The EMBO Journal vol.14 no.9 pp.1961–1969, 1995

PKC ζ is a molecular switch in signal transduction of TNF-α, bifunctionally regulated by ceramide and arachidonic acid

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Communicated by A.Ullrich

Tumor necrosis factor (TNF-α) stimulates a number of signal transduction pathways in which phospholipases produce lipid second messengers. However, the immediate molecular targets of these messengers, in particular those of ceramide and arachidonic acid (AA) and their role in TNF signaling are not well defined. In this study we investigated the relationship of ceramide and AA in regulating an atypical PKC isozyme, PKC ζ. U937 cells responding to TNF-α treatment with NFκB activation displayed enhanced phosphorylation of PKC ζ, which is already detectable 30 s after stimulation. [^{14}C]ceramide specifically binds to and regulates kinase activity of PKC ζ in a biphasic manner. Binding studies indicate high and low affinity binding with b_{max} values of 60 and 600 nM and K_d values of 7.5 and 320 nM respectively. At ceramide concentrations as low as 0.5 nM an up to 4-fold increase in autophosphorylation is obtained, which, at concentrations >60 nM, again declines to basal levels. Interestingly, AA competes for ceramide binding and inhibits basal and ceramide-stimulated PKC ζ kinase activity at <100 nM. Metabolism of [^{14}C]ceramide in cells is slow and is inhibited in the presence of equimolar concentrations of lyso-phosphatidylcholine. Based on the bifunctional modulation of PKC ζ by the lipid messengers ceramide and AA, a model of TNF signal pathways is suggested in which PKC ζ takes a central position, acting as a molecular switch between mitogenic and growth inhibitory signals of TNF-α.

Key words: arachidonic acid/ceramide/protein kinase C ζ/ tumor necrosis factor

Introduction

TNF-α is a pleiotropic cytokine capable of inducing, depending on the cell type, strikingly different cellular reactions, such as cell proliferation, differentiation and necrotic or apoptotic cell death (for reviews see Fiers, 1991; Pfizenmaier *et al.*, 1992; Heller and Krönke, 1994). In many cell types TNF activates a cytosolic phospholipase A_2, which produces arachidonic acid (AA) as a lipid second messenger with a potentially broad spectrum of intracellular targets (for review see Mayer and Marshall, 1993). In addition, it has been shown that TNF receptor-mediated cellular activation is preceded by the rapid activation of sphingomyelinases producing the second messenger ceramide (for reviews see Kolesnick, 1991; Hannun, 1994). Exogenously added short chain ceramides can mimic several TNF actions (Kolesnick, 1991; Obeid *et al.*, 1993; Olivera *et al.*, 1992; Jarvis *et al.*, 1994), emphasising the role of this novel messenger in TNF signaling. However, similarly to arachidonic acid, the immediate intracellular targets of ceramide and their role in conveying diverse TNF actions are largely unknown.

The involvement of PKC in signal transduction of TNF-α has been proposed by several authors, although the typical messengers necessary for activation of conventional PKCs, such as Ca^{2+} and phosphatidylinositol 3,4-biphosphate-derived inositol triphosphate, are not generated by TNF-α (Pfizenmaier *et al.*, 1992). However, rapid production of diacylglycerol (DAG) and TNF-mediated activation of PKC has been demonstrated in various human leukemic cells of distinct lineages (Schütze *et al.*, 1990), but the subtypes of PKC activated by TNF and their role in TNF signaling have not been determined (Schütze *et al.*, 1990). Since the activating lipids of the atypical PKCs are still unknown (Bell and Burns, 1991), we considered novel or atypical PKCs such as PKC ζ as possible candidates involved in the signal transduction of TNF-α, based on the following observations: (i) TNF activates NFκB via a DAG-responsive acidic sphingomyelinase in tumor cells of hematopoetic origin (Schütze *et al.*, 1992). In the same cells, TNF-mediated NFκB activation is independent of PMA-responsive PKCs (Wiegmann *et al.*, 1994). (ii) Over-expression of PKC ζ is by itself sufficient to stimulate a permanent translocation of functionally active NFκB into the nucleus in fibroblasts (Diaz-Meco *et al.*, 1993). (iii) PKC ζ activates an unknown kinase which phosphorylates IκB (Diaz-Meco *et al.*, 1994). (iv) Functional deletion of the endogenous PKC ζ gene by over-expression of a dominant negative mutant results in inhibition of mitogenic signals, indicating that PKC ζ is involved in mitogenic signaling pathways (Berra *et al.*, 1993; Diaz-Meco *et al.*, 1993).

Cloned and purified PKC ζ has been shown to be moderately stimulated by micromolar concentrations of phosphatidylserine and other acidic phospholipids, but not by Ca^{2+}, phorbol ester or DAG (Ono *et al.*, 1989a; Ways *et al.*, 1992; Tsutsumi *et al.*, 1993). The lack of regulation by Ca^{2+} and phorbol esters is consistent with the absence of the Ca^{2+} binding (C2) domain and presence of only one instead of two cysteine-rich zinc finger-like motifs in the C1 domain, responsible for phorbol ester binding (Ono *et al.*, 1989b). Based on this information, PKC ζ was classified as a phorbol ester-independent, atypical PKC that is neither translocated to the membrane fraction nor down-regulated in response to acute or chronic exposure

to phorbol esters (Ways *et al.*, 1992). From a structural point of view, PKC ζ is interesting in that there are significant homologies in the single zinc finger motif with proteins involved in mitogenic signaling, namely the raf-1 kinase and the vav proteins (Ahmed *et al.*, 1991). To date, there is still little information on potentially physiological regulators of PKC ζ. Recent data suggested that phosphatidylinositol 3,4,5-trisphosphate (PIP_3) can function as an activator of PKC ζ in *in vitro* kinase assays (Nakanishi *et al.*, 1993), but the relevance of PIP_3 for *in vivo* regulation of PKC ζ remains unknown. We have here investigated whether two prominent messengers involved in TNF signal transduction, ceramide and AA, exert a direct influence on PKC ζ kinase activity.

