Different roles of protein kinase C α and δ isoforms in the regulation of neutral sphingomyelinase activity in HL-60 cells

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The signalling mechanisms responsible for the hydrolysis of sphingomyelin mediated by 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and interferon γ (IFN-γ) in HL-60 cells were investigated. IFN-γ was found to increase selectively the activity of cytosolic, Mg^{2+}-independent, neutral sphingomyelinase. The treatment of HL-60 cells with the combination of 1,25$(OH)_2D_3$ and IFN-γ had an additive effect on sphingomyelin hydrolysis, ceramide release and the activity of cytosolic, Mg^{2+}-independent, neutral sphingomyelinase. The pretreatment of HL-60 cells with staurosporine, chelerythrine chloride and bisindolylmaleimide abolished the activity of sphingomyelinase in response to 1,25$(OH)_2D_3$ and IFN-γ. Calphostin C, which acts on the regulatory site of protein kinase C (PKC), and Gö 6976, a selective inhibitor of Ca^{2+}-dependent PKC isoforms, inhibited the effect of 1,25$(OH)_2D_3$ but had no effect on the IFN-γ-mediated increase in activity of sphingomyelinase. Isoform-specific antibodies were used to deplete different PKC isoforms from cytosol before the treatment of the cytosolic fraction with 1,25$(OH)_2D_3$, arachidonic acid (AA) and PMA. The depletion of PKC isoforms β_1, β_2, ϵ, η, μ, ζ and λ had no effect on the activation of sphingomyelinase induced by 1,25$(OH)_2D_3$ or by AA. The depletion of PKC α from the cytosol completely abolished the effect of 1,25$(OH)_2D_3$ on sphingomyelinase activity but had no effect on the AA-induced activity of sphingomyelinase. PMA had no effect on the activity of sphingomyelinase in either untreated or α-depleted cytosol but significantly increased the activity of sphingomyelinase when added to cytosol depleted of PKC δ. Moreover, PMA inhibited the effect of 1,25$(OH)_2D_3$ on sphingomyelinase activation but the inhibitory effect was abolished by prior depletion of PKC δ from the cytosol. These studies demonstrate that 1,25$(OH)_2D_3$-induced activation of sphingomyelinase is mediated by PKC α. Furthermore, PKC δ had an inhibitory effect on sphingomyelinase, suggesting that the difference between the 1,25$(OH)_2D_3$- and PMA-mediated effects on sphingomyelin turnover depends on the specific regulation of the PKC α and PKC δ isoforms.

Key words: arachidonic acid, 1,25-dihydroxyvitamin D_3, interferon γ, PMA.

INTRODUCTION

In human promyelocytic leukaemia-derived HL-60 cells, the sphingomyelin cycle has emerged as a key pathway mediating the action of multiple inducers of monocytic cell differentiation. 1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α) cause an early and reversible hydrolysis of sphingomyelin, with a parallel increase in ceramide levels owing to the activation of a sphingomyelinase [1,2]. The role of ceramide as a second messenger for the action of agonists on the growth and differentiation of HL-60 cells was demonstrated by the fact that exogenous cell-permeable ceramides mimic the action of agonists on the induction of Nitro Blue Tetrazolium-reducing ability and non-specific esterase levels [2,3]. Moreover, several intracellular targets for ceramide action have been described, including a specific serine/threonine phosphatase [4], ceramide-activated protein kinase [5], protein kinase C (PKC) ζ [6] and protein kinase c-Raf [7]. Although a number of downstream effects modulated by ceramide have been recognized, little is known of the mechanisms coupling receptor occupancy to sphingomyelinase activation. The structure–function analysis of p55 TNF-α receptor revealed the presence of two distinct domains responsible for the activation of neutral and acid sphingomyelinases; a novel protein that binds the neutral sphingomyelinase activation domain has been isolated [8,9]. In HL-60 cells, TNF-α induces the activation of sphingomyelinase through the activation of phospholipase A_2 and the generation of arachidonic acid (AA) [10]. IFN-γ-mediated hydrolysis of sphingomyelin could be prevented by the pretreatment of HL-60 cells with an inhibitor of phospholipase A_2; this inhibitory effect could be overcome by the addition of AA [11]. The mechanism responsible for the 1,25$(OH)_2D_3$-induced activation of sphingomyelinase has not been examined but it is reasonable to assume that mechanisms coupling receptor occupancy of intracellular receptors might differ from those initiated by the activation of transmembrane receptors for TNF-α and IFN-γ.

The biological actions of 1,25$(OH)_2D_3$ in target tissues have classically been attributed to the interaction of this steroid hormone with specific intracellular receptors and the modulation of gene expression [12]. In the past few years, 1,25$(OH)_2D_3$ has been shown to exert genome-independent actions that occur rapidly and independently of RNA and protein synthesis *de novo*. In various cellular systems, 1,25$(OH)_2D_3$ rapidly stimulates the turnover of phosphoinositides and phosphatidylcholine, leading to increases in the level of Ins(1,4,5)P_3 and 1,2-diacylglycerol (DAG) [13,14]. In HL-60 cells, 1,25$(OH)_2D_3$ induces an early increase in PKC isoenzyme transcription, phorbol ester receptor levels and PKC activity [15]. A possible role for PKC in 1,25$(OH)_2D_3$-induced differentiation has been further indicated by the fact that classical PKC inhibitors as well

Abbreviations used: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; AA, arachidonic acid; DAG, 1,2-diacylglycerol; IFN-γ, interferon γ; PKC, protein kinase C; TNF-α, tumour necrosis factor α.

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as antisense oligonucleotides targeted against PKC β and β_2 blocked $1,25(OH)_2D_3$-mediated differentiation [16,17]. Moreover, Slater et al. [18] suggested that PKC itself might act as a membrane-associated $1,25(OH)_2D_3$ receptor because they showed, with the use of a cell-free assay system with purified PKC, that PKC is directly and potently activated by $1,25(OH)_2D_3$ at physiological concentrations in a manner similar to that by DAG. Our previous study practically ruled out the possible involvement of classical PKC isoforms in IFN-γ-mediated signalling because no increase in either DAG or the intracellular Ca^{2+} concentration (physiological activators of PKC) was observed in HL-60 cells treated with IFN-γ [11]. However, atypical PKC isoforms were described as targets for AA [6] and a rapid release of AA was shown to be a key intermediary step in the IFN-γ-mediated hydrolysis of sphingomyelin [11].

