Selective Inhibition of Juxtanuclear Translocation of Protein Kinase C βII by a Negative Feedback Mechanism Involving Ceramide Formed from the Salvage Pathway*

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In a previous study, we showed that protein kinase C βII (PKC βII) translocated to a novel juxtanuclear compartment as observed in several cell types (Becker, K. P., and Hannun, Y. A. (2003) J. Biol. Chem. 278, 52747–52754). In this study, we noted the absence of this translocation in MCF-7 breast cancer cells, and we examined the mechanisms underlying this selectivity of response. We show that sustained stimulation of PKC βII with 4β-phorbol 12-myristate 13-acetate (PMA) resulted in accumulation of ceramide in MCF-7 cells but not in those cells that showed juxtanuclear translocation of PKC βII. Addition of exogenous ceramides or formation of endogenous ceramide by the action of bacterial sphingomyelase prevented PMA-induced translocation of PKC βII in HEK 293 cells. On the other hand, inhibition of ceramide accumulation with fumonisin B1 restored the ability of PMA to induce translocation of PKC βII in MCF-7 cells. Taken together, the results showed that endogenous ceramide is both necessary and sufficient for preventing juxtanuclear translocation of PKC βII in response to PMA. Investigation of the mechanisms of ceramide generation in response to PMA revealed that PMA activated the salvage pathway of ceramide formation and not the de novo pathway. This conclusion was based on the following: 1) the ability of fumonisin B1 but not myriocin to inhibit ceramide formation, 2) the ability of PMA to induce increases in palmitate-labeled ceramide only under chase labeling but not acute pulse labeling, 3) the induction of the levels of sphingosine but not dihydrosphingosine in response to PMA, and 4) induction of sphingomyelin hydrolysis in response to PMA. Together, these results define a novel pathway of regulated formation of ceramide, the salvage pathway, and they define a role for this pathway in regulating juxtanuclear translocation of PKC βII.

The isoenzymes of protein kinase C (PKC),1 designated as the classical PKCs, comprise a subfamily of closely related isoenzymes that share similar structural features and mechanisms of action and regulation. This group consists of PKC α, PKC βI, PKC βII, and PKC γ that share highly homologous regulatory domains and are activated by diacylglycerol (DAG) and calcium. Thus, they serve as signal transducers for the action of extracellular agonists that activate phospholipases C, which results in the generation of DAG and calcium. 3, 4

Activation of PKC requires the translocation of PKC to the plasma membrane in response to acutely generated DAG (3, 4). This translocation also places PKC in proximity to specific membrane substrates. Thus, the dynamic interaction of PKC with membranes has emerged as a key mechanism in regulating its function.

Previous studies from our laboratory (1), supported by the recent results from Hu and Exton (5), have demonstrated a novel translocation of PKC α and βII to a juxtanuclear region. This novel translocation is seen with prolonged stimulation of these two isoenzymes of PKC by PMA (for at least 30 min) and follows the initial translocation of PKC to the plasma membrane (seen as early as 2–5 min following stimulation with PMA). Most interestingly, this novel translocation operates selectively on PKC α and βII in that it is not observed with PKC βI or any of the nonclassical PKCs (1, 6). Mechanistically, this translocation involves activation of PLD (6) and the interaction of PKC with PLD (5). This novel juxtanuclear compartment is centered around and beyond the centrosome and the microtubule-organizing center, and therefore we have described it as the "pericentriol."

In the course of studying this novel translocation of PKC, we noted that although it is seen in most cell types, we could not observe it in a few cell lines such as MCF-7 breast carcinoma cells and LnCAP prostate cancer cells. Because PKC activation in LnCAP was previously associated with formation of ceramide (7), we wondered if ceramide could regulate this translocation of PKC.

Ceramide is a neutral lipid that serves as the hub of sphingolipid synthesis, analogous to the role of DAG in glycerolipid metabolism. However, unlike DAG, which appears to be involved in the regulation of mitogenesis and tumor promotion, ceramide has been implicated in regulating cell cycle arrest, apoptosis, and cell senescence (8–10).

