Selective Ceramide Binding to Protein Kinase C-α and -δ Isoenzymes in Renal Mesangial Cells†

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ABSTRACT: Ceramide is an important lipid second messenger produced by sphingolipid metabolism in cells exposed to a limited number of agonists and in turn triggers several cell responses in a protein kinase C (PKC)-dependent manner. Stimulation of mesangial cells with a radioiodinated photoaffinity labeling analogue of ceramide, (*N*-[3-[[[2-(125I)iodo-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl]oxy]-carbonyl]propanoyl]-D-*erythro*-sphingosine) ([125I]-TID-ceramide), defines PKC-α and PKC-δ as direct targets of ceramide. No binding of ceramide to PKC-ε and PKC-ζ could be detected. Moreover, TID-ceramide selectively binds to recombinant PKC-α and -δ but not to PKC-ε and -ζ isoenzymes. In vitro kinase activity assays reveal that only the binding of ceramide to PKC-α is accompanied by an increase in kinase activity. In contrast, there is no change in in vitro kinase activity of the other isoforms tested, i.e., PKC-δ, -ε, and -ζ, toward any of the conventional substrates tested. However, it is noteworthy that PKC-δ shows a decreased autophosphorylation upon ceramide binding. In vivo, activation of PKC-α by ceramide is monitored by a delayed translocation of the isoform from the cytosol to the membrane fraction, detectable after 1 h of stimulation. In contrast, neither PKC-δ, nor -ε nor -ζ is redistributed by ceramide. One functional cell response mediated by PKC-α in mesangial cells is a negative feedback regulation of ligand-stimulated phosphoinositide hydrolysis. When cells are pretreated with ceramide, ATP-induced inositol trisphosphate formation is time-dependently reduced. A maximal inhibition is observed after 2 h of ceramide exposure. In summary, these results suggest that ceramide selectively interacts with the α- and δ-isoforms of PKC in mesangial cells. Whereas PKC-α is activated with pronounced inhibition of hormone-stimulated phosphoinositide signaling, PKC-δ displays a decrease in its autophosphorylation, suggesting a negative role of ceramide binding on PKC-δ activity.

Interleukin-1 (IL-1)[1] and tumor necrosis factor α (TNFα) are prototypes of proinflammatory cytokines that induce the expression of a variety of protein factors that in turn trigger acute and chronic inflammatory processes (*1*). The local release of factors such as IL-1 and TNFα from monocytes is an early event associated with inflammatory reactions and may be an important pathogenic determinant of structural and functional alterations accompaining immune injury in many organs including the kidney (*1*). However, the signaling pathways of IL-1 and TNFα linking receptor occupancy to cellular responses are still not completely defined (*2*).

Recently, a novel signal transduction pathway, the sphingomyelin pathway, has received increasing attention since it mediates the action of various stress stimuli including IL-1 and TNFα (*3*−*6*). Stimulation of mammalian cells with these agents triggers the rapid hydrolysis and subsequent resynthesis of sphingomyelin. By the action of neutral and/or acidic sphingomyelinases, ceramide is generated, a lipid second messenger which mediates important cell responses such as differentiation, cell growth, or apoptosis. With respect to immediate targets of ceramide, suggestions for several ceramide-activated enzymes have been forwarded: These include the kinase c-Raf (*7*), a putative ceramide-activated membrane-associated protein kinase (*8*) which has been suggested to be identical with kinase suppressor of Ras (*9*), a ceramide-activated protein phosphatase belonging to the family of protein phosphatase 2A (*10*) and PKC-ζ (*11*).

Protein kinase C (PKC) is a key enzyme in several signal transmission systems in a cell, activated by a wide variety of agonists, including hormones, growth factors, antigens, and neurotransmitters. External stimuli trigger the activation

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[1] Abbreviations: PKC, protein kinase C; [125I]-TID-ceramide, [125I]-3-trifluoromethyl-3-(*m*-iodophenyl)diazirine-ceramide; IL-1, interleukin 1; TNFα, tumor necrosis factor α; DAG, 1,2-diacylglycerol; ERK, extracellular regulated kinase; TLC, thin-layer chromatography; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; PS, phosphatidylserine; MBP, myelin basic protein; $InsP_3$, inositol trisphosphate; CaLB domain, Ca^{2+}-dependent phospholipid binding domain.

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of phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (DAG). DAG is the physiological activator of PKC (for review, see *12*−*15*).