In the present study we investigated further the mechanism by which $1,25(OH)_2D_3$ and IFN-γ exert their effects on sphingomyelin hydrolysis. Evidence is provided that the $1,25(OH)_2D_3$-induced activation of cytosolic, Mg^{2+}-independent neutral sphingomyelinase is mediated by PKC α. Furthermore, we showed that signalling mechanisms responsible for activation of the sphingomyelinase mediated by IFN-γ and AA depend on the presence of ATP but kinase activity is not due to the activity of classical, novel or atypical (λ and ζ) PKC isoforms. In addition, PKC isoform δ was found to have an inhibitory effect on sphingomyelinase, suggesting that the difference in $1,25(OH)_2D_3$- and PMA-mediated effects on sphingomyelin turnover depends on the specific regulation of PKC α and PKC δ isoforms.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: IFN-γ, PMA, AA, CHAPS, EGTA, EDTA, Hepes, leupeptin, PMSF, dioleoyl L-α-phosphatidyl-L-serine, diolein, lipid standards, Triton X-100, Protein G–Sepharose, RPMI-1640, fetal bovine serum, penicillin, streptomycin, insulin and transferrin were from Sigma (St. Louis, MO, U.S.A.); Herbimycin A, $1\alpha,25(OH)_2D_3$, staurosporine, bisindolylmaleimide, calphostin C, chelerythrine chloride, Gö 6976 and wortmannin were purchased from Calbiochem (Nottingham, Notts., U.K.); [γ-^{32}P]ATP, [^{3}H]choline chloride, [^{14}C]sphingomyelin and enhanced chemiluminescence kit were from Amersham International (Little Chalfont, Bucks., U.K.); anti-PKC anti-peptide antibodies (against α, δ, and ϵ) and Protein A–agarose were from Gibco BRL (Gaithersberg, MD, U.S.A.); antibodies against PKC β_1, β_2, η, ζ and λ were from Boehringer (Mannheim, Germany) and antibody against PKC μ was from Signal Transduction, Alexis Corporation (Nottingham, Notts., U.K.). All other chemicals were of analytical grade.

Cell culture

HL-60 cells (ECCACC no. 88112501) were obtained from the European Collection of Animal Cell Cultures (PHLS, Porton, Salisbury, U.K.). The cells were maintained in exponential growth in RPMI 1640 medium with 15 % (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified air/CO_2 (19:1) atmosphere at 37 °C. The cells were washed twice with PBS and resuspended in serum-free medium containing insulin (5 mg/l) and transferrin (5 mg/l) before treatment with various compounds. Unless stated otherwise, all incubations were performed with the concentration of the cells adjusted to 0.5×10^6 cells/ml.

Determination of sphingomyelin level

HL-60 cells were incubated with [^{3}H]choline chloride (0.5 μCi/ml; specific radioactivity 80 Ci/mmol) for 48 h in serum-free medium containing insulin (5 mg/l) and transferrin (5 mg/l). After labelling, cells were washed with PBS and resuspended in serum-free medium for 2 h. Cells were then treated as indicated and harvested. The lipids were extracted by method of Folch et al. [19] and dissolved in 50 μl of chloroform. A 20 μl sample was then applied to silica-gel 60 TLC plates and chromatographed with the solvent system chloroform/methanol/acetic acid/water (60:30:8:5, by vol.). Lipids were detected with iodine vapour; the spot corresponding to the sphingomyelin standard was scraped off and its ^{3}H content determined by scintillation counting.

Mass assay of ceramide

Ceramide was extracted from the cells with 1.5 ml of chloroform/methanol (1:2, v/v). Further extraction was performed as described by Folch et al. [19]. Mass measurement for ceramide was performed with DAG kinase, whose purification was achieved in a single step from rat brain with a DEAE-Sepharose column as described by Divecha and Irvine [20]. The mass measurement for ceramide was performed in the following manner. Dried lipid was dissolved by adding 20 μl of CHAPS (9.2 mg/ml) and sonicated at room temperature for 15 s. After the addition of 80 μl of buffer [50 mM Tris/acetate/80 mM KCl/10 mM magnesium acetate/2 mM EGTA (pH 7.4)], the reaction was started by adding 20 μl of DAG kinase followed by 80 μl of buffer containing 20 μM ATP and 1 μCi of [^{32}P]ATP. After incubation for 1 h at room temperature, the reaction was stopped by adding 750 μl of chloroform/methanol/HCl (80:160:1, by vol.). Ceramide phosphate was separated on TLC plates by using the solvent system butanol/acetic acid/water (6:2:2, by vol.) [4]. After autoradiography, the spots corresponding to ceramide phosphate were scraped off and their ^{32}P content was determined by scintillation counting.

Preparation of enzyme source

The harvested cells were homogenized in 0.3 ml of buffer [20 mM Tris/HCl (pH 7.5)/1 mM EDTA] containing 10 mM 2-mercaptoethanol, 1 μg/ml leupeptin and 1 mM PMSF [21] by using a Potter–Elvehjem glass homogenizer, which was then washed with the same amount of buffer. The homogenate was centrifuged at 104000 ***g*** for 60 min in a Beckman L8-M ultracentrifuge; the cytosolic and microsomal fractions were obtained.

Assay method for sphingomyelinases

The enzyme (30–50 μg of cytosolic or microsomal protein) was incubated with 10 nmol of [^{14}C]sphingomyelin (the specific radioactivity was adjusted to 10000 d.p.m./nmol by the addition of unlabelled sphingomyelin)/0.1 M Tris/HCl (pH 7.5)/0.12 % Triton X-100/2 mg/ml bovine albumin in a final volume of 200 μl at 37 °C for 2 h for Mg^{2+}-independent, neutral sphingomyelinase. For Mg^{2+}-dependent, neutral sphingomyelinase, 10 mM $MgCl_2$ was added to the reaction mixture and incubation was performed for 60 min. For acid sphingomyelinase, 0.1 sodium acetate, pH 5.0, was used instead of Tris/HCl [21]. The reaction was stopped by the addition of 6 ml of chloroform/methanol (2:1, v/v); then 1 ml of doubly distilled water was

added to the mixture, vortex-mixed and centrifuged at 1000 ***g*** for 5 min to separate the two phases. The clear aqueous phase was removed to a glass scintillation vial and its ^{14}C content was determined by scintillation counting.

Measurement of PKC-mediated sphingomyelinase activation

The assay was performed in 50 μl of buffer containing 20 mM Tris/HCl, pH 7.4, 120 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, $CaCl_2$ (to give a free Ca^{2+} concentration of 100 nM) and 100 μM ATP, with or without 24 μg of phosphatidylserine and 0.8 μg of diolein; liposomes were made by sonication with a Decon ultrasonic water bath. Cytosolic protein (50–100 μg) was then added and the mixture was incubated for 60 min at 37 °C. The sphingomyelinase assay was then performed as described above; PKC-mediated sphingomyelinase activation was taken as the difference in sphingomyelinase activity between samples with activators (phosphatidylserine and diolein) and without them (control).