Results

Immunoprecipitation of PKC ζ

Two antisera specific for PKC ζ, a commercially available and a C-terminal peptide-specific antiserum prepared in our laboratory, were employed for immunoprecipitation of PKC ζ. Both antisera precipitated a single protein from U937 cell lysates of molecular weight ~76 kDa, as revealed by SDS−PAGE and subsequent Western blotting (Figure 1A). This is in accordance with the molecular weight of 76 kDa deduced from the open reading frame of the human cDNA (Kochs *et al.*, 1993). Specificity of the antisera for PKC ζ was verified by competition with the immunizing peptide in Western blot analysis. Since the immunizing peptide was derived from the C-terminal region of the protein, which has almost identical sequence homology to the recently identified PKC ι (Selbie *et al.*, 1993), we performed immunoprecipitations in U937 cells with a monoclonal antibody against PKC ι directed against a peptide comprising the C1 and V3 regions. However, PKC ι could not be detected in U937 cells by immunoprecipitation and Western blot analysis (Figure 1B). Further, immunoprecipitates prepared with PKC ζ-specific antiserum gave no signal with the PKC ι-specific monoclonal antibody on Western blots, indicating no cross-reactivity with PKC ζ (data not shown). Therefore, we conclude that in U937 cells PKC ζ is expressed in significant amounts, whereas the structurally closely related isozyme PKC ι is either not expressed or is expressed at a level below the detection limit (Figure 1B).

TNF-α and C_6-ceramide stimulate and arachidonic acid inhibits the phosphorylation of PKC ζ in U937 cells

Activation of PKCs is accompanied by intramolecular autophosphorylation, which reflects the activity state of the kinase (Newton and Koshland, 1987). Immunoprecipitation of PKC ζ from $^{32}P_i$-labeled U937 cells revealed that PKC ζ exhibits a basal kinase activity which may result from unknown cellular stimulants and messengers or occurs during the process of immunoprecipitation (Figure 2A). Pre-incubation of $^{32}P_i$-labeled cells with 100 nM TNF-α for 30 s or 10 min resulted in enhanced phosphorylation of PKC ζ (Figure 2A), suggesting, that PKC ζ participates in TNF-induced signaling pathways. In parallel with the phosphorylation, the protein level of PKC ζ in the Triton-soluble fraction was estimated to be ~100 ng/4×10^7 cells. No change was observed upon TNF treatment (Figure 2B), indicating that PKC ζ is neither translocated nor up- nor down-regulated by TNF-α within a 10 min treatment time. Extracellular addition of 20 μM C_6-ceramide to $^{32}P_i$-labeled cells mimicked TNF action (Figure 2C). When *in vitro* kinase assays with PKC ζ immunoprecipitated from TNF- or C_6-ceramide-treated unlabled U937 cells were performed, similar results were obtained (data not shown). Scanning of autoradiographs revealed an average 3-fold increase in PKC ζ phosphorylation after 10 min TNF stimulation (Figures 2A and C). Together, these data suggest that both TNF and the membrane permeable C_6-ceramide stimulate kinase activity of PKC ζ. Interestingly, when U937 cells were pre-treated with 20 μM arachidonic acid, basal phosphorylation of PKC ζ was reduced and subsequent stimulation with TNF or C_6-ceramide no longer resulted in activation of PKC ζ (Figure 2C). The protein levels of PKC ζ did not change during the experiment shown in Figure 2C (data not shown).

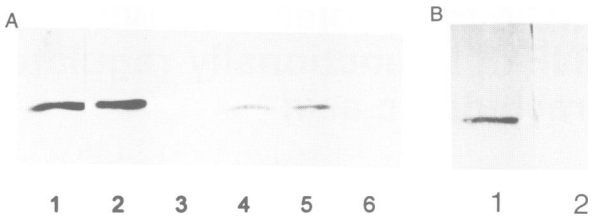

Fig. 1. Western blots of immunoprecipitates of PKC ζ performed with different antisera against PKC ζ. **(A)** Lanes 1–3: immunoprecipitates of PKC ζ of 3×10^7 U937 cells/lane performed with antiserum I detected on Western blots with antisera I and II (lanes 1 and 2); immunoprecipitation in the presence of the immunizing peptide (5 μg/ml) (lane 3). Lanes 3–6: immunoprecipitates of PKC ζ of 3×10^7 cells/lane with the antiserum II detected on Western blots with antisera I and II (lanes 4 and 5); immunoprecipitation in the presence of the immunizing peptide (5 μg/ml) (lane 6). **(B)** Western blot of immunoprecipitates of PKC ζ and PKC ι from U937 cells. Immunoprecipitations were performed with antiserum I against PKC ζ (lane 1) and a monoclonal antibody against PKC ι (lane 2) from 4×10^7 U937 cells. PKC ζ in (A) and (B) was visualized by staining with a goat anti-rabbit secondary antibody using the AuroProbe BL system.

Stimulation of NFκB translocation by TNF and C_{16}-ceramide

In order to verify that the U937 cell line employed in our studies show a typical TNF response, we have investigated TNF activation of NFκB. As shown by the electrophoretic mobility shift assay, a 20 min incubation of U937 cells with 50 ng/ml TNF resulted in activation and translocation of NFκB to the cell nucleus (Figure 2D). Further, employing permeabilized U937 cells, C_{16}-ceramide was also shown to activate NFκB, with effective concentrations as low as 50 nM (Figure 2E). Accordingly, for the cell line under study the TNF signal pathway leading to NFκB activation is functional.

Concentration-dependent activation of PKC ζ autophosphorylation by ceramide

As the above data indicated that the TNF-induced messenger ceramide is involved in regulation of PKC ζ kinase activity, a direct activation of PKC ζ by ceramide was investigated in an *in vitro* kinase assay employing