Molecular cloning and sequence analysis of PKC indicated that the enzyme exists as a family of at least 11 isoforms, which are classified into 3 groups according to their cofactor requirement: the conventional calcium-dependent PKC subtypes (cPKCs: α, β1, β2, γ); the nonconventional or novel calcium-independent isoforms (nPKCs: δ, ϵ, η, θ, and μ); and the atypical calcium-independent and DAG- or phorbol ester-unresponsive PKC subtypes [aPKCs: ζ and ι(λ)].

Several physiological functions have been linked to PKC activation such as cell proliferation, secretion, exocytosis, modulation of ion conductances, and gene expression. One can immediately anticipate that the different isoenzymes of PKC may each have distinct roles in the control of these and other cellular functions.

Previously we have reported that mesangial cells express four PKC isoforms, PKC-α, -δ, -ϵ and -ζ, but not PKC-β, -γ, -η, -θ, or -μ (*16*, *17*). In these cells, PKC-α was found to negatively regulate phosphoinositide turnover (*16*−*19*), whereas PKC-ϵ stimulates phospholipase A_2 activity and prostaglandin synthesis (*16*), the activity of phospholipase D, and the activity of extracellular signal-regulated kinases (ERKs) (*20*−*22*).

In this study, we investigated the effect of ceramide on the PKC isoenzymes expressed in mesangial cells. By using a photoactivatable analogue of ceramide, we demonstrate that ceramide directly binds to PKC-α and -δ, but not to PKC-ϵ and -ζ isoenzymes. Furthermore, this ceramide binding leads to an activation of PKC-α in vitro and in vivo. PKC-δ shows a decreased autophosphorylation upon ceramide binding. The activation of PKC-α by ceramide is accompanied by an inhibition of hormone-induced polyphosphoinositide turnover, a cell response that has earlier been attributed to PKC-α in glomerular mesangial cells (*16*).

EXPERIMENTAL PROCEDURES

Reagents. [γ-^{32}P]ATP was from Amersham International, Bucks, U.K.; *N*-palmitoylsphingosine (C16-ceramide), phosphatidylserine, diacylglycerol, myelin basic protein, and Histon III S were purchased from Sigma Chemical Co., St. Louis, MO; IL-1β and TNFα were provided by Novartis Ltd., Basel, Switzerland; protein A−Sepharose 4B-CL was from Pharmacia Fine Chemicals, Uppsala, Sweden; Dowex 1 × 8 was from Biorad, Glattbrugg, Switzerland; the photoaffinity labeling reagent [^{125}I]-TID-ceramide (*N*-[3-[[[2-(^{125}I)iodo-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl]oxy]carbonyl]-propanoyl]-D-*erythro*-sphingosine) was synthesized as described (*23*). Monoclonal anti-PKC-α (MC5) was from Amersham International (Bucks, U.K.). Synthetic peptides based on the C-terminal sequence of PKC-δ (KGFSFVN-PKYEQFLE), PKC-ϵ (NQEEFKGFSYFGEDLMP), and PKC-ζ (GFEYINPLLLSAEESV) were synthesized on an ARI 431 peptide synthesizer, coupled to keyhole limpet hemocyanin by glutaraldehyde, and used to immunize rabbits. The detailed characteriziation of anti-PKC-δ, -ϵ, and -ζ antibodies is described in detail elsewhere (*16*, *17*, *24*, *25*).

Cell Culture. Rat renal mesangial cells were cultivated as described previously (*26*). In a second step, single cells were cloned by limited dilution in 96-microwell plates (*27*). Clones with apparent mesangial cell morphology were used for further processing. The cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and bovine insulin (0.66 unit/mL). For the experiments, passages 7−20 of the cells were used.

TID Labeling Studies and Immunoprecipitation. Confluent mesangial cells were incubated for 2 days in DMEM containing 0.1 mg/mL fatty acid-free BSA. Thereafter cells were stimulated for 5 min with 50 nM [^{125}I]-TID-ceramide (0.1 mCi/mL) and subjected to photolysis for 30 s using a high-pressure mercury lamp (Osram HBO 350 W) mounted in a SUSS LH 1000 lamp house equipped with a shutter to control exposure time. Cells were then washed twice in ice-cold buffer containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, and scraped into lysis buffer [50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 2 mM EDTA, 2 mM EGTA, 40 mM β-glycerophosphate, 50 mM NaF, 10 mM sodium pyrophosphate, 25 μg/mL leupeptin, 200 units/mL aprotinin, 1 μM pepstatin A, 1 mM PMSF] and homogenized by 10 passes through a 26 gauge needle. The homogenate was centrifuged for 10 min at 14000*g*, and the supernatant was taken for immunoprecipitation. Immunoprecipitation was performed exactly as previously described (*7*) using specific monoclonal (PKC-α) or polyclonal antibodies (PKC-δ, -ϵ, and -ζ), diluted as indicated in the figure legends. Immunoprecipitates were separated on a 8% SDS−PAGE and exposed to Hyperfilm MP at −70 °C for 3−7 days.