Indeed, the regulation of ceramide metabolism has received increasing attention over the past 15 years with increasing appreciation that many of the enzymes of ceramide metabolism are regulated and that this regulation may subserve specific small interfering ribonucleic acid, siRNA; SMase, sphingomyelinase; SPT, serine palmitoyltransferase; LC/MS, liquid chromatography/mass spectrometry; PBS, phosphate-buffered saline; IIs, internal standards.

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1 The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; GFP, green fluorescent protein; GCS, glucosylceramide synthase; PMA, 4β-phorbol 12-myristate 13-acetate; FB1, fumonisin B1;
De novo pathway

Serine + Palmitoyl-CoA → 3-Ketosphinganine → Dihydrosphingosine → Ceramide synthase → Ceramide

Salvage pathway

Sphingomyelinase → Sphingosine → Ceramide synthase → Ceramide

Sphingomyelin/Complex SLs

PKC-βII was described previously (15).

Experimental Procedures

Materials—Eagle’s minimal essential media, RPMI, and HEPES were from Invitrogen. The MCF-7 and HEK 293 cell lines were purchased from American Tissue Culture Collection (Manassas, VA). [3H]Palmitic acid was from PerkinElmer Life Sciences. 4-β-Phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochem. Ceramide was from Avanti Polar Lipids (Alabaster, AL). Phospholipid standards were from Avanti Polar Lipids. Fumonisin B1 was from Alabaster, AL. Phospholipid synthase, and sphingomyelin synthase (14).

In this study, we provide evidence for a specific role for endogenous ceramide in inhibiting the novel juxtanuclear translocation of PKC to the pericentrum. Most importantly, we show that sustained activation of PKC over 30–60 min activates the salvage pathway of sphingolipid metabolism (Fig. 1) by which sphingoid bases generated from hydrolysis of complex sphingolipids are recycled into ceramide synthesis. This is the first description of regulation of this pathway in response to an agonist. Thus, the results define a negative feedback mechanism in the activation of PKC involving PKC-induced formation of endogenous ceramide through the salvage pathway.

Cell Culture—MCF-7 cells were maintained in RPMI, and HEK 293 cells were maintained in Eagle’s minimal essential media supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO₂ incubator at 37 °C. Cells were passaged every 3–4 days to maintain cells in logarithmic growth.

Plasmid Construction—All recombinant DNA procedures were carried out following standard protocols. The wild type pBK-CMV-GFP-PKC-βII was described previously (15).

Confocal Microscopy—Transient transfection of DNA (1.5 μg/dish) was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Transfected cells were grown for 12 h. 10% fetal bovine serum media. A transfection efficiency of 40–60% was obtained in these experiments. Cells expressing green fluorescent (GFP) fusion proteins alone were viewed live in 10 mM HEPES-buffered media or viewed immediately following a 10-min fixation with 3.7% paraformaldehyde, 10% methanol pre-warmed to 37 °C. All confocal images were taken with an Olympus UltraView Spinning Disk Confocal IX-70 system with an Olympus 60× 1.4 NA lens and krypton/argon laser line. Each microscopic image is representative of 20 fields over a minimum of three experiments, and all images were taken at the equatorial plane of the cell. GFP fusion proteins were excited at 488 nm. All fluorescent images were captured sequentially and combined within the UltraView software (PerkinElmer Life Sciences). Raw data images were cropped in Adobe Photoshop® 5.0 for publication.

Choline Labeling for Measuring Sphingomyelin—Chase labeling experiments using [3H]Choline chloride were performed to measure changes in sphingomyelin levels. Cells were incubated with 3 μCi/ml [3H]Choline for 72 h and then replaced in RPMI 1640 containing 0.1% bovine serum albumin. After PMA treatment, lipids were extracted as specified times and at the noted concentrations. All incubations were performed at 37 °C. For pulse labeling experiments, cells were placed in RPMI 1640 containing 0.1% bovine serum albumin for 24 h and then treated with PMA in concurrence with labeling with 3 μCi/ml [3H]Palmitic acid for 1 h.

