 8, F-11).

C₁₆-serinol was studied for its potential as an inhibitor or substrate for glucosyltransferase and acid ceramidase as determined with the solubilized enzymes from rat brain using different substrate and analog concentrations. The Kᵦ value for glucosyltransferase determined with ceramide as substrate was found to be 52 μM. The C₁₆-serinol showed almost no
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FIG. 2. Alteration of ceramide levels in neuroblastoma cells upon incubation with C16-serinol. NG108-15 or F-11 cells were incubated with 100 μM C16-serinol overnight and endogenous ceramide analyzed by HPTLC of neutral lipids corresponding to 200 μg of cellular protein applied per lane. The HPTLC plate was developed in CHCl/ HOAc (9:1, v/v) and sphingolipids stained with the cupric acetate/ phosphoric acid reagent. Lanes 1 and 2, control NG108-15 cells without effector incubation, 20% (1) or 100% (2) confluence, respectively; lanes 3 and 4, NG108-15 cells with effector incubation, 20% (3) or 100% (4) confluence, respectively; lanes 5 and 6, control F-11 cells without effector incubation, 20% (5) or 100% (6) confluence, respectively; lanes 7 and 8, F-11 cells with effector incubation, 20% (7) or 100% (8) confluence, respectively; lane 9, standard non-hydroxyceramide; lane 10, standard ganglioside mixture.

inhibition of the enzyme; however, it was accepted as a substrate for the glucosylation reaction with a $K_m$ value of 0.8 mM. The synthesis of glucosylated C16-serinol was analyzed by HPTLC of the radiolabeled product and proceeded with a 10 times lower rate than that of glucosylceramide synthesis. A potential inhibition of ceramidase from rat brain lysosomes was analyzed with N-palmitoyl sphingosine at 150 μM corresponding to the $K_m$ value of the enzyme as described in literature (26). It was found that 500 μM C16-serinol inhibited the enzyme from rat brain by 50%, indicating that it is only a moderate inhibitor of ceramidase.

Analysis of Cell Growth and Apoptosis—The effect of incubation of neuroblastoma cells with N-acylated serinols on cell growth and development was evaluated by determination of cell number and apoptosis. Treatment of subconfluent F-11 cells with 100 μM C16-serinol resulted in the cell death of the entire culture within 25–30 h of incubation. Cell death was concomitant with clear morphological indications of apoptosis, e.g. blebbing of the plasma membrane. In situ detection of apoptosis using an assay system with terminal nucleotidyl transferase revealed that about 50–60% of the NG108-15 or F-11 cells were apoptotic if cells were up to 50% confluent. Fig. 3 shows the dependence of the number of apoptotic cells on the density of the cells incubated with C16-serinol. It can be seen that the degree of apoptosis was decreased markedly when cell density was below 20% or above 50% confluence. This was confirmed by determination of cell density-dependent DNA fragmentation (laddering) upon incubation with 100 μM C16-serinol for 15 h. As shown in Fig. 4, the typical laddering of 200-base pair fragments was increased markedly for 30% confluent cells (lane 2, NG108-15; lane 4, F-11) compared with cells with 100% confluence (lane 3, NG108-15; lane 5, F-11). The dependence of apoptosis on the cell cycle was analyzed by detection of cyclin E using a specific antibody for immunofluorescence microscopy. Apoptotic cells stained with Hoechst dye or identified by terminal nucleotidyl transferase assay were concomitantly immunoreactive with anti-cyclin E antibody, indicating an onset of apoptosis at the G1 to S phase transition.

The apoptotic potential of C16-serinol in dependence on cell differentiation was analyzed by incubation of undifferentiated PC12 cells and cells induced to differentiate by preincubation with 10 μM forskolin for 48 h. It was found that only undifferentiated cells showed the typical staining with Hoechst dye 33258 after incubation with C16-serinol for 24 h.