Cell Fractionation and Immunodetection of PKC Isoenzymes. Confluent mesangial cells were incubated for 2 days in DMEM containing 0.1 mg/mL fatty acid-free BSA, and stimulated as indicated. Thereafter cells were washed and scraped into 1 mL of homogenization buffer (20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, 25 μg/mL leupeptin, 200 units/mL aprotinin, 10 mM benzamidine, 1 μM pepstatin A, 1 mM PMSF), lysed by sonication (3 × 10 s, setting 4.0, using a Branson B15 Sonifier), and centrifuged for 1 h at 100000*g* at 4 °C. Supernatants were used as a source of cytosolic protein. Pellets were resonicated in 1 mL of homogenization buffer containing 1% (v/v) Triton X-100 and centrifuged for 1 h at 100000*g*, yielding the solubilized particulate fractions. Protein concentration was determined by the method of Bradford (*28*). The PKC fractions were subjected to SDS−PAGE (8% acrylamide gel), proteins were transferred to nitrocellulose, and immunodetection was carried out exactly as previously described (*16*).

In Vitro PKC Isoenzyme Activity Assay. PKC isoenzyme activity was measured by incorporation of ^{32}P from [γ-^{32}P]-ATP into Histon III S, myelin basic protein, or α-pseudosubstrate peptide in the presence or absence of phosphatidylserine and diolein exactly as described previously (*29*).

In Vitro Autophosphorylation Assay. One microgram of purified recombinant PKC-δ was incubated in a total volume of 100 μL containing 20 mM Tris/HCl, pH 7.4, 50 mM KCl, 10 mM $MgNO_3$, 100 μg/mL PS, 10 μg/mL DAG, varying concentrations of C16-ceramide, 0.25 mM $CaCl_2$, 20 μM ATP, and 0.5 μCi pf [γ-^{32}P]ATP for 10 min at 32 °C. Thereafter SDS buffer was added to stop the reaction, and the samples were separated on an 8% SDS−PAGE. Phos-

phorylated bands corresponding to PKC-δ were analyzed on a phosphoimager.

Determination of Inositol Phosphates. Confluent cells in 35 mm diameter dishes were labeled for 72 h with myo[2-^{3}H]inositol (10 μCi/mL) in RPMI 1640 medium free of inositol, containing 2% dialyzed fetal calf serum. Therafter, the medium was removed, and the cells were rinsed several times to remove free [^{3}H]inositol. Then cells were preincubated for the indicated time periods with ceramide prior to stimulation with ATP (100 μM) or vehicle for 30 s. Therafter, the reaction was terminated by rapid aspiration of the medium and addition of 20% (w/v) trichloroacetic acid. The trichloroacetic acid was removed with diethyl ether. The final extract was neutralized and applied to anion-exchange columns containing Dowex 1 $\times$ 8 (100−200 mesh). Free inositol and the inositol phosphates were eluted exactly as described (*30*). The ratios of the phosphorylated compounds relative to total water-soluble [^{3}H]inositol were calculated for each sample as described previously (*31*).