Lipid Extraction and Analysis—Following stimulation, the culture media were removed, and the cells were washed rapidly three times with 1 ml of ice-cold PBS. Total cellular lipids were extracted via the method of Bligh and Dyer (16). Lipids were dried down and resuspended in 75 μl chloroform/methanol (2:1, v/v). Fifty μl of the 75-μl volume was loaded per lane. The TLC solvent system consisted of ethyl acetate/isooctane/acetic acid (9:5:2, v/v/v) for resolving ceramides and chloroform/methanol/acetic acid/H₂O (50:30:8.5, v/v/v/v) for resolving sphingomyelin. The plates were sprayed with ENHANCE Spray (PerkinElmer Life Sciences) to amplify the tritium signal and then exposed for autoradiography for 24 h. The major ceramide species were...
identified by running C14-, C16-, C24-ceramide standards along side each sample. Each experiment was repeated 3–5 times. The area corresponding to each lipid was scraped off, and the radioactivity was measured by liquid scintillation.

**RESULTS**

**Translocation of GFP-PKC βII to a Subset of Recycling Endosomes in HEK 293 but not in MCF-7 Cells**—The novel translocation of classical PKC isoenzymes, α and βII, to a subset of recycling endosomes concentrated around the microtubule-organizing center/centrosome (the pericentrion) was observed in several cell types including HEK 293, HeLa, HT-1080, DLD-1, A549, and COS-1 cells (1). Further studies into the translocation of a GFP-tagged PKC βII revealed that this novel juxtanuclear translocation was not evident in all cell lines. Stimulation of HEK 293 cells with 100 nM PMA for 60 min induced an alteration in the localization of GFP-PKC βII from the cytoplasm to the plasma membrane and to the juxtanuclear compartment (Fig. 2A). In contrast, similar stimulation of MCF-7 resulted in translocation of GFP-PKC βII to the plasma membrane only with no detectable pericentriolar translocation (Fig. 2B). Time course experiments in MCF-7 cells revealed that this difference between the two cell lines was not because of a difference in the rate of translocation because further incubation with PMA for 3 or 6 h did not produce juxtanuclear translocation (data not shown). These results demonstrate cell-specific heterogeneity in the response of PKC to long term stimulation.

**PMMA Induces the Formation of Ceramide in MCF-7 Cells but Not in HEK 293 Cells**—It has been reported that the bioactive sphingolipid ceramide can inhibit both the activity and translocation of various classical PKC isoenzymes in both a short and long term manner (17–19). Separately, it was reported that phorbol ester stimulation leads to a selective increase in ceramide in LnCap cells but not in all prostate cancer cell lines (7). To investigate if phorbol ester stimulation leads to a selective increase in ceramide in MCF-7 cells but not in HEK, both cell types were labeled overnight with [3H]palmitate and then stimulated for 1 h with 100 nM PMA. As seen in Fig. 3A, 60 min of PMA led to an increase in ceramide in MCF-7 cells, and this was not seen in HEK 293 cells. The effects of PMA on ceramide in MCF-7 cells were also analyzed using LC/MS. The time course studies revealed that ceramide was increased to ~130% of basal levels within 30 min of stimulation, and this ceramide increase remained sustained through 2 h of stimulation (Fig. 3B). As seen in Table I, levels of all of the ceramide species except C16-ceramide were increased under PMA stimulation, and notably the elevation of C16-ceramide was particularly pronounced with a greater than
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MCF-7 cells were treated with 100 nM PMA for the indicated times, and then the levels of individual ceramide species were determined as described under "Experimental Procedures" by LC/MS. The data represent mean ± S.E. of three values. C14-Cer, C18-ceramide; dhC16-Cer, dihydro C16-ceramide; C18:1-Cer, C18:1-ceramide; C18-Cer, C18-ceramide; C20-Cer, C20-ceramide; C24:1-Cer, C24:1-ceramide; and C24-Cer, C24-ceramide.