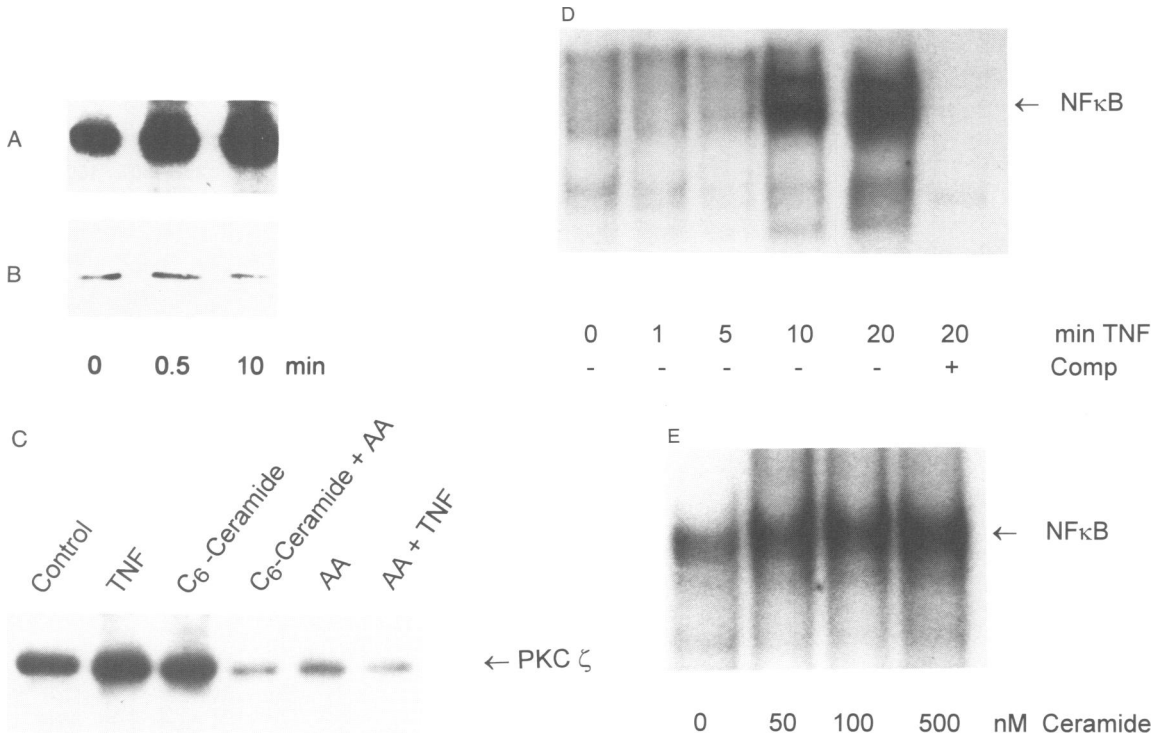

Fig. 2. TNF-α and ceramide stimulate the phosphorylation of PKC ζ and the translocation of NFκB to the cell nucleus. (**A**) U937 cells were labeled for 4 h with $^{32}P_i$ (50 μCi/ml) and incubated for 0.5 or 10 min with TNF-α (100 ng/ml) prior to lysis of cells and subsequent immunoprecipitation of PKC ζ. Immunoprecipitates of 10×10^6 cells/lane were separated on SDS−PAGE, blotted to nitrocellulose and autoradiographed for 7 days. (**B**) Western blots of immunoprecipitates of PKC ζ from U937 cells (4×10^7 cells/lane) pre-incubated with TNF-α for 0.5 or 10 min. (**C**) Autoradiograph of PKC ζ immunopreciptates of $^{32}P_i$-labeled cells (procedure as in A). Prior to lysis cells were incubated for 10 min with TNF (100 ng/ml), C_6-ceramide (20 μM) and AA (20 μM) or with C_6-ceramide and TNF after pre-incubation of cells for 10 min with AA. No factor was added in the control. (**D**) Activation of NFκB by TNF. After stimulation of U937 cells with 50 ng/ml TNF, nuclear extracts were prepared at different times and assayed for NFκB binding activity by electrophoretic mobility shift assay. Specificity of oligonucleotide binding to NFκB was determined by incubating the 20 min extracts in the presence of 100-fold excess of unlabeled oligonucleotide (Comp). The same results were obtained in three independent experiments. (**E**) Activation of NFκB by C_{16}-ceramide. Permeabilized U937 cells (see Materials and methods) were incubated with increasing concentrations of C_{16}-ceramide for 10 min. Nuclear extracts were prepared and analyzed for NFκB binding activity by electrophoretic mobility shift assays as described in Materials and methods.

immunoprecipitated PKC ζ. The assay was performed in the absence of detergents and phosphatidylserine.

Dose−response curves were established with ceramides from different sources to investigate their ability to modulate the autophosphorylation activity of PKC ζ. C_{16}-ceramide was found to be a potent activator of PKC ζ autophosphorylation, starting at concentrations as low as 500 pM (Figure 3A). A 4-fold activation of PKC ζ is sustained up to 60 nM. At higher concentrations, however, C_{16}-ceramide becomes less effective and at 250 nM−1 μM ceramide, no stimulation of autophosphorylation activity was seen (Figure 3B and C). C_{16}-ceramide from a different source and ceramide type III also produced biphasic responses and stimulated PKC ζ kinase activity with maxima at 5 and 25 nM respectively. As before, at concentrations above 200 and 100 nM, respectively, these ceramide preparations also completely inhibited autophosphorylation (data not shown). The bifunctional effect of ceramide on autophosphorylation was paralleled by a similar modulation of substrate phosphorylation *in vitro*; phosphorylation of myelin basic protein (50 μg/ml kinase buffer) was stimulated at low concentrations and inhibited at high levels of ceramide (data not shown), indicating that stimulation of autophosphorylation of PKC ζ is a measure of its activity state. The dose-dependent positive and negative regulatory actions of the different ceramides on PKC ζ could potentially be due to differing compositions of fatty acid moieties of the ceramides, depending on the source and isolation procedure, or could be due to the presence of biologically inactive or inhibitory stereoisomers of ceramide. However, analysis of the various ceramide preparations by high performance thin layer chromatography (HPTLC), as described in Materials and methods, resulted in only a single spot and thus revealed no contamination with other lipids. In *in vitro* assays based on the conventional method of kinase activation by mixed lipid micelles there was no detectable activation of PKC ζ by ceramide (data not shown).

Action of sphingosine on PKC ζ

Sphingosine is a metabolic product of ceramide and could be a minor contaminant of ceramide preparations. As sphingosine is known as an inhibitor of conventional PKCs (Hannun and Bell, 1989), it could also potentially interfere with ceramide-stimulated PKC ζ kinase activity. We found that sphingosine stimulated PKC ζ kinase activity, although in a rather narrow range between 25 and 50 nM. Higher concentrations (>75 nM) were less effective or inhibitory (Figure 4). Ceramide activates PKC ζ at ~10- to 50-fold lower concentrations, therefore minor sphingosine contamination of the ceramide preparations could not contribute to the observed kinase activation.