RESULTS

*Ceramide Directly Binds to PKC-*α *and PKC-*δ. To identify possible molecular targets of ceramide signaling in mesangial cells, we used a photoaffinity labeling analogue of ceramide of high [^{125}I]iodine-specific radioactivity (>2000 Ci/mmol) (*23*). Quiescent mesangial cells were incubated with [^{125}I]-TID-ceramide for 5 min followed by photolysis at 364 nm for 30 s. Immunoprecipitation of the different PKC isoenzymes from cell lysates with specific polyclonal antibodies revealed selective labeling of PKC-α (Figure 1A, lane 2) and PKC-δ (Figure 1B, lane 2) after photolysis. Neither PKC-α nor PKC-δ showed significant labeling under unphotolyzed conditions (Figure 1A,B, lanes 1). No labeling of PKC-ϵ and PKC-ζ was detected (data not shown). All antibodies used in this study have been characterized and shown to quantitatively immunoprecipitate their respective antigens (*16*, *17*, *30*, *31*). To investigate the specificity of the observed ceramide binding, competition experiments were performed with cold exogenous or endogenous ceramide. Labeling of PKC-α and PKC-δ by [^{125}I]-TID-ceramide was inhibited by addition of increasing concentrations of unlabeled exogenous ceramide (Figure 1A, lanes 3 and 4 for PKC-α; Figure 1C, lanes 2 and 3 for PKC-δ). Since cytokines have been reported to increase intracellular ceramide levels in mesangial cells (*7*), this regimen was used to compete for [^{125}I]-TID-ceramide binding with endogenous ceramide. Pretreatment of cells with either IL-1β (2 nM) or TNFα (2 nM) prior to [^{125}I]-TID-ceramide incubation led to a complete reduction in PKC-α and -δ labeling, comparable to that observed with exogenous ceramide (Figure 1A, lane 5 for PKC-α; Figure 1B, lanes 3 and 4 for PKC-δ). Having demonstrated that TID-ceramide selectively binds to immunoprecipitated PKC-α and -δ isoenzymes in vivo in intact mesangial cells, we next attempted to directly evaluate TID-ceramide binding to purified recombinant PKC isoenzymes in order to exclude binding of ceramide to some unknown proteins coimmunoprecipitating with PKC-α and -δ. As shown in Figure 2A, TID-ceramide bound to recombinant PKC-α and-δ isoforms, and this binding could be competed for by addition of cold ceramide (Figure 2A). In contrast, TID-ceramide did not bind to recombinant PKC-β, -γ, -ϵ, and -ζ isoenzymes (Figure 2B), thus clearly confirming the in vivo binding data in mesangial cells and demonstrating specific binding of ceramide to PKC-α and -δ isoenzymes.

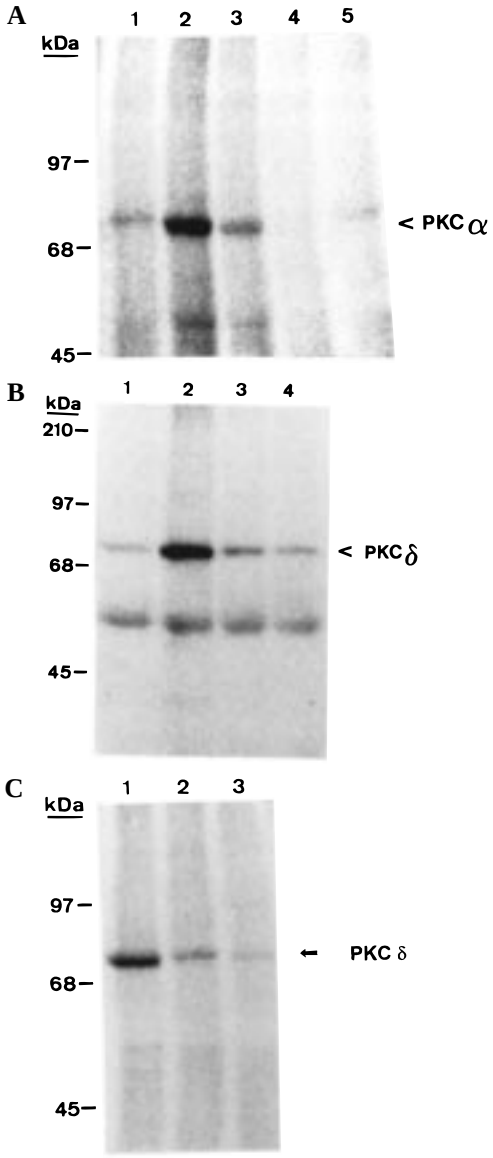

FIGURE 1: [^{125}I]-TID-ceramide labeling of PKC isoenzymes in mesangial cells. (A) Quiescent mesangial cells were incubated for 5 min with 50 nM [^{125}I]-TID-ceramide alone (lanes 1 and 2) or in the presence of cold C16-ceramide in a equimolar ratio (lane 3) or in a 10-fold excess (lane 4), or after preincubation for 5 min with 2 nM IL-1β (lane 5). Thereafter cells were either kept nonphotolyzed (lane 1) or subjected to photolysis for 30 s (lanes 2−5). Cell homogenates were prepared, and immunoprecipitations were performed using a monoclonal anti-PKC-α (MC5) antibody at a dilution of 1:100. (B) Quiescent mesangial cells were incubated for 5 min with 50 nM [^{125}I]-TID-ceramide alone (lanes 1 and 2) or after preincubation for 5 min with 2 nM IL-1β (lane 3) or 2 nM TNFα (lane 4). Thereafter cells were either kept nonphotolyzed (lane 1) or subjected to photolysis for 30 s (lanes 2−5). Cell homogenates were prepared, and immunoprecipitations were performed using a polyclonal anti-PKC-δ antibody at a dilution of 1:100. (C) Quiescent mesangial cells were incubated for 5 min with 50 nM [^{125}I]-TID-ceramide alone (lane 1) or in the presence of cold C16-ceramide in an equimolar ratio (lane 2) or in a 10-fold excess (lane 3). Thereafter cells were photolyzed for 30 s, and cell homogenates were prepared and subjected to immunoprecipitation using a polyclonal anti-PKC-δ antibody at a dilution of 1:100.