|                | C14-Cer | dhC16-Cer | C18-Cer | C18:1-Cer | C18:2-Cer | C20-Cer | C24:1-Cer | C24-Cer |
|----------------|---------|-----------|---------|-----------|-----------|---------|-----------|---------|
| Control (0 min)| 16.3 ± 0.8 | 1.8 ± 0.3 | 178.8 ± 3.4 | 3.9 ± 0.4 | 6.4 ± 0.4 | 8.8 ± 0.6 | 246.8 ± 14.4 | 235.1 ± 17.4 |
| Control (15 min)| 15.9 ± 1.0 | 2.1 ± 0.3 | 165.8 ± 14.0 | 4.1 ± 0.3 | 6.6 ± 0.4 | 8.6 ± 0.4 | 259.6 ± 10.5 | 243.7 ± 11.8 |
| PMA (15 min)   | 15.8 ± 0.5 | 2.5 ± 0.1 | 170.9 ± 5.5 | 4.1 ± 0.2 | 7.1 ± 0.7 | 9.1 ± 0.3 | 296.9 ± 17.5 | 253.7 ± 14.3 |
| Control (30 min)| 15.2 ± 0.5 | 2.1 ± 0.3 | 143.6 ± 3.8 | 4.0 ± 0.2 | 7.0 ± 0.4 | 8.4 ± 0.4 | 288.1 ± 18.4 | 251.3 ± 12.0 |
| PMA (30 min)   | 23.6 ± 1.4 | 7.0 ± 0.7 | 257.6 ± 22.4 | 6.5 ± 0.4 | 10.4 ± 0.3 | 11.8 ± 0.2 | 375.3 ± 14.9 | 315.6 ± 7.7 |
| Control (60 min)| 15.6 ± 0.3 | 2.5 ± 0.1 | 133.9 ± 1.3 | 4.3 ± 0.1 | 7.1 ± 0.2 | 8.7 ± 0.6 | 331.0 ± 12.7 | 281.5 ± 13.2 |
| PMA (60 min)   | 27.8 ± 0.5 | 9.7 ± 0.1 | 304.1 ± 9.4 | 6.5 ± 0.1 | 11.8 ± 0.1 | 13.9 ± 0.1 | 377.5 ± 2.7 | 295.2 ± 19.2 |
| Control (120 min)| 17.3 ± 0.3 | 5.0 ± 0.4 | 152.3 ± 12.2 | 4.1 ± 0.2 | 6.6 ± 0.4 | 7.7 ± 0.7 | 380.8 ± 45.3 | 252.4 ± 39.2 |
| PMA (120 min)  | 30.0 ± 0.1 | 14.1 ± 0.2 | 322.9 ± 0.5 | 6.1 ± 0.2 | 13.3 ± 0.1 | 13.5 ± 0.2 | 398.9 ± 20     | 266.2 ± 4.3 |

2-fold induction at 60 min. Furthermore, as shown in Figs. 2 and 5, these alterations of very long (C24- ceramide) or long chain (C16- ceramide) ceramide levels upon PMA stimulation in MCF-7 cells are consistent with what was observed in the lower or higher ceramide bands separated by thin layer chromatography, which correspond to C16- ceramide and C24- ceramide, respectively. These results demonstrate that PMA is able to induce the formation of ceramide in MCF-7 cells but not in HEK cells.

Juxtunuclear Translocation Is Inhibited by Endogenous and Exogenous Ceramide—As noted, PMA has also been shown to induce ceramide formation in LnCAP prostate cancer cells (7), and we could not detect juxtunuclear translocation of PKC in response to PMA in LnCap cells (data not shown). These results suggested a correlation between ceramide formation and prevention of juxtunuclear translocation. Therefore, to investigate if ceramide could inhibit translocation of PKC βII to the juxtunuclear compartment, HEK 293 cells transfected with GFP-PKC βII were preincubated with 40 μM C6-ceramide for 8 h followed by 60 min of 100 nM PMA. In cells treated with PMA alone, there was translocation of GFP-PKC into the juxtanuclear compartment (Fig. 4A, panel 1). In contrast, pretreatment of cells with C6-ceramide prevented the juxtunuclear translocation in response to PMA (Fig. 4A, panel 2). To determine whether endogenous ceramide could accomplish a similar effect as exogenously added ceramide, HEK 293 cells transfected with GFP-PKC βII were pretreated for 30 min with 100 milliunits/ml bacterial SMase followed by 60 min PMA. Unlike cells treated with PMA alone, pretreatment with bacterial SMase led to an inhibition of juxtunuclear translocation (Fig. 4B, panels 1 and 2). These data show that endogenous ceramide can inhibit translocation of PKC βII to the pericentrum, raising the possibility that endogenous ceramide may be responsible for the lack of the PMA effect in MCF-7 cells.