Depletion of PKC isoforms from cytosol

Isoform-specific antibodies were used to deplete different PKC isoforms from the cytosol. Cytosolic protein (50 μg) in 50 μl of homogenization buffer was incubated on ice for 4 h with 6 μl of a 1:10 dilution of non-specific rabbit IgG1 or isoform-specific rabbit anti-PKC IgG1 in 0.9 % saline, resulting in a final antibody dilution of 1:500. Protein A–agarose or G-Sepharose (20 μl) was added for 4 h to clear the samples of immunoreactive PKC isoforms and any remaining unbound antibody. After precipitation of this material, supernatant was used for the measurement of PKC-mediated sphingomyelinase activation as described above.

Western blot analysis of PKC isoforms

Proteins for electrophoresis were prepared so that the concentration of each sample was 50 μg per 25 μl of sample loading buffer [22]; PAGE was performed with a Bio-Rad Minigel apparatus at a gel concentration of 10 % (w/v). After electrophoresis, the proteins were transferred to nitrocellulose with a Bio-Rad wet-blotting system. The blot was blocked with buffer containing 4 % (w/v) dried milk (Marvel), 20 mM Tris, 140 mM NaCl and 0.05 % (v/v) Tween 20. It was then probed for 2 h with primary antibody (diluted 1:1000), then washed with blocking buffer and incubated with the secondary antibody conjugated with horseradish peroxidase. Detection was performed with the ECL® kit (Amersham).

Statistical evaluation

Results are shown as means ± S.E.M. For statistical analyses, Student's *t*-test for unpaired samples at a level of significance of 0.05 was used.

RESULTS

Additive effect of the combination of $1,25(OH)_2D_3$ and IFN-γ on sphingomyelin hydrolysis and ceramide release

Both $1,25(OH)_2D_3$ and IFN-γ cause an early sphingomyelin turnover, with a peak response observed at 2 h [1,2,11]. To test the possible additive effect of the two agonists on sphingomyelin hydrolysis, HL-60 cells were labelled with ^{3}[H]choline for 48 h, washed, resuspended and treated with $1,25(OH)_2D_3$ (500 nM), IFN-γ (2000 units/ml), a combination of $1,25(OH)_2D_3$ (500 nM) and IFN-γ (2000 units/ml), or vehicle alone. A similar profile of sphingomyelin hydrolysis was detected in HL-60 cells treated with either $1,25(OH)_2D_3$ or IFN-γ alone: approx. 15 % sphingomyelin hydrolysis was evident by 30 min and a 30 % decrease in sphingomyelin level was detected after 2 h. In HL-60 cells treated with the combination of agents a significant decrease to 82.7 % of control was observed as early as 20 min after the beginning of the treatment, approx. 30 % sphingomyelin hydrolysis was evident by 30 min, and the level of sphingomyelin decreased further to 64.5 ± 4.2 % of control after 2 h (Figure 1, upper panel).

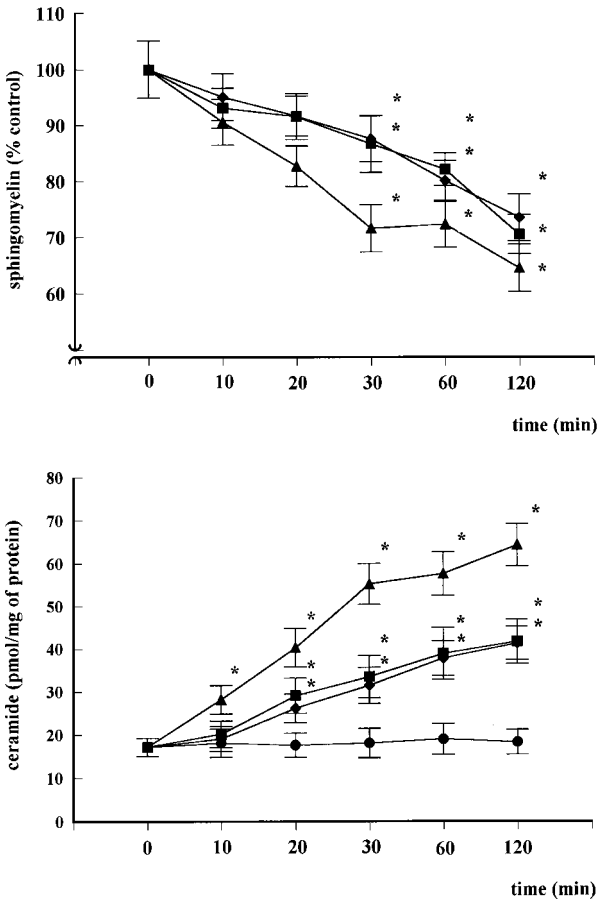

Figure 1 Effects of $1,25(OH)_2D_3$, IFN-γ and a combination of these agents on sphingomyelin (upper panel) and ceramide (lower panel) levels in HL-60 cells

[^{3}H]Choline-labelled or unlabelled cells were treated with either 500 nM $1,25(OH)_2D_3$ (◆), 2000 units/ml IFN-γ (■), a combination of 500 nM $1,25(OH)_2D_3$ and 2000 units/ml IFN-γ (▲), or vehicle alone (●). At the indicated times, cells were harvested, lipids were extracted and sphingomyelin levels were determined by TLC (the 100 % level is 2850 d.p.m. per mg of protein), whereas ceramide levels were determined with a DAG kinase assay as described in the Materials and methods section. Results are means ± S.E.M. for three different experiments, each performed in duplicate. $^*P < 0.05$ (Student's *t*-test) with respect to controls.

The additive effect of the combination of agents was confirmed by mass measurement for ceramide by using the DAG kinase assay. As shown in Figure 1 (lower panel), the time course and extent of the amount of ceramide released in response to $1,25(OH)_2D_3$ (500 nM) and IFN-γ (2000 U/ml) mirrored the observed decrease in sphingomyelin level. The ceramide response to the combination of $1,25(OH)_2D_3$ (500 nM) and IFN-γ (2000 units/ml) resembled that seen with either agent alone but differed in having an earlier onset (20 min compared with 30 min) and a

Table 1 Effects of IFN-γ and 1,25(OH)$_2$D$_3$ on the activity of acid and neutral sphingomyelinases in cytosolic and membrane fractions of HL-60 cells

HL-60 cells were treated for 60 min in the presence of 1,25(OH)$_2$D$_3$ (500 nM) or IFN-γ (2000 units/ml) or of vehicle alone. At the end of the incubation, cytosolic and microsomal fractions were separated, labelled sphingomyelin was added and enzyme activity was assayed as described in the Materials and methods section. The results are means ± S.E.M. for three different experiments, each performed in duplicate. *$P < 0.05$ (Student's *t*-test) with respect to controls.