*Ceramide Activates PKC-*α *and Decreases PKC-*δ *Autophosphorylation in Vitro.* In the next step, we investigated

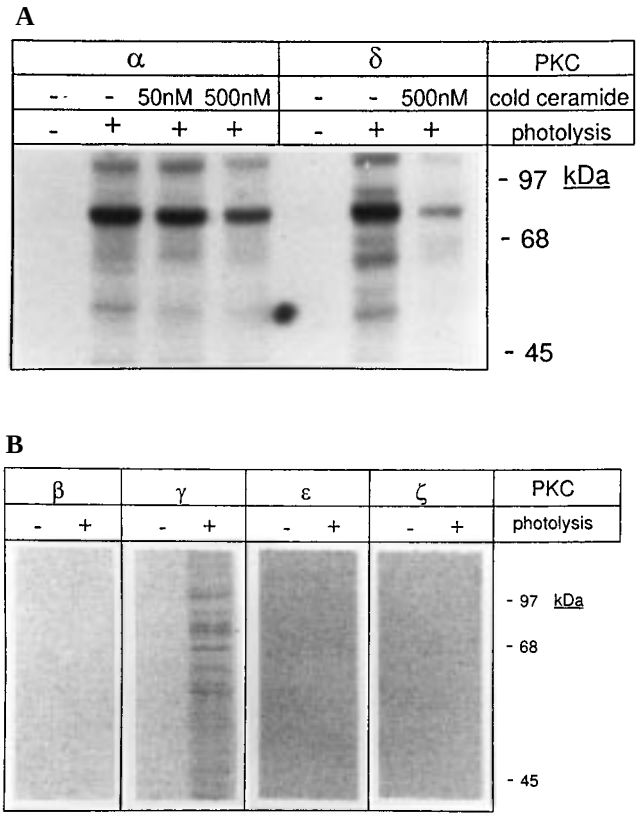

FIGURE 2: Binding of [^{125}I]-TID-ceramide to recombinant PKC isoenzymes in vitro. (A) Three micrograms of purified recombinant PKC-α and -δ isoenzymes was incubated for 15 min at room temperature with 50 nM [^{125}I]-TID-ceramide alone or in the presence of the indicated concentrations of cold C16-ceramide. Thereafter protein−lipid mixtures were either kept nonphotolyzed (−) or subjected to photolysis for 30 s at 360 nm (+). (B) Ten micrograms of PKC-*β*, -*γ*, -*ϵ*, and -ζ was incubated for 15 min at room temperature with 50 nM [^{125}I]-TID-ceramide. Thereafter protein−lipid mixtures were either kept nonphotolyzed (−) or subjected to photolysis for 30 s at 360 nm (+). Proteins were separated on SDS−PAGE and exposed to autoradiography. Data are representative of 4 experiments giving similar results.

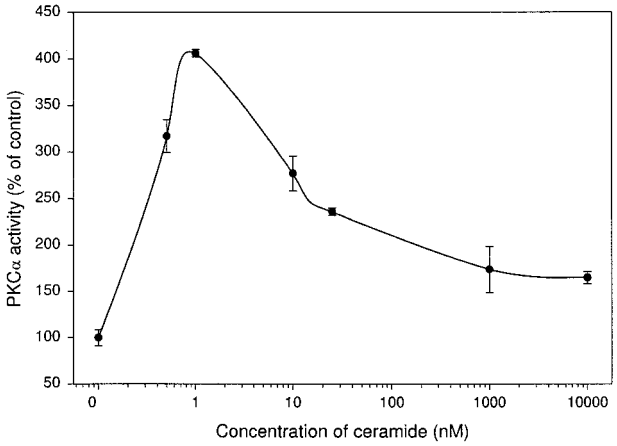

FIGURE 3: Effect of ceramide on PKC-α activity in vitro. Three micrograms of purified recombinant PKC-α was incubated with the indicated concentrations of C16-ceramide in the presence of 100 μg/mL PS and 0.25 mM calcium chloride, using 0.5 mg/mL histone III S as exogenous substrate in a total volume of 100 μL, as described under Experimental Procedures. Results are expressed as percentage of respective control values and are means of triplicates ± SD.