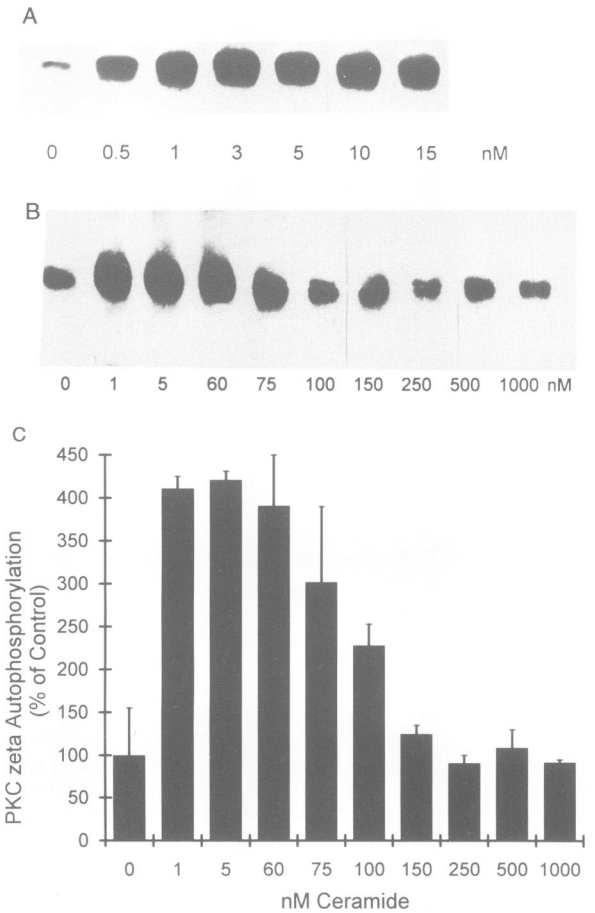

Fig. 3. Bifunctional modulation of PKC ζ by ceramide. (**A**) Ceramide-dependent stimulation of autophosphorylation of PKC ζ at low concentrations of ceramide. Immunoprecipitates of PKC ζ from U937 cells (3×10^6 cells/lane) were pre-incubated for 5 min with increasing concentrations of ceramide (0.5–15 nM) and subjected to *in vitro* autophosphorylation as described in Materials and methods. The reaction mixture was fractionated by SDS–PAGE (7.5%), dried and exposed to overnight autoradiography. Similar results were obtained in five independent experiments. (**B**) Bifunctional modulation of the autophosphorylation of PKC ζ. Immunoprecipitates of PKC ζ were pre-incubated with different ceramide concentrations and the kinase assay was performed as in (A). (**C**) Densitometric analysis of the autoradiograph shown in (B). Basal autophosphorylation of PKC ζ without stimulation was set as 100%. Standard deviations were calculated from three independent experiments.

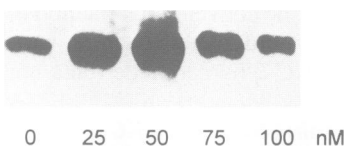

Fig. 4. Bifunctional modulation of PKC ζ by sphingosine. Modulation of the autophosporylation of PKC ζ by sphingosine. The experimental protocol was as described in Figure 3A. Similar results were obtained in five independent experiments.

Inhibition of basal and ceramide-stimulated autophosphorylation of PKC ζ by arachidonic acid

Because we have noted a strong inhibition of PKC ζ phosphorylation in U937 cells pre-treated with AA (Figure 2C), it was of interest to investigate whether this is a direct effect on PKC ζ in *in vitro* kinase assays with PKC

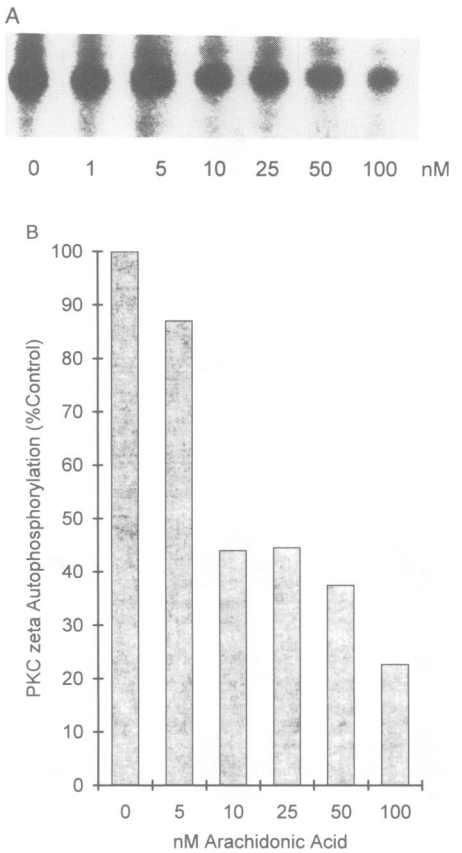

Fig. 5. Concentration-dependent inhibition of autophosphorylation of PKC ζ by arachidonic acid. (**A**) Immunoprecipitates of PKC ζ from U937 cells were pre-incubated with increasing concentrations of AA for 5 min prior to the kinase assay. Kinase activity of PKC ζ (3×10^6 cell aliquots/lane) was analyzed by SDS–PAGE and autoradiography. Identical results were obtained in three independent experiments. (**B**) Densitometric analysis of the autoradiopraph shown in (A). The basal autophosphorylation of PKC ζ was set as 100%.

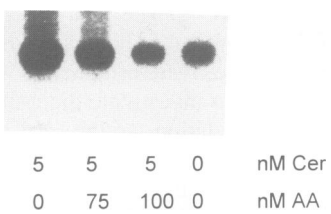

Fig. 6. Inhibition of ceramide-stimulated autophosphorylation of PKC ζ by arachidonic acid. Autoradiograph of Western blots of PKC ζ immunoprecipitates from 3×10^6 cells/lane. Immunoprecipitates were pre-incubated with 5 nM ceramide in the presence of 75 or 100 nM AA before the kinase assay was performed, as described in Materials and methods. Experiments were repeated three times with similar results.

ζ immunoprecipitates. As shown in Figure 5A, there was a concentration-dependent inhibition of basal autophosphorylation of PKC ζ, detectable at concentrations above 10 nM AA and >75% at 100 nM AA (Figure 5B). Likewise, PKC ζ activated by 5 nM ceramide, was efficiently inhibited by the simultaneous presence of 100 nM AA (Figure 6).

Binding characteristics of [^{14}C]ceramide to PKC ζ

The activation of PKC ζ by ceramide in a cell-free assay system had already suggested a direct interaction of PKC ζ