Treatment		Sphingomyelinase activity (nmol/h per mg)		
		Neutral Mg^{2+}-independent	Neutral Mg^{2+}-dependent	Acid
None	Cytosol	0.073 ± 0.013	0.173 ± 0.029	1.573 ± 0.197
	Membrane	1.431 ± 0.176	1.508 ± 0.179	4.832 ± 0.459
1,25(OH)$_2$D$_3$	Cytosol	0.305 ± 0.076*	0.207 ± 0.034	1.678 ± 0.186
	Membrane	1.571 ± 0.207	1.609 ± 0.207	4.541 ± 0.478
IFN-γ	Cytosol	0.369 ± 0.085*	0.196 ± 0.050	1.702 ± 0.204
	Membrane	1.459 ± 0.194	1.571 ± 0.196	4.941 ± 0.409

significantly greater extent of ceramide release at any time point than that in response to either 1,25(OH)$_2$D$_3$ or IFN-γ alone.

Additive effect of the combination of 1,25(OH)$_2$D$_3$ and IFN-γ on the activity of the cytosolic, Mg^{2+}-independent, neutral sphingomyelinase

To test for the possibility that the additive effect of the combination of 1,25(OH)$_2$D$_3$ and IFN-γ was due to the activation of different sphingomyelinases, we next attempted to characterize the sphingomyelinase species activated by IFN-γ. Neither 1,25(OH)$_2$D$_3$ (500 nM) nor IFN-γ (2000 units/ml) caused any change in the activity of acidic (pH 5.0) sphingomyelinase in either the cytosolic or the particulate fraction (Table 1). The absolute activity of cytosolic, Mg^{2+}-dependent, neutral sphingomyelinase was 2.4-fold that of cytosolic, Mg^{2+}-independent, neutral sphingomyelinase but the activity of the Mg^{2+}-dependent enzyme did not change after treatment with either 1,25(OH)$_2$D$_3$ (500 nM) or IFN-γ (2000 units/ml). In contrast, the activity of

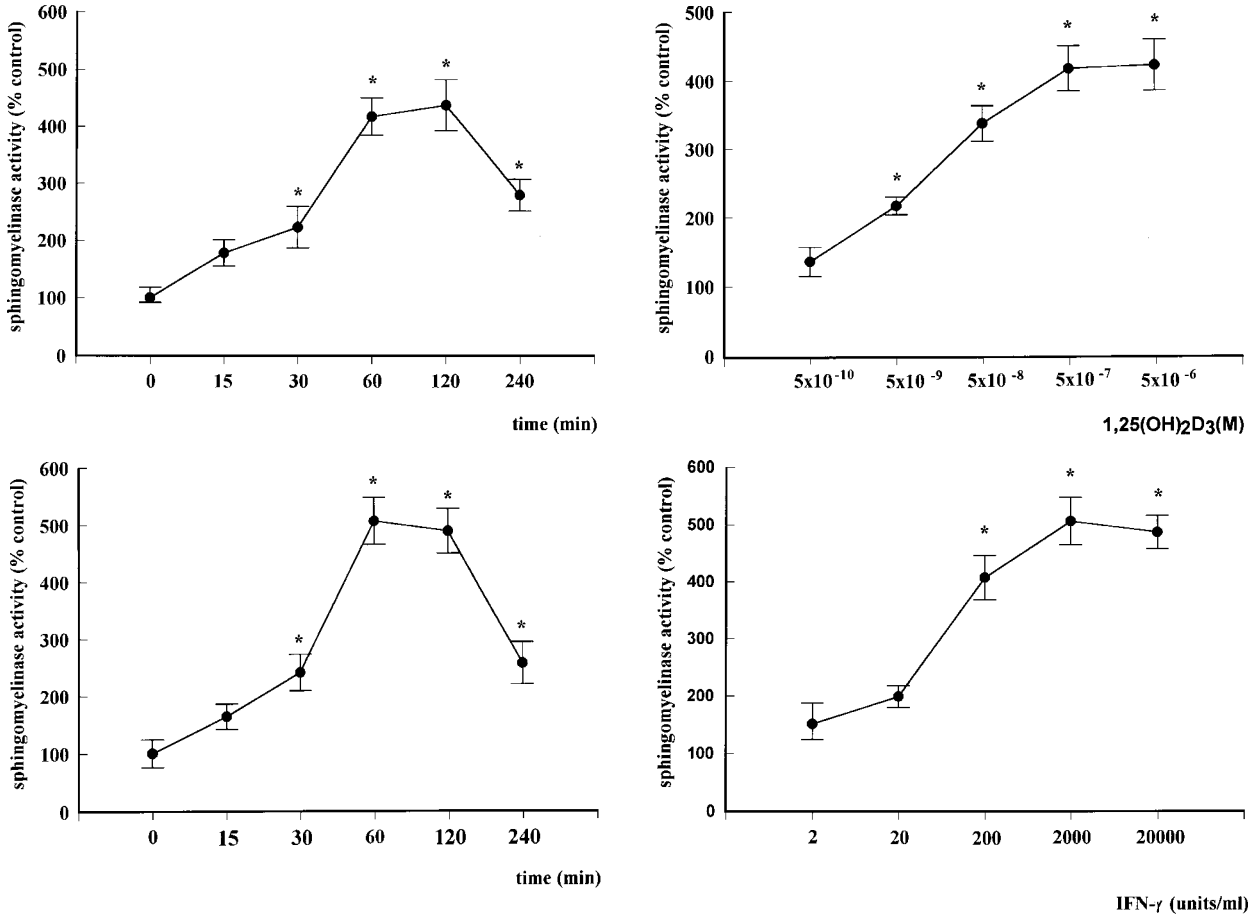

Figure 2 Time course and dose response of the activity of cytosolic, Mg^{2+}-independent neutral sphingomyelinase in HL-60 cells stimulated by 1,25(OH)$_2$D$_3$ (upper panels) and IFN-γ (lower panels)

