

# Ceramide inhibits PKC $\theta$ by regulating its phosphorylation and translocation to lipid rafts in Jurkat cells

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**Abstract** Protein kinase C theta (PKC $\theta$ ) is a novel, calcium-independent member of the PKC family of kinases that was identified as a central player in T cell signaling and proliferation. Upon T cell activation by antigen-presenting cells, PKC $\theta$  gets phosphorylated and activated prior to its translocation to the immunological synapse where it couples with downstream effectors. PKC $\theta$  may be regulated by ceramide, a crucial sphingolipid that is known to promote differentiation, growth arrest, and apoptosis. To further investigate the mechanism, we stimulated human Jurkat T cells with either PMA or anti-CD3/anti-CD28 antibodies following induction of ceramide accumulation by adding exogenous ceramide, bacterial sphingomyelinase, or Fas ligation. Our results suggest that ceramide regulates the PKC $\theta$  pathway through preventing its critical threonine 538 (Thr538) phosphorylation and subsequent

activation, thereby inhibiting the kinase's translocation to lipid rafts. Moreover, this inhibition is not likely to be a generic effect of ceramide on membrane reorganization. Other lipids, namely dihydroceramide, palmitate, and sphingosine, did not produce similar effects on PKC $\theta$ . Addition of the phosphatase inhibitors okadaic acid and calyculin A reversed the inhibition exerted by ceramide, and this suggests involvement of a ceramide-activated protein phosphatase. Such previously undescribed mechanism of regulation of PKC $\theta$  raises the possibility that ceramide, or one of its derivatives, and may prove valuable in novel therapeutic approaches for disorders involving autoimmunity or excessive inflammation—where PKC $\theta$  plays a critical role.

**Keywords** PKC $\theta$  · T cell activation · Lipid rafts · Ceramide

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## Introduction

T cell activation is induced by triggering of the T cell antigen receptor (TCR)/CD3 complex with costimulatory signals in a process involving multiple enzymes, adaptors, and other molecules. Protein kinase C theta (PKC $\theta$ ) is a novel, calcium-independent member of the PKC family of kinases [1] that was identified as a central player in this pathway [2]. PKC $\theta$  is unique among PKC isozymes in its translocation to the immunological synapse (IS)—also known as the supramolecular activation complex (SMAC)—upon T cell activation by antigen-presenting cells [3–5]. Its presence there is crucial for mediation of TCR signal transduction [6, 7].

Early studies revealed that TCR engagement leads to activation of phospholipase C  $\gamma$  (PLC $\gamma$ ), which hydrolyzes

membrane inositol phospholipids to produce inositol phosphates and diacylglycerol (DAG) [8]. DAG binds to the C1 domain of PKC $\theta$  inducing a conformational change that enhances the enzyme's membrane association [2]. Villalba et al. [9, 10] proposed a novel phosphatidylinositol 3-kinase- (PI3-K) and Vav-dependent mechanism for selective TCR-induced activation of PKC $\theta$  and its recruitment to the cell membrane and lipid rafts. PI3-K-generated lipid products result in activation and membrane recruitment of Vav, which causes reorganization of the T cell actin cytoskeleton and TCR capping thereby leading to PKC $\theta$  activation and its translocation to the T cell SMAC [9, 10]. More recently, it has been suggested that PKC $\theta$  participates in T cell activation through its indirect association with the cytoplasmic domain of the costimulatory receptor CD28 through the lymphoid-specific Src family protein-tyrosine kinase Lck [11], and that a proline-rich region within the V3 hinge domain of PKC $\theta$  is required for this interaction [12].

The PKC $\theta$  pathway culminates in the induction of the growth-promoting lymphokine interleukin 2 (IL-2), a key regulator of T cell proliferation and differentiation. This is accomplished by activation of the factors necessary for stimulating the IL-2 gene regulatory region including the activator protein (AP-1) transcription factor complex [13, 14] through the MAP kinase JNK/SAPK [15, 16] and nuclear factor of activated T cells (NFAT) [17]. PKC $\theta$  is also responsible for CD28- and TCR-initiated stimulation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) [18] through activation of I $\kappa$ B kinase  $\beta$  (IKK $\beta$ ) [14, 19] in cooperation with Akt1, a serine/threonine kinase known to participate in T cell survival [2, 20, 21]. PKC $\theta$  phosphorylates CARMA1 [22] which associates with Bcl10–MALT1 complex and recruits these proteins to the lipid rafts in the pathway leading to activation of IKK through TNF receptor-associated factor 6 (TRAF6) [16, 19, 23, 24]. However, the reversal of CARMA1 phosphorylation, by PP2A, negatively regulates T cell activation [25]. PKC $\theta$  also promotes longevity of effector and memory T cells through activation of NF- $\kappa$ B in a TCR-independent pathway initiated by the TNFR family member OX40 (CD134) and involving CARMA1, Bcl10, MALT1, and TRAF2 [26].