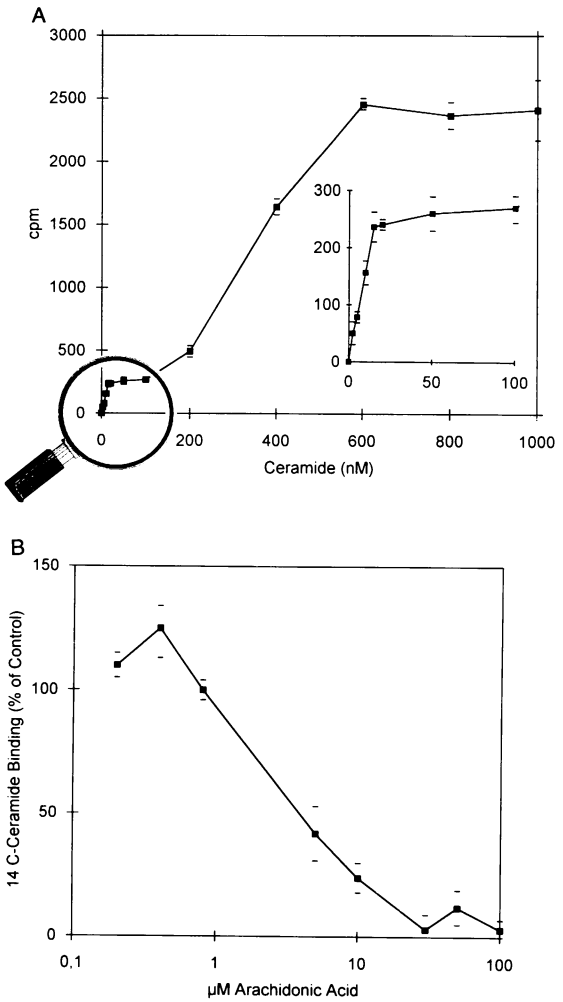

Fig. 7. Binding of [^{14}C]ceramide to PKC ζ. (**A**) Biphasic binding of [^{14}C]ceramide to PKC ζ. Immunoprecipitates of 3×10^7 cells/data point were incubated with [^{14}C]ceramide for 2 h in the presence or absence of a 100-fold excess of unlabeled ceramide at 4°C, extensively washed and radioactivity was determined as described in Materials and methods. Mean values were obtained from triplicate determinations. Similar data were obtained in two independent experiments. Insert: magnification of the high affinity binding between 0 and 100 nM ceramide. (**B**) Competition of [^{14}C]ceramide binding by AA. Immunoprecipitates of PKC ζ of 3×10^7 cells/data point were incubated with 320 nM [^{14}C]ceramide for 2 h in the absence (100%) or presence of increasing concentrations of AA or 32 μM ceramide as a control for non-specific binding, which was subtracted. Immunoprecipitates were washed and processed as described in Materials and methods. Mean values were obtained from triplicate determinations.

with this lipid messenger. In order to prove a direct interaction and to determine binding characteristics, saturation binding studies of [^{14}C]ceramide were performed with immunoprecipitates of PKC ζ. A biphasic saturation binding curve was observed with b_{max1} at 60 and b_{max2} at 600 nM and apparent K_d values of 7.5 and 320 nM, respectively (Figure 7A). To exclude non-specific binding of ceramide to antibodies and protein A beads present in the assay, parallel experiments were performed with immunoprecipitates of pre-immune serum. Under these conditions the values of total [^{14}C]ceramide binding were similar to those of non-specific binding in the presence of

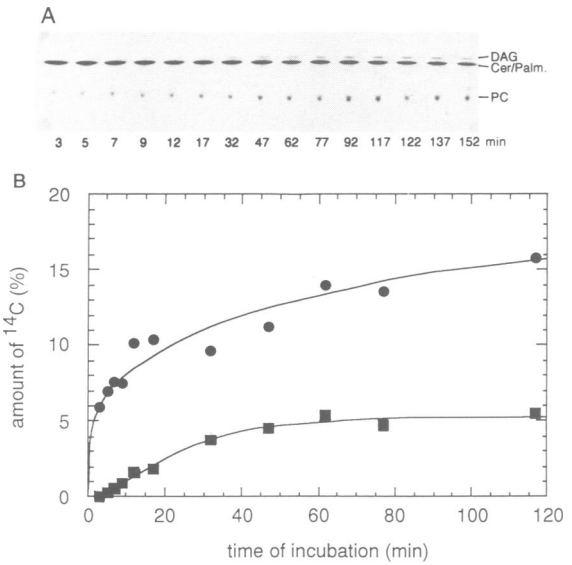

Fig. 8. Metabolism of [^{14}C]ceramide in U937 cells. (**A**) U937 cells (10×10^7) were incubated with 1.5 μCi [^{14}C]palmitoylceramide in 15 ml of RPMI. Aliquots of 0.5 ml were removed, washed three times with ice-cold PBS and subjected to lipid extraction. Lipid extracts were separated on HPTLC plates and autoradiographed. (**B**) Laser densitometric evaluation of the autoradiograph in (A). Phosphatidylcholine (●); DAG (■).

excess (100×) unlabeled ceramide (data not shown). This indicates that [^{14}C]ceramide binding is specific for PKC ζ.

As AA was found to down-regulate constitutive and ceramide-stimulated PKC ζ kinase activity (Figures 5 and 6), we investigated whether or not ceramide and AA compete for the same binding site(s). Competition studies were performed employing a fixed concentration of [^{14}C]ceramide and increasing concentrations of unlabeled AA. The data obtained show that AA is an inhibitor of ceramide binding, with half maximum inhibition at an ~20-fold molar excess (Figure 7B). Interestingly, at a similar molar excess of AA, inhibition of ceramide-stimulated PKC ζ activity was observed (Figure 6), suggesting that AA-mediated inhibition of PKC ζ kinase activity could be due to displacement of the activating messenger ceramide from the regulatory domain.

Metabolism of [^{14}C]ceramide

To investigate the potential metabolic route of the [^{14}C]palmitoyl moiety of C_{16}-ceramide, the fate of [^{14}C]palmitoylsphingosine was investigated over a time period of 2.5 h. Interestingly, the metabolism of ceramide was extremely slow and no labeled lipids other than DAG, ceramide and phosphatidylcholine were detectable even after 2 h (Figure 8A). Ceramide was not subjected to sphingomyelin synthesis. After 2 h, only 15% of the total accumulated [^{14}C]palmitoyl label occured in the phosphatidylcholine fraction and 5% in the DAG fraction, as determined by two-dimensional laser densitometry (Figure 8B). Since activation of phospholipase A_2 by TNF implies not only the formation of AA, but also the generation of lyso-phosphatidylcholine as a late messenger (Schütze *et al.*, 1992), it was of interest to note that metabolism of ceramide was completely inhibited in the presence of lyso-phosphatidylcholine (Figure 9A and B).