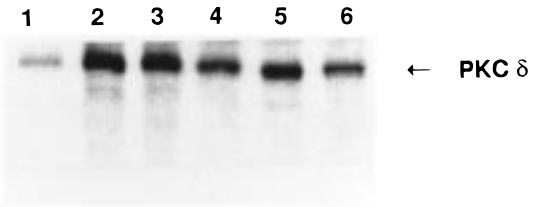

FIGURE 4: Effect of ceramide on PKC-δ autophosphorylation in vitro. One microgram of purified recombinant PKC-δ was incubated in the absence (lane 1) or in the presence of 100 μg/mL PS and 10 μg/mL DAG (lanes 2−6) with either vehicle (lane 2) or 10 nM (lane 3), 100 nM (lane 4), 1 μM (lane 5) or 2 μM (lane 6) C16-ceramide. Autophosphorylation was measured as described under Experimental Procedures. Results are representative of two independent experiments.

the effect of ceramide on the in vitro kinase activity of the different PKC isozymes. As shown in Figure 3, ceramide was able to activate PKC-α in a dose-dependent manner, when applied together with the cofactor phosphatidylserine (PS). Significant activation was observed already at 0.5 nM ceramide, and maximal activation was obtained at 1 nM and declined again at higher concentrations of ceramide. The extent of activation by ceramide and PS is comparable to the maximal activation of the enzyme induced by PS and DAG (data not shown). In the absence of PS, ceramide had no stimulatory effect on the activity of PKC-α, clearly indicating that PS is an essential cofactor required for activation, whereas DAG may be replaced by structurally related compounds, like ceramide. Stimulation of PKC-α with PS and DAG caused full activation of the kinase, which cannot be further increased by addition of ceramide. Moreover, when intact cells were exposed to ceramide, PKC-α displayed an increased activity as measured by an immunocomplex kinase assay (data not shown).

In contrast, no activation of PKC-*δ*, -*ϵ*, and -ζ isoenzymes by ceramide was found, using either histone III S, α-pseudosubstrate peptide, or myelin basic protein (MBP) as substrates (data not shown). However, PKC-*δ* showed decreased autophosphorylation after ceramide binding (Figure 4). In the absence of the cofactors PS and DAG, no significant autophosphorylation of PKC-*δ* was detected (lane 1). Addition of the cofactors PS and DAG caused a potent autophosphorylation of PKC-*δ* (lane 2) which was reversed by C16-ceramide (lanes 3−6). Maximal inhibition of PKC-*δ* autophosphorylation was obtained at 2 μM ceramide (51% inhibition).

Ceramide Selectively Translocates PKC-α to the Membrane Compartment. Translocation of PKC from the cytosol to the particulate fraction of a cell is usually considered to reflect activation of the enzyme. To see whether activation of PKC-α by ceramide is accompanied by translocation of the isoenzyme, Western blot analyses were performed. As shown in Figure 5, in unstimulated cells PKC-α is predominantly localized in the cytosolic fraction, whereas PKC-*δ* and PKC-*ϵ* are already present in a membrane-bound state under basal conditions. Upon addition of ceramide, there is a delayed translocation of PKC-α from the cytosol to the membrane which is clearly detectable after 1 h of ceramide stimulation. No redistribution of PKC-*δ*, PKC-*ϵ*, and PKC-ζ isoenzymes was observed upon ceramide exposure (Figure 5). A caveat that should be noted is that it would be difficult to observe translocation of PKC-*δ* and PKC-*ϵ* to membranes, as they are largely recovered in this fraction in the unstimu-