The effect of the alkyl chain length on the apoptotic potential of N-acylated serinols was evaluated by incubation of neuroblastoma cells with C8- and C18-serinol compared with C16-serinol. Furthermore, the contribution of the second β-hydroxymethyl group of C16-serinol was analyzed by comparison with the effect of C16-EA on apoptosis. Fig. 5 shows the dependence of cell death on the concentrations of the various effectors. Cell death was monitored by determination of the number of floating and trypan blue-stained cells and corresponded to the degree of staining of adherent cells with condensed chromatin.

FIG. 3. Cell density-dependent rate of apoptosis of NG108-15 cells on incubation with C16-serinol. NG108-15 cells were grown to the degree of confluence (100% = 1.0 × 10^5 cells/cm^2) as indicated and then incubated overnight with 100 μM C16-serinol. The floating cells were harvested from the medium and the amount of cellular protein determined for calculation of dead cells. In addition, cell death was quantified by staining with trypan blue. The attached cells were stained with Hoechst dye 33258 for calculation of apoptotic cells with condensed chromatin. The number of apoptotic cells was determined by counting 100 stained cells in 20 different areas on tissue culture dishes from three independent experiments.

PC12 cells and cells induced to differentiate by preincubation with 10 μM forskolin for 48 h. It was found that only undifferentiated cells showed the typical staining with Hoechst dye 33258 after incubation with C16-serinol for 24 h.
the palmitoyl residue. C8-serinol was of extremely low apoptotic potential and that of C18-serinol was reduced by about 50% at a concentration of 100 μM. In addition, there was almost no apoptosis observable with C16-EA, indicating the significance of a second β-hydroxymethyl group in C16-serinol.

Signal Pathways Involved in C16-Serinol-induced Apoptosis—The signal pathway by which C16-serinol may have affected ceramide-induced apoptosis was evaluated by use of effectors antagonizing the apoptotic signal cascade for ceramide. Simultaneous incubation with PMA, known to counteract ceramide-induced apoptosis in chicken embryo astrocytes by activation of PKC (27), only partially suppressed the apoptotic effects of C16-serinol (less than 20% reduction of the number of apoptotic cells). The different isoforms of PKC, including the classical PKC (cPKCα) and β as well as the novel isoenzymes (nPKC) were specifically affected by the inhibitors Gö 6983, Gö 6976, and staurosporine. Novel PKC isoforms, in particular PKCζ, have been shown to be modulated by binding to ceramide (28, 34). Its involvement in C16-serinol-induced apoptosis was evaluated by inhibition with Gö 6983 compared with Gö 6976 and staurosporine, two PKC inhibitors that do not affect PKCζ. Only Gö 6983 suppressed C16-serinol-induced apoptosis by about 70%. Inhibition with Gö 6976 and staurosporine resulted in an amplification of apoptosis.

Analysis of Protein Kinases Eluted from C16-Serinol-Sepharose—The binding specificity of C16-serinol was analyzed by chromatography of protein solubilized from rat brain on affinity gels containing the immobilized analog as ligand. As shown in Fig. 6, protein eluted from C16-serinol-Sepharose was characterized by SDS-PAGE and a potential kinase activity determined by a gel activity assay. Elution with buffer only (lane 1) showed only minute amounts of protein, indicating the specificity of the elution with C16-serinol (lane 2). Characterization of the protein was attempted by a gel activity assay for serine/threonine-specific protein kinases. The activity was monitored by phosphorylation of myelin basic protein using [γ-32P]ATP as a substrate. The protein eluted from C16-serinol-Sepharose contained at least three different protein kinases with molecular masses of 50, 70, and 95 kDa (lane 3). The protein kinase species were analyzed further by immunoblotting using a PKCζ-specific antibody for immunodetection. As shown in lane 4, the protein kinase of 70 kDa was immunostained, and the other protein kinase species were not identified yet.