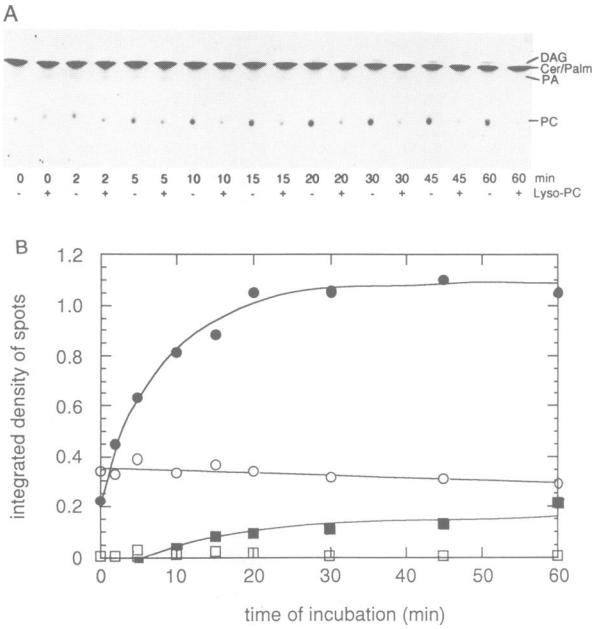

Fig. 9. Metabolism of [^{14}C]ceramide in the presence of equimolar concentrations of lyso-phosphatidylcholine. (**A**) U937 cells (5×10^7) were incubated with 2 μCi [^{14}C]palmitoylceramide in the presence and absence of equimolar concentrations of lyso-phosphatidylcholine. Aliquots (0.5 ml) of each cell suspension were removed, washed three times with ice-cold PBS and the lipids were extracted, separated on HPTLC plates and autoradiographed. (**B**) Laser densitometric evaluation of the autoradiographs shown in (A). Phosphatidylcholine, control (●); phosphatidylcholine + lyso-phosphatidylcholine (○); DAG control (■); DAG + lyso-phosphatidylcholine □).

The slow metabolism of ceramide is different from that of other ^{14}C-containing lipids, such as [^{14}C]lyso-phosphatidylcholine, degradation of which starts immediately (data not shown) and the fatty acid [^{14}C]palmitate is found within 3 min equally distributed in all membrane phospholipids (data not shown).

Discussion

The present study describes, for the first time, PKC ζ as a direct target of two lipid messengers implicated in TNF signaling pathways, ceramide and AA, and shows that PKC ζ kinase activity is under the bifunctional control of ceramide and AA. Our data suggest that PKC ζ could take a central position in TNF receptor-induced signal pathways, acting as a molecular switch between mitogenic and non-mitogenic pathways.

We have shown here that TNF-α and ceramide treatment of cells rapidly stimulates phosphorylation of PKC ζ (Figure 2) and subsequently induces translocation of NFκB to the cell nucleus. As ceramide specifically bound to and strongly activated PKC ζ in *in vitro* kinase assays (Figures 3 and 7), our data provide experimental evidence that PKC ζ is an immediate target of the TNF messenger ceramide. Of note is that PKC ζ was activated by sub-nanomolar concentrations of ceramide, with a maximum in the range 3–10 nM (Figure 3A). Activation occurs even in the absence of mixed lipid micelles, otherwise a prerequisite for PKC activation (Newton, 1993). This is in accordance with the finding that PKC ζ is not associated with cellular membranes, but is located in the cytosol and particulate fraction of cells (unpublished results). Moreover, at the activating concentrations of the messenger ceramide, this lipid is soluble in aqueous buffers and possibly also in the cytosolic compartment. Thus, physiologically produced ceramide should be accessible to PKC ζ. The unexpected finding, however, was that ceramide looses its stimulating activity at concentrations exceeding 60 nM (Figure 3B).

This bifunctional effect of ceramide on PKC ζ kinase activity correlates well with the biphasic binding characteristics of [^{14}C]ceramide to PKC ζ (Figure 7). Two alternative mechanisms could account for this biphasic binding and activation profile. First, assuming an interaction of ceramide and PKC ζ at a 1:1 molar ratio, the data are compatible with two distinct binding sites, binding ceramide with high and low affinity, respectively. Interestingly, the high affinity binding of ceramide correlates with its stimulatory potential, whereas the less effective concentrations correlate with the low affinity binding of ceramide. Alternatively, assuming a single ceramide binding site, the biphasic binding could be a result of changes in the molar ratio of ceramide–PKC ζ interactions at higher concentrations of ceramide. Independent of the underlying molecular events, the biphasic binding could reflect an intrinsic negative feedback mechanism of ceramide on PKC ζ autophosphorylation, in the sense that TNF stimulation of a sphingomyelinase and activation of PKC ζ by ceramide is followed by an intrinsic inhibitory phase when certain ceramide concentrations are exceeded.

Addition of [^{14}C]ceramide to cells resulted in fast accumulation, but very slow metabolism, of ceramide (Figure 8). This indicates that ceramide can function as a long-term messenger and can, upon continuous activation of sphingomyelinases by TNF, accumulate in the cells. Another interesting regulatory principle of ceramide metabolism was revealed in studies where [^{14}C]ceramide and lyso-phosphatidylcholine were added to cells together. This resulted in complete inhibition of ceramide degradation (Figure 9), indicating that lyso-phosphatidylcholine might function as an independent regulator of intracellular ceramide levels, eventually leading to high levels of ceramide in the cells in response to TNF-α.

Ceramide has been shown to mimick TNF action in many respects (Hannun and Linardic, 1993) and has in fact been implicated in positive and negative growth control in various cell types, including mitogenesis in fibroblasts (Olivera *et al.*, 1992), induction of differentiation and of tumor cell apoptosis (Okazaki *et al.*, 1989; Obeid *et al.*, 1993; Jayadev *et al.*, 1994; Liu *et al.*, 1994; Wolff *et al.*, 1994) Although ceramide is not the only lipid messenger system induced by TNF-α (Pfizenmaier *et al.*, 1992; Heller and Krönke, 1994), a bifunctional role of ceramide can be envisaged, since ceramide can induce both cell growth and growth inhibition. It can be postulated that in growth stimulatory signaling, components of mitogenic signaling pathways are activated by ceramide, whereas, during induction of apoptosis, the mitogenic signaling cascade would be inhibited by higher concentrations of ceramide. We have shown here that higher concentrations of ceramide no longer activate PKC ζ and thus potentially interrupt the link to mitogenic signaling cascades.

Apart from the potential down-regulating function of

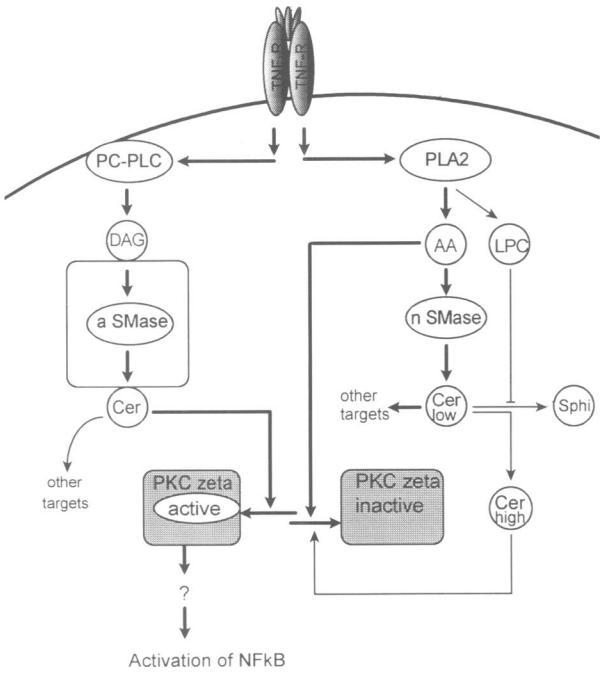

Fig. 10. Model of PKC ζ as a bifunctional molecular switch in TNF-α signaling cascades. For details see text. Abbreviations: TNF-R, TNF receptor; PC-PLC, phosphatidylcholine-specific phospholipase C; PLA2, phospholipase A_2; DAG, diacylglycerol; AA, arachidonic acid; LPC, lyso-phosphatidylcholine; a SMase, acidic sphingomyelinase; n SMase, neutral sphingomyelinase; Cer, ceramide; Sphi, sphingosine.