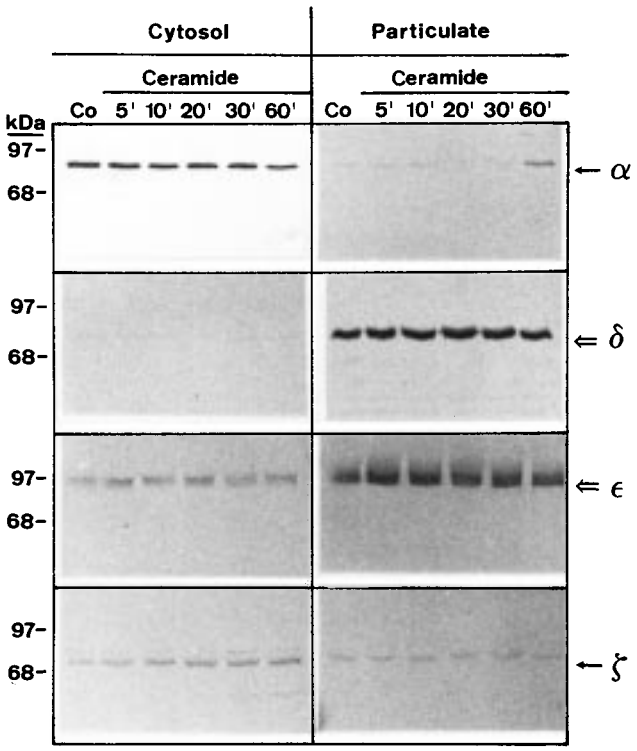

FIGURE 5: Effect of ceramide on PKC isoenzyme translocation in mesangial cells. Quiescent mesangial cells were stimulated with either vehicle (Co) or 30 μM C16-ceramide for the indicated time periods. Thereafter cells were fractionated into cytosolic and particulate fractions, subjected to SDS−PAGE, and transferred to nitrocellulose, and Western blot analyses were performed using a monoclonal antibody against PKC-α (at a dilution of 1:100) or polyclonal antibodies against PKC-δ, -ϵ, and -ζ (at a dilution of 1:1000). Bands were visualized with alkaline phosphatase.

lated state. Also, it is generally difficult to observe translocation of PKC-ζ.

Ceramide Triggers PKC-α-Mediated Feedback Inhibition of Phosphoinositide Turnover. One important functional cell response that has been appointed to PKC-α is the negative feedback regulation of hormone-stimulated phosphoinositide turnover in mesangial cells (*16*, *19*). Since ceramide has a stimulatory effect on PKC-α activity, we wanted to test whether this activation is physiologically relevant and followed by inhibition of inositol phosphate formation in mesangial cells. Stimulation of mesangial cells for 30 s with the extracellular nucleotide ATP leads to a 4-fold increase in inositol trisphosphate formation, thus confirming previously published data (*32*). Preincubation of cells for up to 2 h with ceramide followed by addition of ATP caused a time-dependent reduction of inositol trisphosphate ($InsP_3$) formation, as shown in Figure 6. Comparable data were obtained for $InsP_2$ and $InsP_1$. A significant decrease of $InsP_3$ was observed as early as 30 min after ceramide addition (48% inhibition), and almost complete inhibition was obtained after 2 h of ceramide incubation (94% inhibition). Ceramide had no effect on basal inositol phosphate levels (data not shown).

DISCUSSION

Ceramide has gained increasing attention as an important signaling molecule regulating fundamental biological processes such as cell proliferation and differentiation, oncogenesis, and immune and inflammatory reactions. However,

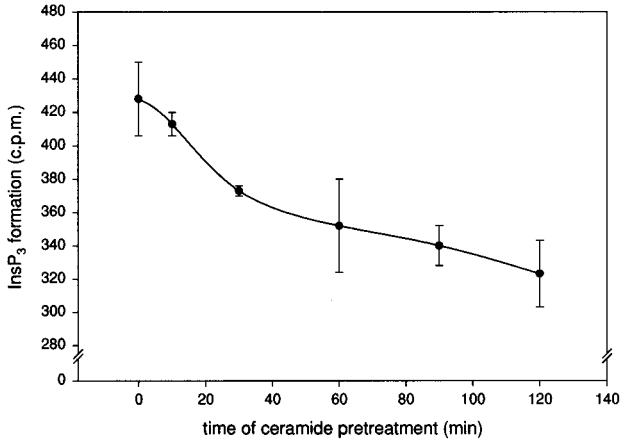

FIGURE 6: Effect of ceramide on ATP-induced inositol trisphosphate formation in mesangial cells. [^{3}H]Inositol-labeled mesangial cells were pretreated for the indicated time periods with 30 μM C16-ceramide followed by stimulation for 30 s with ATP (100 μM). Inositol trisphosphate was separated as described under Experimental Procedures. Inositol trisphosphate in unstimulated cells was 317 ± 20. Results are expressed as means ± SD (n = 4).

the mechanism of action of ceramide is still ill-defined. Ceramide is produced either by the hydrolysis of sphingomyelin by both neutral and acidic sphingomyelinases or from sphinganine by the action of ceramide synthase (*4*−*6*). With respect to direct targets of ceramide, suggestions for several ceramide-activated enzymes have been forwarded, including the kinase c-Raf (*7*), a 97 kDa membrane-associated protein kinase (*8*) which was suggested to be identical to kinase suppressor of Ras (*9*), a protein phosphatase 2A-related enzyme (*10*), and PKC-ζ (*11*). However, for most of these studies, the evidence provided was circumstantial, and with the exception of c-Raf, direct proof for ceramide binding is still lacking.

In this study, we used a photoaffinity labeling analogue of ceramide of high [^{125}I]iodine-specific radioactivity (>2000 Ci/mmol) which we previously have used successfully to identify ceramide binding to c-Raf (*7*), to screen another important class of potential direct ceramide targets, the family of PKC isoenzymes.