The effect of C16-serinol on the activity of PKCζ was analyzed in vitro using a peptide substrate as phosphate acceptor and by determination of PKCζ autophosphorylation in neuroblastoma cells. The in vitro phosphorylation assay was performed with human recombinant PKCζ optionally activated by the addition of phosphatidylserine to 180% of the activity determined without phosphatidylserine. Incubation of phosphatidylserine-activated PKCζ with 100 μM C16-serinol resulted in a slight reduction of enzyme activity by about 20 ± 5%. The activity of PKCζ without phosphatidylserine activation, however, was activated considerably by C16-serinol to 130 ± 7% at a concentration of 100 μM and 165 ± 10% at 150 μM. Intracellular activation of PKCζ was evaluated by the analysis of autophosphorylation upon incubation of F-11 cells with 100 μM C16-serinol for 8 h. The cells were then metabolically labeled with [32P]P, and after solubilization, PKCζ was isolated by immunoprecipitation and analyzed by SDS-PAGE and autoradiography. In Fig. 6, lanes 5 and 6, it is shown that incubation with C16-serinol enhanced the amount of phosphorylated PKCζ by about 50%, which is consistent with the activation of the enzyme observed in the in vitro phosphorylation assay.

DISCUSSION

The design and synthesis of N-acylated serinol as a new ceramide analogs were based on the rationale to mimic the hydrophilic β-hydroxyamide motif derived from serine and the hydrophobic aliphatic moieties of the ceramide/sphingosine moiety. The effect of the N-acyl chain length was analyzed by introduction of different fatty acid residues ranging from C8 to C18. In particular, C16-serinol was studied for its potential to serve as a substrate or an inhibitor of enzymes involved in ceramide metabolism. An enzyme kinetic characterization, however, revealed that its affinity to glucosyltransferase or lysosomal ceramidase was weak compared with ceramide.

The biological effects observed with different N-acylated derivatives of serinol were evaluated by the analysis of cell growth and apoptosis in neuroblastoma cells. Incubation of NG108-15 or F-11 cells with C16-serinol elevated the concentration of endogenous ceramide to 150–180% of the value found in unaffected control cells and eventually resulted in apoptotic cell death. Ceramide elevation may have been caused by mod-
erate inhibition of acid ceramidase. The effects on other species of ceramidase have not been analyzed yet. However, previous studies have reported a ceramide elevation by more than 2-fold for induction of apoptosis in F-11 cells (8). Most recently, we have found evidence that induction of apoptosis in murine neuroblastoma cells by incubation with PDMP was concomitant with an elevation of endogenous ceramide by 3–4-fold (18). Thus, induction of apoptosis by C16-serinol very likely involved an activity in addition to elevation of endogenous ceramide. A putative mechanism underlying this additional activity of C16-serinol could be given by direct binding of C16-serinol to target proteins that are regulated by ceramidase. This assumption is consistent with the observation that the apoptotic potential of N-acylated serinol was critically dependent on the chain length of the fatty acid residue and was found to be maximal with C16-serinol. Recently, we have reported that the major species of ceramide elevated in apoptotic neuroblastoma cells was C16:0 ceramide (18). In this species the lengths of the alkyl chain of sphingosine and the fatty acid are very similar and may be functionally substituted by a single N-acyl chain as given in C16-serinol for effective binding of ceramide target proteins (Fig. 1). Binding of N-acylated serinol to sphingosine targets cannot be excluded because the structural features necessary for the effectiveness of C16-serinol may have also mimicked those found in sphingosine, which has been reported to induce apoptosis by inhibition of PKC (3, 35). In particular, the observation of a second β-hydroxy group was indispensable for apoptotic activity as demonstrated by the ineffectiveness of C16-EA.

To analyze potential binding proteins C16-serinol was immobilized on a Sepharose gel matrix and used for affinity chromatography of protein solubilized from rat brain. Among the protein species eluted from C16-serinol-Sepharose there were at least three different serine/threonine-specific protein kinases as determined by a gel activity assay. In particular, immunodetection of PKCζ in the C16-serinol-Sepharose eluate indicated that this protein kinase showed considerable affinity to C16-serinol. Recently, it has been found that modulation of PKCζ by binding to ceramide may be one trigger switch for elicitation of apoptosis and other effects of tumor necrosis factor (34, 36). Ceramide can also specifically activate another protein kinase (ceramide-activated protein kinase) or phosphatase (ceramide-activated protein phosphatase) (1, 5, 35–36). It is known that stress factors or cytokines activate a protein kinase cascade (stress-activated protein kinase) via ceramide released by plasma membrane-bound neutral sphingomyelinas

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