high ceramide concentrations, evidence for an independent negative regulatory mechanism of PKC ζ kinase activity, mediated by AA, has been obtained. AA has been implicated as a messenger for TNF with several potential functions (Heller and Krönke, 1994). In the context discussed here, it has been shown that AA is an activator of neutral sphingomyelinase (Jayadev *et al.*, 1994). On the other hand, we here show that AA acts as an inhibitor of constitutive and ceramide-induced PKC ζ autophosphorylation. AA was found to inhibit ceramide-induced NFκB translocation (unpublished results), as well as TNF and C_{16}-ceramide-induced PKC ζ activation in whole cells (Figure 2), and thus may inhibit mitogenic signals induced via ceramide. As discussed above, this inhibitory function may be enforced by the concomitant release of lysophosphatidylcholine, an inhibitor of ceramide metabolism.

Based on the published data and those presented here, a model of TNF signaling is proposed in which diverse TNF-induced signal pathways converge at the level of PKC ζ (Figure 10): PKC ζ is a molecular switch with three 'positions', directly modulated by two lipid messengers, ceramide and AA, which compete for the same binding site on PKC ζ: (i) PKC ζ is activated by low levels of ceramide; (ii) high levels of ceramide revert the activation state to basal kinase activity and may potentially cause insensitivity of PKC ζ to other messengers; (iii) AA inhibits basal and ceramide-induced kinase activity of PKC ζ.

The biological relevance of this switch function of PKC ζ can be deduced from the mechanisms of TNF activation of acidic and neutral sphingomyelinases: TNF induces the activation of a phosphatidylcholine-specific phospholipase C and the activation of a cytosolic phospholipase A_2 (Pfizenmaier *et al.*, 1992; Heller and Krönke, 1994). The lipid messengers produced by these phospholipases, DAG and AA, are responsible for the selective activation of a lysosomal/endosomal acidic sphingomyelinase and a neutral cytosolic sphingomyelinase, respectively. It has been demonstrated that only the acidic and not the neutral sphingomyelinase is able to activate NFκB (Wiegmann *et al.*, 1994). Since PKC ζ stimulates NFκB activation (Beg *et al.*, 1993; Lozano *et al.*, 1994), it may be concluded that PKC ζ is the target of ceramide derived from the acidic sphingomyelinase. Experimental evidence for this sequence of events in the proposed cascade is provided by recent results showing that extracellular phosphatidylcholine-specific phospholipase C can mimic TNF-mediated NFκB translocation in fibroblasts transfected with different mutants of PKC ζ (Diaz-Meco *et al.*, 1993). In support of this, D 609, a selective inhibitor of phosphatidylcholine-specific phospholipase C (Schütze *et al.*, 1992) inhibits TNF-induced PKC ζ kinase activity completely (unpublished results).

The second major arm of the presently known TNF signal pathways involves the activation of a phospholipase A_2, which generates two messengers from phospholipids within minutes upon TNF stimulation, AA and lysophosphatidylcholine (Yanaga *et al.*, 1992). It has been proposed that AA stimulates the activation of a neutral cytosolic sphingomyelinase (Jayadev *et al.*, 1994), which, in contrast to the acidic sphingomyelinase, is not involved in NFκB activation (see above). The model described here resolves this apparently controversial finding that the very same second messenger produced by distinct phospholipase cascades is active only in one case and not in the other. We propose that independently activated TNF signal pathways converge at a common target, PKC ζ, which is subject to dual control by different messenger cascades. In the PLA_2 arm of the TNF signal cascade, AA serves not only as an activator of the neutral sphingomyelinase but, at the same time, an inhibitor of PKC ζ kinase activity and thus of NFκB activation.

Materials and methods

Reagents

Semisynthetic C_{16}-ceramide and sphingosine were purchased from Biomol (Hamburg, Germany) and ceramide type III, sphingosine (from bovine brain sphingomyelin), C_{16}-ceramide (semisynthetic from bovine brain cerebrosides) and arachidonic acid were from Sigma (St Louis, MO). The rabbit antiserum against PKC ζ was generated as described (McGlynn *et al.*, 1992) from a peptide derived from the C-terminal sequence of PKC ζ (antiserum I). A second polyclonal antiserum against PKC ζ (antiserum II), the immunizing peptide and T4 polynucleotide kinase were from Boehringer Mannheim (Mannheim, Germany). The monoclonal antibody against PKC ι was from Signal Transduction, Nottingham, UK. The Trans-Port transient cell permeabilization kit was from GIBCO/BRL, Eggenstein, Germany. Oligonucleotides were from MWG-Biotech (Ebersberg, Germany). [^{14}C]Palmitoylsphingosine (C_{16}-ceramide), [γ-^{32}P]ATP and $^{32}P_i$ were from Amersham International (Amersham, UK).

Cell culture

For binding assays and maximal expression of PKC ζ, U937 cells were incubated in RPMI supplemented with 5% fetal calf serum. To keep the cells continuously in the logarithmic growth phase the medium was changed daily. Prior to the experiment, cells were incubated in fresh medium for 6 h, harvested by centrifugation and washed in ice-cold phosphate-buffered saline (PBS).

Immunoprecipitation

U937 cells were washed with cold PBS and lysed for 1 h on ice in 1 ml of buffer A [50 mM Tris−HCl, pH 7.4, 150 mM NaCl, 2 mM phenylmethylsulfonyl flouride (PMSF), 100 μg/ml leupeptin, 0.5% Triton X-100 and 5 mM EDTA]. The extracts were cleared in an Eppendorf centrifuge (15 000 *g* for 15 min at 4°C) and the particulate fraction was discarded. The supernatant (Triton-soluble fraction) was used for immunoprecipitation and 2 μl of rabbit anti-PKC ζ antiserum or pre-immune serum was added for 12 h at 4°C. Resulting precipitates were removed by centrifugation and 30 μl of protein A−Sepharose beads were added to the supernatant. After 1 h incubation at 4°C, the immunoprecipitates adsorbed to the beads were washed 3–4 times for 10 s with ice-cold washing buffers (see below).