PMA-induced Ceramide Production in MCF-7 Cells Requires PKC Activity and Involves the Sphingolipid Salvage Pathway but Not de Novo Synthesis—It is well established that the major cellular receptor for PMA is PKC and that PMA exerts many of its cellular effects through stimulation of DAG/PMA-responsive PKC isoenzymes. Therefore, to determine whether PKC activity was required for the formation of ceramide, cells were steady-state labeled with [3H]palmitate and incubated in the presence of the PKC inhibitor, bisindolylmaleimide I. In cells treated with 3 μM bisindolylmaleimide I, there was complete abolition of ceramide production (Fig. 5A, panels 1–3). These data suggest that the ceramide increase is mediated by PKC and that this ceramide then modulates translocation of PKC.

In previous studies in LnCAP cells, it was concluded that PMA induced ceramide formation through activation of the de novo pathway based on studies with FB1, an inhibitor of ceramide synthase (7). Therefore, to determine the metabolic source and mechanism involved in ceramide generation, [3H]palmitate-labeled MCF-7 cells were treated with FB1, and the results showed that FB1 inhibited ceramide formation in response to PMA (Fig. 5B). Thus, the formation of ceramide in response to PMA requires activity of ceramide synthase.

FIG. 4. Juxtunuclear translocation of PKC βII is inhibited by endogenous and exogenous ceramide. A, representative confocal images of HEK 293 cells transfected with GFP-PKC βII for 12 h and pretreated with either vehicle alone (0.01% ethanol) (panel 1) or 40 μM C6-ceramide (panel 2) for 8 h followed by 60 min with 100 nM PMA. B, representative confocal images of HEK 293 cells transfected with GFP-PKC βII for 12 h and then pretreated with either vehicle alone (PBS) (panel 1) or 100 milliunits/ml bacterial SMase (panel 2) for 30 min followed by 60 min with 100 nM PMA.

FIG. 5. PMA-induced ceramide production in MCF-7 cells is inhibited by PKC inhibitors and fumonisin B1. Autoradiography of ceramide from MCF-7 cells labeled overnight with [3H]palmitate and then treated with 3 μM bisindolylmaleimide (BIS, A), 100 nM myriocin (Myc, A), or 200 μM fumonisin B1 (FB1, B) for 1 h, ctrl, control. Cells were then stimulated with 0.01% MeSO (DMSO) or 100 nM PMA for 60 min. Total labeled lipids were run on a thin layer chromatography gel silica plate in ethyl acetate/iso-octane/acetic acid (9:5:2, v/v/v). Both bands represent ceramide species.
Because ceramide synthase acts on free sphingosine by catalyzing its acylation to ceramide, the action of FB1 in inhibiting this reaction does not distinguish between activation of the \textit{de novo} pathway of sphingoid base formation or the generation of sphingosine from breakdown of complex sphingolipids, i.e. the salvage pathway (Fig. 1) (20). We undertook several approaches to distinguish the two possibilities.

First, we employed myriocin which inhibits the initial step of the \textit{de novo} pathway of sphingoid base formation or the generation of sphingosine from breakdown of complex sphingolipids, i.e. the salvage pathway (Fig. 1) (20). We undertook several approaches to distinguish the two possibilities.

To further discern between these two possibilities, the effects of PMA on palmitate labeling of ceramide were compared under a pulse protocol \textit{versus} a chase condition. If PMA stimulates \textit{de novo} synthesis of ceramide, then PMA should stimulate the acute incorporation of palmitate into ceramide when cells are simultaneously incubated with radiolabeled palmitate (pulse label). On the other hand, if PMA stimulates turnover of sphingolipids resulting in the formation of sphingosine as a breakdown product which is then acylated back to ceramide, then PMA would not stimulate the acute incorporation of palmitate. In that case, steady-state labeling of sphingolipids with palmitate (chase conditions) would then demonstrate enhanced labeling of ceramide in response to PMA. Therefore, MCF-7 cells were acutely labeled with palmitate and simultaneously treated with PMA (pulse conditions). Under these conditions, PMA did not enhance the incorporation of label into ceramide (Fig. 6A). In contradistinction, when cells were labeled with palmitate to steady state (24 h), PMA significantly induced ceramide formation after 60 min of treatment (Fig. 6A), demonstrating that PMA induces the accumulation of ceramide from pre-labeled precursors.