Cytosolic enzyme activity was assayed after pretreatment of cells with the indicated concentrations of 1,25(OH)$_2$D$_3$ or IFN-γ or with vehicle alone. At the end of the pretreatment period, labelled sphingomyelin was added; enzyme activity was assessed as described in the Materials and methods section. (Upper left panel) Time course of cytosolic sphingomyelinase activity in HL-60 cells treated with 500 nM 1,25(OH)$_2$D$_3$. (Upper right panel) Cytosolic sphingomyelinase activity in HL-60 cells treated with various concentrations of 1,25(OH)$_2$D$_3$ for 60 min. (Lower left panel) Time course of sphingomyelinase activity in HL-60 cells treated with 2000 units/ml IFN-γ. (Lower right panel) Cytosolic sphingomyelinase activity in HL-60 cells treated with various concentrations of IFN-γ for 60 min. Results are expressed as percentages of the cytosolic, Mg^{2+}-independent, neutral sphingomyelinase activity in the control (0.073 nmol/h per mg) and are means ± S.E.M. for three different experiments, each performed in duplicate. *$P < 0.05$ (Student's *t*-test) with respect to controls.

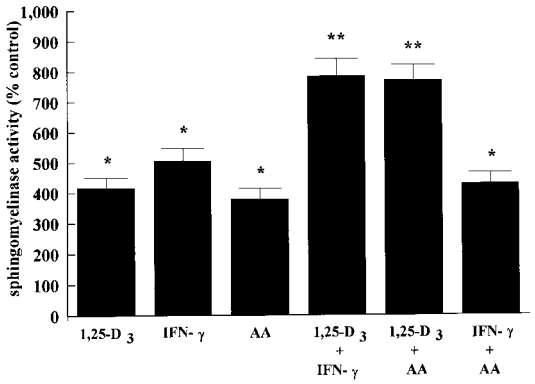

Figure 3 Effects of 1,25(OH)$_2$D$_3$, IFN-γ, AA and a combination of these agents on the activity of cytosolic, Mg^{2+}-independent neutral sphingomyelinase in HL-60 cells

HL-60 cells were treated for 60 min in the presence of 1,25(OH)$_2$D$_3$ (500 nM), IFN-γ (2000 units/ml) or AA (10 μM), or a combination of these agents. At the end of the incubation period, cytosolic fractions were separated, labelled sphingomyelin was added, and enzyme activity was assayed as described in the Materials and methods section. Results are expressed as the percentage of cytosolic, Mg^{2+}-independent, neutral sphingomyelinase activity in the control (0.081 nmol/h per mg) and are means ± S.E.M. for three different experiments, each performed in duplicate. *$P < 0.05$ (Student's *t*-test) with respect to controls; **$P < 0.05$ (Student's *t*-test) with respect to the activity in cells treated with either agent alone.

cytosolic, Mg^{2+}-independent, neutral sphingomyelinase was increased significantly after treatment with 1,25(OH)$_2$D$_3$ (420 % increase) and IFN-γ (500 % increase). Activities of both types of neutral sphingomyelinase in the microsomal fractions did not change after treatment with either 1,25(OH)$_2$D$_3$ or IFN-γ.

As shown in Figure 2, there was a dose-dependent increase in the activity of cytosolic, Mg^{2+}-independent, neutral sphingomyelinase, with maximal activity observed with 2000 units/ml IFN-γ and 500 nM 1,25(OH)$_2$D$_3$. At higher concentrations the activity did not show any further increase. Time-course studies (Figure 2) revealed that both 1,25(OH)$_2$D$_3$ (500 nM) and IFN-γ (2000 units/ml) stimulated the activity of cytosolic, Mg^{2+}-independent neutral sphingomyelinase as early as 15 min after the beginning of the treatment [178 ± 23 % for 1,25(OH)$_2$D$_3$; 164 ± 22 % for IFN-γ]. The peak response was observed at 60–120 min [417 ± 33 % and 437 ± 45 % for 1,25(OH)$_2$D$_3$; 506 ± 41 % and 488 ± 39 % for IFN-γ] and the activity at 3 h was still increased approx. 2.5-fold compared with the control. The time course of sphingomyelinase activation closely paralleled the kinetics of sphingomyelin hydrolysis and ceramide release observed in response to 1,25(OH)$_2$D$_3$ (500 nM) and IFN-γ (2000 units/ml). When HL-60 cells were incubated with the combination of 1,25(OH)$_2$D$_3$ (500 nM) and IFN-γ (2000 units/ml), the activity of neutral Mg^{2+}-independent sphingomyelinase in the cytosolic fraction was increased significantly compared with the level of the enzyme activity in cytosolic fractions of cells treated with either agent alone (Figure 3). The activity of neutral, Mg^{2+}-independent sphingomyelinase in the microsomal fraction, as well as the activities of both neutral, Mg^{2+}-dependent and acid sphingomyelinase in the cytosolic and particulate fractions did not change after the treatment of cells with the combination of agents (results not shown).

AA was found to provide an early biochemical signal in mediating the induction of sphingomyelin hydrolysis in HL-60 by IFN-γ; 10 μM AA mimicked the effect of IFN-γ on sphingomyelin hydrolysis in [^{3}H]choline-labelled HL-60 cells [11]. When HL-60 cells were treated with 10 μM AA for 1 h, the activity of cytosolic, Mg^{2+}-independent, neutral sphingomyelinase increased to a level similar to that observed in response to 2000 units/ml IFN-γ; no further increase in activity was observed when cells were incubated in the presence of both AA (10 μM) and IFN-γ (2000 units/ml). In contrast, the treatment of HL-60 cells with the combination of AA (10 μM) and 1,25(OH)$_2$D$_3$ (500 nM) caused a marked increase in activity compared with that in cytosolic fractions of cells treated with 1,25(OH)$_2$D$_3$ (500 nM) alone. These results suggested that the early biochemical events responsible for mediating the effects of 1,25(OH)$_2$D$_3$ and IFN-γ on sphingomyelinase activity might be different.

Table 2 Effects of different inhibitors on the activity of neutral sphingomyelinase activated by 1,25(OH)$_2$D$_3$ or IFN-γ

HL-60 cells were pretreated for 60 min with cycloheximide (50 μM), chelerythrine chloride (6 μM), bisindolylmaleimide (100 nM) or Gö 6976 (25 nM), for 30 min with calphostin C (500 nM), staurosporine (10 nM) and wortmannin (50 nM), or for 12 h with herbimycin A (1 μg/ml). After the preincubation the cells were treated for 60 min with 1,25(OH)$_2$D$_3$ (500 nM) or IFN-γ (2000 units/ml) or with vehicle alone. At the end of the treatment, cells were harvested, cytosolic fractions were separated, and neutral, Mg^{2+}-independent sphingomyelinase activity was assayed as described in the Materials and methods section. *$P < 0.05$ (Student's *t*-test) with respect to controls.