The present data clearly demonstrate that ceramide specifically binds to PKC-α and -δ isoenzymes but not to PKC-ϵ and -ζ isoenzymes in mesangial cells. Binding of ceramide to PKC-α is accompanied by activation of the enzyme in vitro and in vivo and subsequent inhibition of hormone-stimulated phosphoinositide signaling, thus establishing a novel type of cross-communication between cytokine signaling pathways (i.e., ceramide production) and classical G protein-coupled receptor signaling via phospholipase C-mediated second-messenger formation.

Moreover, it is noteworthy that ceramide has been reported to inhibit DAG-kinase (*33*) and to cause accumulation of DAG in HL-60 human leukemia cells which may contribute to further activation of PKC isoenzymes.

By contrast, binding of ceramide to PKC-δ correlates with a decreased autophosphorylation of the enzyme, thus suggesting possible inhibition of its catalytic activity. However, in vitro kinase assays using conventional PKC substrates such as histone, MBP, or α-pseudosubstrate peptide did not show a reduced activity of PKC-δ. In this context, it needs to be mentioned that so far no physiological substrates for PKC-δ have been identified. This makes it difficult to judge

regulatory functions of mediators such as ceramide by using artificial substrates such as histone, MBP, and α-pseudosubstrate peptide. In addition, a physiological consequence of the postulated ceramide-induced inhibition of PKC-δ is not yet known and will require additional studies. In any case, the approach chosen in this study has unequivocally identified PKC-α and -δ as direct targets of ceramide action in mesangial cells. In previous studies using more indirect approaches, Jones and Murray (*34*) using mouse epidermal (HEL-37) and human skin fibroblasts (SF3155) and Lee et al. (*35*) using Molt-4 human leukemia cells also suggested PKC-α as a putative ceramide target. In contrast to our data, these authors concluded that ceramide inhibits bradykinin-stimulated PKC-α activity (*34*) or indirectly affects in a delayed fashion PKC-α activity (*35*). The discrepancy between these reports may be due to the different experimental procedures applied or may be attributed to different responses of the cell lines employed. Recently, Sawai et al. (*36*) demonstrated that ceramide induces an inverse translocation, i.e., from the particulate fraction to the cytosol, of PKC-δ and -ϵ isoenzymes in HL-60 cells which would correspond to the inhibition of PKC-δ activity observed in our study. Furthermore, Müller et al. (*11*) have reported that ^{14}C-labeled ceramide binds to PKC-ζ immunoprecipitates obtained from U937 cells. The increased autophosphorylation of PKC-ζ immunoprecipitates in the presence of ceramide led the authors conclude that ceramide causes a direct activation of PKC-ζ isoenzyme. Our results are in apparent contrast to these observations in that we definitely observed no TID-ceramide binding to PKC-ζ in intact mesangial cells nor to purified recombinant PKC-ζ isoenzyme (*7*, and Figures 1 and 2). In this context, it is worth noting that the PKC-ζ antibodies used by Müller et al. (*11*) have been reported to cross-react with PKC-α (*37*−*39*), and therefore in cells expressing high levels of PKC-α, considerable contamination of PKC-ζ immunoprecipitates with PKC-α might occur and in part be responsible for the reported data.

An important question that immediately arises is concerned with the molecular mechanism of ceramide binding to its direct targets identified so far, i.e., PKC-α, PKC-δ (this study), and c-Raf (*7*). Is there a specific binding motif for ceramide that is common in these molecules? The conserved C1 and C2 domains in the regulatory part of PKC isoenzymes are candidates for lipid interacting motifs. The C2 part present in the conventional cPKCs contains a Ca^{2+}-phospholipid binding domain (CaLB domain) that is responsible for interaction with phospholipids. The C1 part present in all PKC isoenzymes contains tandem repeats of cysteine-rich motifs, a so-called zinc butterfly, that is thought to be responsible for binding of DAG and phorbol esters (*12*, *13*). Preliminary data using a ceramide−Sepharose column to purify ceramide-target proteins demonstrate that DAG is able to compete for ceramide binding almost as efficient as cold ceramide does (Huwiler and Pfeilschifter, unpublished observations). A homologous cysteine-rich motif is present in c-Raf (*40*). Although these lipid binding motifs share certain common characteristics, they are not functionally equivalent (*41*) which may explain why only PKC-α and -δ but not PKC-ϵ or -ζ are able to directly bind ceramide. We are presently examining this hypothesis and try to identify a minimal sequence motif required for ceramide binding.

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