Coincident with ceramide accumulation, PMA induced the
accumulation of free sphingosine, which primarily derives from breakdown of complex sphingolipids. In contrast, free dihydrosphingosine, which arises predominantly through the de novo pathway, was depleted in response to PMA (Fig. 6B), suggesting selective enhancement of availability of free sphingosine/dihydrosphingosine for ceramide synthesis upon PMA stimulation. Taken together, the results implicate induction of the salvage pathway in response to PMA.

Because the above studies suggested turnover of complex sphingolipids, we investigated the effects of PMA on sphingomyelin. Transient degradation of sphingomyelin was observed as early as 15 min following PMA treatment, and a significant change in the turnover was seen at the time point of 60 min (Fig. 6C). These very small changes in sphingomyelin are expected because the levels of sphingomyelin are ∼10-fold those of ceramide. Therefore, the results suggest induction of sphingomyelin turnover as the source of the formed ceramide.

**Fumonisin B1 Restores Juxtanuclear Translocation in MCF-7 Cells**—The inhibition of PMA-induced ceramide formation by FB1 suggested that the absence of PKC translocation to the pericentriol in MCF-7 cells may be reversible. MCF-7 cells transfected with GFP-PKC βII were either treated with vehicle alone (PBS) or 200 μM FB1 for 6 h and then 60 min of 100 nM PMA. In cells pretreated with PBS alone, there was a translocation of GFP-PKC βII to the plasma membrane only (Fig. 7, panels 1 and 2). An 8-h preincubation with FB1 resulted in the restoration of translocation of PKC βII to the pericentriol (Fig. 7, panel 3), thus suggesting a role for ceramide as the endogenous inhibitor of PKC translocation. However, ceramide may also get converted to glucosylceramide through the action of glucosylceramide synthase (GCS), and the resulting glucosylceramide then serves as a precursor for the formation of complex glycosphingolipids. To examine for a possible role for glucosylceramide or glycosphingolipids in the inhibition of PKC βII juxtanuclear translocation, GCS was inhibited by using small interfering RNA (siRNA). As shown in Fig. 7, panel 6, PMA-induced translocation of GFP-PKC βII to the plasma membrane was not influenced by treatment with siRNA for GCS (under conditions where it inhibited formation of glucosylceramide). Therefore, downstream products of GCS do not appear to be involved in the juxtanuclear translocation of PKC βII. These data implicate the endogenous ceramide induced by PMA in the prevention of juxtanuclear translocation of PKC in MCF-7 cells.

**DISCUSSION**

The results from this study define a negative feedback mechanism activated by PKC that results in inhibition of translocation of PKC βII to the juxtanuclear region/pericentriol. Thus, the results demonstrate the activation of PKC by PMA in MCF-7 cells causes the formation of ceramide, which in turn appears to be the mediator that inhibits the translocation of PKC βII to the juxtanuclear region but not to the plasma membrane.

Specifically, the results from this study shed important light on a novel mechanism of ceramide formation. The results also provide evidence for a specific role of this pathway in negative regulation of PKC. Finally, the results add a new dimension to the emerging appreciation of pathway-specific actions of ceramide.

Although the formation of ceramide through the activation of SMases or the de novo pathway has come under intense investigation, there are many outstanding questions as to the mechanisms that regulate these pathways. In particular, many of the studies implicating the de novo pathway have relied heavily on the ability of FB1 to inhibit ceramide synthase, a downstream enzyme along this pathway. However, it should be noted that ceramide synthase can act on substrates (dihydrosphingosine or sphingosine) that are generated either from activation of the de novo pathway or that are generated from hydrolysis of complex sphingolipids, a pathway termed the “salvage” pathway (Fig. 1) (20).