	Sphingomyelinase activity (% of control)		
Treatment	Inhibitor alone	Inhibitor + 1,25(OH)$_2$D$_3$	Inhibitor + IFN-γ
Cycloheximide	100 ± 24	482 ± 36*	463 ± 36*
Chelerythrine chloride	88 ± 17	103 ± 15	97 ± 21
Bisindolylmaleimide	107 ± 19	94 ± 18	79 ± 28
Calphostin C	89 ± 17	107 ± 14	473 ± 51*
Gö 6976	107 ± 6	96 ± 17	455 ± 36*
Staurosporine	103 ± 14	111 ± 19	93 ± 16
Wortmannin	113 ± 7	467 ± 36*	505 ± 26*
Herbimycin A	106 ± 12	427 ± 36*	483 ± 29*

Role of PKC in the 1,25(OH)$_2$D$_3$- or IFN-γ-induced activation of neutral sphingomyelinase

As shown in Table 2, the pretreatment of HL-60 cells with 50 μM cycloheximide for 60 min had no effect on the activation of cytosolic, Mg^{2+}-independent neutral sphingomyelinase mediated by either 1,25(OH)$_2$D$_3$ or IFN-γ. The 1,25(OH)$_2$D$_3$-mediated activation of neutral sphingomyelinase was completely abolished by pretreatment with different inhibitors of PKC. Whereas the pretreatment of HL-60 cells with staurosporine (10 nM), chelerythrine chloride (6 μM) or bisindolylmaleimide (100 nM) abolished the IFN-γ-mediated activation of sphingomyelinase, pretreatment with calphostin C (500 nM), which acts on the regulatory site of PKC, and Gö 6976 (25 nM), a selective inhibitor of Ca^{2+}-dependent PKC isoforms, had no effect on the IFN-γ-mediated increase in activity of sphingomyelinase. The pretreatment of HL-60 cells with wortmannin (50 nM), an inhibitor of phosphoinositol 3-kinase, had no effect on the increase in the activity of sphingomyelinase mediated by either 1,25(OH)$_2$D$_3$ or IFN-γ. Furthermore, protein tyrosine kinase phosphorylation seemed not to be involved in the signalling pathways leading to sphingomyelinase activation in response to either 1,25(OH)$_2$D$_3$ or IFN-γ because herbimycin A (1 μg/ml), a potent inhibitor of tyrosine kinase, had no effect on the agonist-stimulated increase in the activity of cytosolic sphingomyelinase.

To investigate further a role for PKC in effects on sphingomyelinase mediated by 1,25(OH)$_2$D$_3$ and IFN-γ, a further set of experiments were performed to measure the PKC-mediated

Table 3 Effects of the presence of PKC coactivators on sphingomyelinase activity in cytosolic fraction incubated with $1,25(OH)_2D_3$, IFN-γ, AA or a combination of $1,25(OH)_2D_3$ and AA

HL-60 cells were disrupted, particulate and cytosolic fractions were separated, and cytosolic fractions were treated for 60 min with $1,25(OH)_2D_3$ (500 nM), IFN-γ (2000 U/ml), AA (10 μM) or a combination of $1,25(OH)_2D_3$ (500 nM) and AA (10 μM) in the presence or absence of phosphatidylserine (24 μg) and DAG (0.8 μg). At the end of the incubation period, labelled sphingomyelin was added and the enzyme activity was assayed as described in the Materials and methods section. The results are means ± S.E.M. for three different experiments, each performed in duplicate. *$P < 0.05$ (Student's t-test) with respect to controls; †$P < 0.05$ (Student's t-test) with respect to cytosolic fractions treated with agents alone.

Treatment	Phosphatidylserine + diolein	Sphingomyelinase activity (% of control)		
		100 nM ATP, 100 nM Ca^{2+}	0 nM ATP, 100 nM Ca^{2+}	100 nM ATP, 0 nM Ca^{2+}
IFN-γ	−	106 ± 12	108 ± 6	104 ± 6
	+	111 ± 12	114 ± 7	109 ± 7
AA	−	328 ± 26*	107 ± 11	339 ± 19*
	+	361 ± 19*	107 ± 23	372 ± 29*
$1,25(OH)_2D_3$	−	106 ± 13	111 ± 12	96 ± 7
	+	291 ± 19*	103 ± 4	101 ± 4
$1,25(OH)_2D_3$ + AA	−	315 ± 17*	112 ± 7	351 ± 22*
	+	514 ± 41†	109 ± 9	362 ± 17*

activation of sphingomyelinase. HL-60 cells were disrupted and cytosolic fractions were separated; 50–100 μg of cytosolic proteins was resuspended in buffer containing ATP (0–100 nM) and 0–100 nM Ca^{2+} and then treated with agonists in the presence or absence of 24 μg of phosphatidylserine and 0.8 μg of diolein. As expected, treatment of the cytosolic fraction with 2000 units/ml IFN-γ caused no increase in the level of cytosolic sphingomyelinase activity, in either the presence or the absence of the physiological PKC coactivators ATP and Ca^{2+}. However, when the cytosolic fraction was exposed to 10 μM AA, a mediator of IFN-γ effects, for 60 min, an increase in sphingomyelinase activity was observed similar to that recorded after the treatment of the whole cells. As shown in Table 3, the AA-induced activity of sphingomyelinase did not depend on either the presence of PKC coactivators or the level of cytosolic Ca^{2+} but was abolished in the absence of ATP. In contrast with the lack of effect of IFN-γ, treatment of the cytosolic fraction with $1,25(OH)_2D_3$ (500 nM) resulted in a significant increase in the level of sphingomyelinase activity that was dependent on the presence of PKC coactivators and was abolished by the presence of EGTA. When the cytosolic fraction was treated with the combination of $1,25(OH)_2D_3$ (500 nM) and 10 μM AA, the additive effect was observed only in the presence of the PKC co-activators ATP and Ca^{2+}. In the absence of PKC co-activators and/or Ca^{2+}, the activity of the enzyme after treatment with the combination of agents was similar to that observed in the cytosolic fraction treated with AA alone.