Western blotting of PKC ζ

Immunoprecipitates of PKC ζ from 4×10^7 U937 cells/lane were subjected to electrophoreses on 7.5 or 10% polyacrylamide gels and transferred to nitrocellulose sheets on a semi-dry transfer unit with a buffer system according to Towbin (Towbin *et al.*, 1979). Western blots were visualized using the immunogold silver staining kit AuroPobe™ BL plus (Amersham).

PKC ζ kinase assays

For PKC ζ kinase assays, the immunoprecipitates were washed once in buffer A, three times in buffer B (buffer A without EDTA) and once in buffer C (50 mM Tris−HCl, pH 7.4, 150 mM NaCl and 1 mM PMSF) to remove non-specific background activity. In a last step, the immunoprecipitates were washed and resuspended in a final volume of 50 μl of kinase buffer (20 mM Tris−HCl, pH 7.4, 50 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin and 1 mM sodium orthovanadate). PKC ζ was dissociated from the immune complex by incubation of the immunoprecipitate with the immunizing peptide (20 μg/ml) for 12 h at 4°C and subsequent centrifugation. The kinase reaction for autophosphorylation was started by addition of 4 μCi [γ-^{32}P]ATP at 37°C and the phosphorylation was stopped by addition of 50 μl of reducing Laemmli sample buffer. Stock solutions of lipids in ethanol and dilutions in kinase buffer were freshly prepared for each experiment from lyophilized aliqots. The samples were pre-incubated with lipids for 5 min. The proteins were separated on 7.5 or 10% SDS−PAGE and autoradiography was performed with membranes of Western blots. Protein loading of the gels and efficiency of transfer to membranes was controlled by immunglobulin staining with the AuroProbe staining kit (Amersham).

In vivo labeling of cells

U937 cells (4×10^7) were incubated in phosphate-free medium for 4 h. Then 50 μCi/ml $^{32}P_i$ was added for an additional 4 h. Cells were washed and resuspended in medium for equilibration. After addition of TNF (100 ng/ml) the cells were lysed in buffer A containing phosphatase inhibitors (20 mM *p*-nitrophenylphosphate, 1 mM sodium fluoride and 1 mM sodium orthovanadate) and subjected to immunoprecipitation for PKC ζ. PKC ζ was separated on SDS−PAGE and autophosphorylation was analyzed on autoradiograms of Kodak X-OMAT films.

Nuclear extract preparation

For preparation of nuclear extracts 5×10^6 cells were left untreated or were stimulated either with TNF-α (50 ng/ml) or C_{16}-ceramide (50–500 nM) after permeabilization of cells with the Trans-Port transient cell permeabilization kit (GIBCO/BRL), according to the manufacturer's protocol. Cells were washed twice with ice-cold PBS and resuspended in 400 μl of buffer A (10 mM KCl, 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF). After 15 min on ice, 25 μl of 10% Nonidet P-40 was added for 2 min and nuclei were pelleted and resuspended in buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT). After 20 min of shaking and subsequent centrifugation, the lysates containing the nuclear proteins in the supernatant were used for the electrophoretic mobility shift assay after protein determination (Bio-Rad), with bovine serum albumin as standard.

Electrophoretic mobility shift assays

HPLC-purified NFκB-specific oligonucleotides (5′-ATCAGGGSCTTT-CCGCTGGGGACTTTCCG-3′) were from MWG-Biotech and were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Elecrophoretic mobility shift assays were performed by incubating 5 μg of nuclear extracts with 5 μg poly(dI−dC) in a binding buffer (5 mM HEPES, pH 7.8, 5 mM $MgCl_2$, 50 mM KCl, 0.2 mM EDTA, 5 mM DTT and 10% glycerol). The double-stranded, end-labeled, purified oligonucleotide probe ($2\times10^4-5\times10^4$ c.p.m.) was added and the reaction mixture incubated for 15 min at room temperature. The samples were separated by native PAGE in low ionic strength buffer.

[^{14}C]Ceramide binding assays

For the [^{14}C]ceramide binding assays, immunoprecipitates of 3×10^7 U937 cells for each sample, adsorbed to 30 μl of protein A beads, were washed three times for 10 s in 1 ml of buffer C (50 mM Tris−HCl, pH 7.4, 150 mM NaCl and 1 mM PMSF) and resuspended in 1 ml of buffer C. To this suspension were added different concentrations of [^{14}C]ceramide with and without a 100-fold excess of unlabeled *N*-hexadecanoyl ceramide (C_{16}-ceramide), for determination of total and non-specific binding respectively. Prior to the assay, the stock solutions of unlabeled ceramide (100×) in buffer C were treated for 10 min in an ultrasonic water bath at 37°C. Incubation was performed for 2 h at 4°C and unbound [^{14}C]ceramide was removed by washing the beads three times for 10 s in buffer C. Non-specific binding was dependent on the number of protein A beads present but, under the conditions employed here, did not exceed 15% of total binding. The immunoprecipitates were measured by liquid scintillation counting.

[^{14}C]Ceramide labeling of cells

U937 cells (10×10^7) were incubated for 1 h in 15 ml of RPMI medium prior to the addition of 1.5 μCi [^{14}C]palmitoylceramide. Over a period of 2.5 h, 0.5 ml was removed at short time intervals, metabolism of lipids stopped by the addition of ice-cold PBS and the cells were washed three times and subjected to lipid extraction according to Bligh and Dyer (1959). The lipids were separated in a horizontal electrophoresis chamber on HPTLC plates. For the investigation of the presence of lyso-phosphatidylcholine, 5×10^7 cells were incubated with 2 μCi [^{14}C]-ceramide with and without equimolar concentrations of lyso-phosphatidylcholine. At the same time intervals, 0.5 ml of the cell suspensions with and without lyso-phosphatidylcholine were removed, washed, extracted and separated on HPTLC plates as described above.

Thin layer chromatography

The [^{14}C]palmitoyl-labeled lipids were separated by HPTLC using a solvent system containing $CHCl_3$:CHOH:CH_3COOH:H_2O (50:30:8:5). Plates were dried for 20 min at 110°C and were exposed for autoradiography to Hyperfilm β max (Kodak) for 3–5 days. Spots were quantified by two-dimensional laser scanning. Lyso-phosphatidylcholine, phosphatidylcholine, sphingomyelin, ceramide and palmitate (Sigma) were used as standards and visualized using iodine vapor. The purities of ceramide and sphingosine were controlled by separation with the same solvent system as above.

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Received on November 8, 1994; revised on January 17, 1995