The results from this study clearly show that PMA activates the salvage pathway and not the de novo pathway. This conclusion is based on the following criteria. 1) Whereas FB1 inhibited ceramide formation, myriocin, a potent inhibitor of SPT, did not affect the response to PMA. SPT is the first and rate-limiting enzyme in the de novo pathway, and therefore its inhibition should abrogate the formation of downstream sphingolipids including ceramide. 2) The use of differential pulse and chase labeling was particularly revealing. Thus, PMA did not induce incorporation of palmitate into ceramide under pulse conditions, arguing against activation of de novo synthesis, whereas PMA induced significant incorporation of label into ceramide under chase conditions whereby the palmitate label was already incorporated into lipid precursors. 3) PMA caused an accumulation of sphingosine and not dihydrosphingosine. Notably, careful studies by Merrill et al. (20) have provided significant evidence that sphingosine, unlike dihydrosphingosine, arises primarily from the breakdown of complex sphingolipids and ceramide and not as an intermediate in the de novo pathway. This observation also supports activation of the salvage pathway by PMA. At this point, we can only speculate as to the mechanisms by which PMA caused a decrease in dihydrosphingosine. This is unlikely due to inhibition of the de novo pathway as we did not see a decrease in acute incorporation of palmitate into sphingolipids with PMA. It is more likely that PMA may activate ceramide (dihydroroceramide) synthase as a component of activation of the salvage pathway. 4) Finally, the studies with choline labeling also demonstrate an effect of PMA on sphingomyelin levels and suggest activation of a sphingomyelinase. Although the changes in sphingomyelin were very modest, it should be noted that in MCF-7 cells a doubling of ceramide would require a decrease of only ∼10% in sphingomyelin levels.

Taken together, these criteria implicate activation of the salvage pathway and not the de novo pathway. We also propose that these criteria can become valuable in dissecting out these two pathways in other situations where investigators have noted effects of FB1.

It should be noted that other studies have shown the exist-
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ence and operation of the salvage pathway in lipid metabolism (21, 22, 27). The results from this study show that PKC activity is required for activation of this pathway in response to PMA, and thus, this represents the first description of regulation of sphingolipid salvage.

Functionally, the results provide one specific consequence of activation of this pathway, i.e. inhibition of translocation of PKC βII to the juxtanuclear region. Thus, formation of endogenous ceramide by the action of bacterial SMase on membrane sphingomyelin was sufficient to inhibit the ability of PMA to induce juxtanuclear translocation of PKC in a cell line (HEK 293) that otherwise demonstrated this action. In complementing this conclusion, results with FB1 showed that endogenous ceramide formed in response to PMA was necessary for the block of PKC translocation in MCF7 cells. Thus, activation of the salvage pathway is both necessary and sufficient to inhibit juxtanuclear translocation of PKC. Most interestingly, however, this pathway is not activated by PMA/PKC in most cell lines. The mechanisms responsible for this selective activation may reflect roles of specific isoenzymes of PKC, and these are currently under investigation.

The results also implicate ceramide itself and not other key metabolites in this response. For example, sphingosine levels increase with FB1, ruling out a role for further degradation of ceramide to sphingoid bases. Similarly, the response to PMA was not affected by down-regulation of glucosylceramide synthase, thus ruling out a key role for GCS or any of its downstream metabolites. However, at present, a role for other undefined metabolites of ceramide as the direct mediators of this response cannot be ruled out. Nevertheless, it should be noted that formation of ceramide is required for inhibiting the PMA response irrespective of whether ceramide is the mediator per se.

In the context of ceramide-mediated biology, these results begin to define specific pathways of ceramide generation with specific functions. For example, previous studies have shown that de novo generated ceramide in response to stimulation of the BcR receptor in B cells results in selective accumulation of C16-ceramide (23), and this was implicated in the apoptotic response of those cells (24). On the other hand, activation of neutral SMase2 was shown to selectively induce the formation of C24′-ceramide and C24:1-ceramide, and evidence was provided for a role for this pathway in cell cycle regulation (25) and in interleukin-6 formation (26). The current results provide a third specific pathway of ceramide formation with its own distinct function in regulating translocation of PKC. Previous studies have also disclosed additional effects of ceramide on PKC. For example, Lee et al. (18) showed that prolonged action of ceramide results in dephosphorylation and inactivation of PKCa, and this was attributed, at least in part, to the activation of sphingomyelineses. Thus, ceramide generated from different pathways may exert differential effects on PKC.

Taken together, these results define a novel feedback pathway by which PKC activates sphingolipid salvage resulting in ceramide accumulation. This in turn inhibits translocation of PKC βII to the juxtanuclear region.

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Selective Inhibition of Juxtanuclear Translocation of Protein Kinase C βII by a Negative Feedback Mechanism Involving Ceramide Formed from the Salvage Pathway

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