All of these results strongly suggested the involvement of classical PKCs in the $1,25(OH)_2D_3$-mediated activation of sphingomyelinase. To determine the particular PKC isoform involved in the activation of cytosolic sphingomyelinase mediated by $1,25(OH)_2D_3$ and AA, isoform-specific antibodies were used to deplete different PKC isoforms from the cytosol (Figure 4) before treatment of the cytosolic fraction with either $1,25(OH)_2D_3$ or AA. Depletion of the classical PKC isoforms β_1 and β_2, the novel PKC isoforms ϵ, η, μ and the atypical isoforms ζ and λ had no effect on either the $1,25(OH)_2D_3$- or AA-induced activation of cytosolic, Mg^{2+}-independent, neutral sphingomyelinase (results not shown). When cytosol was depleted of classical PKC isoform α, the effect of $1,25(OH)_2D_3$ (500 nM) on sphingomyelinase activity was completely abolished, whereas the stimulatory effect of AA (10 μM) on the activity of sphingomyelinase was preserved (Table 4). PMA, a known activator of classical PKC isoforms,

Figure 4 Western blot analysis of PKC isoform levels in cytosolic fractions after immunodepletion of particular PKC isoforms

Isoform-specific antibodies were used to deplete PKC isoforms from cytosol; the specificity of the depletion was tested by Western blots. Lanes A, cytosolic fraction incubated with non-specific rabbit IgG1 and probed on Western blot with specific anti-PKC antibody; lane B, cytosolic fraction depleted of specific PKC isoform and probed on Western blot with specific anti-PKC antibody.

has an opposite effect on sphingomyelin turnover; at 100 nM it induces an early synthesis of sphingomyelin in HL-60 cells [23]. When PMA (500 nM) was added to the non-depleted cytosolic fraction of HL-60 cells or to the cytosol that had previously been depleted of PKC α, no change in the level of neutral sphingomyelinase activity was detected. However, when cytosolic fraction was depleted of PKC δ and then treated for 60 min with 500 nM PMA, a significant increase in the level of sphingomyelinase was observed. In the absence of PMA, depletion of PKC δ did not change the activity of cytosolic sphingomyelinase in untreated cells and had no influence on the activation of sphingomyelinase mediated by either $1,25(OH)_2D_3$ or AA. To check whether the putative inhibitory effect of PMA-activated PKC δ might influence the $1,25(OH)_2D_3$-mediated activation of sphingomyelinase, cytosolic fractions, depleted or not of PKC α or PKC δ, were stimulated with the combination of $1,25(OH)_2D_3$ (500 nM) and PMA (500 nM). As shown in Table 4, the presence of PMA (500 nM) completely abolished the stimulatory effect of

Table 4 Effects of depletion of various PKC isoforms from cytosol on the activity of neutral sphingomyelinase induced by treatment of the cytosolic fraction with $1,25(OH)_2D_3$ or AA

HL-60 cells were disrupted, cytosolic fractions were separated, and isoform-specific antibodies were used to deplete the α and δ isoforms of PKC from the cytosol as described in the Materials and methods section. After precipitation, the supernatant was incubated with $1,25(OH)_2D_3$ (500 nM), AA (10 μM), PMA (500 nM) or a combination of these agents. At the end of the incubation, PKC-mediated sphingomyelinase activation was measured as described in the Materials and methods section. The results are means ± S.E.M. for three different experiments, each performed in duplicate. *$P < 0.05$ (Student's *t*-test) with respect to controls; †$P < 0.05$ (Student's *t*-test) with respect to cytosolic fractions treated with either agent alone.

	Sphingomyelinase activity (% of control)		
Treatment	Control	Anti-(PKC-α)	Anti-(PKC-δ)
None	100 ± 4	107 ± 12	101 ± 13
$1,25(OH)_2D_3$	376 ± 32*	96 ± 17	326 ± 17*
AA	363 ± 27*	317 ± 32*	369 ± 28*
PMA	103 ± 12	105 ± 18	306 ± 16*
$1,25(OH)_2D_3$ + AA	527 ± 36†	334 ± 21*	517 ± 34†
$1,25(OH)_2D_3$ + PMA	107 ± 14	109 ± 11	351 ± 19*
AA + PMA	362 ± 17*	339 ± 28*	531 ± 21†
$1,25(OH)_2D_3$ + PMA + AA	347 ± 26*	316 ± 31*	528 ± 31*

$1,25(OH)_2D_3$ (500 nM) in both non-depleted cytosol and the cytosolic fraction depleted of PKC α but the inhibitory effect was absent from the cytosolic fraction depleted of PKC δ. Moreover, when the cytosolic fraction depleted of PKC δ was treated with the combination of PMA (500 nM) and AA (10 μM), an additive effect on the level of sphingomyelinase activity was detected, similar to that observed in response to the combination of $1,25(OH)_2D_3$ (500 nM) and AA (10 μM). No further increase in the activity of sphingomyelinase was detected in PKC δ-depleted cytosol treated with the combination of $1,25(OH)_2D_3$ (500 nM), PMA (500 nM) and AA (10 μM).

DISCUSSION

Although the role of the sphingomyelin pathway has been firmly established in response to different agonists, relatively little is known about sphingomyelin-specific forms of phospholipase C, which hydrolyse the phosphodiester bond of sphingomyelin to yield ceramide and phosphocholine [24]. The best-studied isoform is an acid sphingomyelinase that is deficient in some forms of Niemann–Pick disease; the enzyme has been cloned and shown to be activated in cells exposed to radiation [25], Fas [26] and TNF-α [8,27]. A neutral, membrane-associated, Mg^{2+}-dependent sphingomyelinase was shown to be activated by TNF-α [8], Fas [28] and sodium nitroprusside [29]; the sequence of a human enzyme with properties of Mg^{2+}-dependent, neutral sphingomyelinase of the plasma membrane has been published recently [30]. The least is known about the biological relevance of Mg^{2+}-independent, cytosolic, neutral sphingomyelinase, which is specifically activated in response to $1,25(OH)_2D_3$ in HL-60 cells [21]. The results of our study suggest that the Mg^{2+}-independent enzymic activity in the cytosol is not only responsible for the effect of $1,25(OH)_2D_3$ on sphingomyelin turnover but also has a critical role in transducing the effects of IFN-γ in HL-60 cells. Further study is required to explain the additivity of the combination of stimuli that might result from the activities of two distinct pools of sphingomyelinases or distinct isoforms of the same enzyme. Similarly to previous findings [21], the activities of acid sphingomyelinases in control cells were high but were unaffected by the addition of either $1,25(OH)_2D_3$ or IFN-γ. Because the activities of neutral sphingomyelinases in microsomal fractions and the Mg^{2+}-dependent, cytosolic enzyme remained unchanged, these results established the specific role of the cytosolic, cation-independent enzyme in IFN-γ-mediated sphingomyelin hydrolysis and ceramide generation.

Even less is known about the mechanism of activation of sphingomyelinases. A delayed onset of sphingomyelinase activation and sphingomyelin hydrolysis argues against a model of direct interaction of agonist-sensitive sphingomyelinase with activated receptor. The activation of neutral sphingomyelinase in HL-60 cells and promonocytic U937 cells in response to TNF-α is mediated by AA [10], whereas the activation of acidic sphingomyelinase in U937 requires the prior activation of phosphatidylcholine/phospholipase C and the generation of DAG [27]. IFN-γ-induced sphingomyelin hydrolysis has been shown to be mediated by phospholipase A_2 and AA [11]. The present study presents results suggesting the role of different PKC isoforms in the regulation of cytosolic, Mg^{2+}-independent, neutral sphingomyelinase activity: (1) the effect of $1,25(OH)_2D_3$ on the activation of sphingomyelinase was shown to be mediated by PKC-α; (2) the effect of IFN-γ and AA occurred independently of the presence of all the PKC isoforms tested, although the requirement for the presence of ATP as well as the inhibitory effect of less-selective inhibitors of PKC suggested the involvement of some other kinase activity; and (3) experiments with PMA showed the inhibitory effect that PKC-δ exerts on the level of the sphingomyelinase activity.

Several previous studies have provided some evidence that one or more of the individual kinase isoenzymes might have a major role in the differentiation of HL-60 cells in response to $1,25(OH)_2D_3$ and PMA [17,31–34]. Most of the studies indicated that the specific increase in the levels of mRNA for the β isoenzymes was associated with an induction of monocytic marker expression in HL-60 cells treated with either PMA [32] or $1,25(OH)_2D_3$ [17] for several days; however, these effects were clearly distinct from rapid, non-genomic effects of $1,25(OH)_2D_3$ and PMA on the activity of neutral sphingomyelinase. The effect of $1,25(OH)_2D_3$ on the activity of sphingomyelinase was insensitive to cycloheximide, occurred within minutes and was observed when only the cytosolic fraction was exposed to $1,25(OH)_2D_3$, indicating that the effect was not only independent of genomic actions of the steroid hormone but also did not depend on the presence of one or more putative cell-membrane-bound receptors for $1,25(OH)_2D_3$. An interesting study presented the evidence that PKC was directly activated by $1,25(OH)_2D_3$ at physiological concentrations; the effect was demonstrable with single PKC α, γ, and ϵ isoenzymes assayed in a system containing only purified enzyme, substrate, cofactors and lipid vesicles [18]. The putative role of PKC isoforms in mediating rapid, non-genomic effects of $1,25(OH)_2D_3$ on cellular phospholipases has recently been confirmed in other cell systems because PKC α was found to be involved in the stimulation of phospholipase D in Caco-2 cells by $1,25(OH)_2D_3$ [35].

None of the PKC isoforms tested was shown to be important for the induction of sphingomyelinase activity mediated by IFN-γ and AA, although some atypical isoforms such as PKC ζ were described as a downstream target for AA [6]. The activation of sphingomyelinase in the cytosolic fraction depleted of PKC-α, β, δ or ϵ in response to either IFN-γ or AA would be expected from the results of experiments with Gö 6976, a specific inhibitor of PKC α and β_1, and calphostin C, which by acting on the regulatory domain of the enzyme inhibits only isoforms that are dependent on DAG. However, bisindolylmaleimide and chelerythrine chloride, which act on the catalytic domain of PKC, and staurosporine, a broad-spectrum inhibitor of protein

kinases, inhibited the stimulatory effect of IFN-γ and AA on the sphingomyelinase activity, suggesting the involvement of one or more protein kinases. Our immunoprecipitation studies ruled out the possible role of atypical PKC isoforms ζ and λ in mediating the effects of IFN-γ and AA on the sphingomyelinase activity. There is a possibility that some novel, as yet unidentified, isoform of PKC or PKC-related kinase(s) is responsible for the AA-mediated activation of sphingomyelinase.

Several studies have indicated a role for PKC as a negative regulator of the apoptotic effect of ceramide; however, little is known about the role of PKC in the regulation of ceramide production [24]. The rapid translocation and activation of PKC α was observed in HL-60 cells treated with phorbol esters [33], some PMA-resistant HL-60 lines have substantially lower levels of PKC α and PKC δ expression than the PMA-sensitive lines [34], and the role of PMA-responsive PKC α and δ isoforms in the induction of differentiation has been reported in other cell systems [36]. In HL-60 cells, PMA was previously reported to have no effect on basal sphingomyelinase activity but to inhibit daunorubicin-triggered sphingomyelinase activity, ceramide generation and apoptosis [37]. In other cell systems, the cytokine-induced activation of neutral sphingomyelinase was also found to be inhibited by PMA [38]. In the cytosolic fraction of HL-60 cells, PMA itself had no effect on the level of Mg^{2+}-independent, neutral sphingomyelinase, but inhibited the $1,25(OH)_2D_3$-mediated activation of the enzyme, raising the possibility that some of the PMA-activated PKC isoforms might exert an inhibitory effect on the enzyme. In the WEHI-231 cell line, chelerythrine chloride and calphostin C, inhibitors of PKC isoforms α, β, δ and ϵ, triggered neutral sphingomyelinase, suggesting that PKC might regulate basal sphingomyelinase activity [39]. Our immunoprecipitation studies indicated that of all classical and novel isoforms tested, only PKC δ was important for the PMA-mediated inhibition of sphingomyelinase activity. Although the depletion of PKC δ itself had no effect on basal sphingomyelinase activity, the incubation of PKC δ-depleted cells with PMA increased the sphingomyelinase activity to a level similar to that observed in response to $1,25(OH)_2D_3$. No further increase in the level of sphingomyelinase activity was observed in PKC δ-depleted cytosol in response to the combination of PMA and $1,25(OH)_2D_3$, suggesting that the increase in the level of sphingomyelinase activity in PKC δ-depleted cytosol in response to PMA alone might be due to the activation of PKC α. The inhibitory effect of PMA on the $1,25(OH)_2D_3$-mediated activation of sphingomyelinase was absent from cytosol depleted of PKC δ, confirming the inhibitory role of activated PKC δ in the regulation of Mg^{2+}-independent, neutral sphingomyelinase. On the basis of the results of our study, a model can be proposed in which the activity of sphingomyelinase is regulated by different PKC isotypes: whereas the activity of classical, Ca^{2+}-dependent isoform α is responsible for the activation of sphingomyelinase, the novel, Ca^{2+}-independent isoform δ negatively regulates the activity of the enzyme.

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