Dysregulation of PKC $\theta$  has been implicated in several pathologies including allergies and autoimmune diseases through its regulation of effector T cells and regulator T cells [11, 27]. It promotes inflammation by enhancing effector function and decreasing suppressor function [27] and is thus considered a potential therapeutic target particularly in autoimmune and alloimmune diseases [11, 27–31].

PKC $\theta$  activity is regulated by phosphorylation [32]; two phosphorylation sites have been identified in its N-terminal regulatory domain and four in the C-terminal catalytic domain. Lck phosphorylates Tyr90 in the regulatory

domain of PKC $\theta$ , thereby positively regulating T cell proliferation [33]. A Thr219 autophosphorylation site was identified as essential for proper cross talk between PKC $\theta$  and Akt/PKB $\alpha$  and possibly for membrane localization of PKC $\theta$  in T cells [34]. In the catalytic domain, there is Thr538 at the activation loop, Ser676 and Ser685 at the turn motif, and Ser695 at the hydrophobic motif [32, 35]. The Thr538 site is the best studied and understood thus far and is believed to be the most critical in PKC $\theta$  activation. AMP-activated protein kinase (AMPK) was recently shown to phosphorylate PKC $\theta$  at Thr538 and inhibition of PKC $\theta$  caused reduction in AMPK-mediated transcriptional activation of NFAT and AP-1 in Jurkat cells [36]. The kinase germinal center kinase-like kinase (GLK/MAP4K3), a regulator of cell growth and inflammation, was also shown to act directly upstream of PKC $\theta$  in TCR signaling by phosphorylating its Thr538 residue upon CD3 ligation [31, 37]. Although phosphorylation of this Thr538 residue is possibly essential for the enzyme's intracellular localization [38], a study suggested that it is not necessary for its translocation to lipid rafts [34]. Based on these findings, it appears that GLK may induce another phosphorylation site such as Thr219 or a novel S/T residue that regulates PKC $\theta$  translocation to the lipid rafts [31].

Dbaibo et al. have previously shown that PKC $\theta$  may be regulated by ceramide [39], a bioactive sphingolipid that is known to induce differentiation, growth arrest, and apoptosis [40–42]. Ceramide accumulation causes growth suppression and G0/G1 arrest by inducing dephosphorylation of the retinoblastoma protein (pRb) [43, 44]. Tumor necrosis factor (TNF)- $\alpha$  stimulates the generation of ceramide [45] through both the de novo synthesis pathway and through the sphingomyelinase pathway [46]. This mechanism allows ceramide to act as a mediator for a subset of TNF- $\alpha$ 's biochemical and biological effects that include stress-induced apoptosis through stress-activated protein kinases (SAPKs/JNKs) [47, 48]. Ceramide accumulation also mediates the cytotoxic effect of Fas (CD95) signaling in an initiator caspase-dependent manner [49–51].

On the other hand, ceramide is implicated in inducing cell death in pathologies related to oxidative stress including various retinopathies, neurodegeneration, cardiovascular diseases, eryptosis, and pulmonary disease [52–56]. In fact, ceramide toxicity is believed to be due to triggering accumulation of mitochondrial reactive oxygen species [57–59]. Ceramide also mediates lytic effects of adenoviral infection through inducing dephosphorylation of serine/arginine rich (SR) proteins by protein phosphatase 1 (PP1) and regulating the shift in adenovirus alternative RNA splicing [60].

Treatment of Jurkat cells with exogenous ceramide decreases the activity of PKC $\theta$ , NF- $\kappa$ B activation, and IL-2 production [39, 61]. Moreover, Fas ligation reduces the

activity and inhibits the translocation of PKC $\theta$  thereby suppressing TCR/CD3 signaling and attenuating T cell activation [39, 62]. The aim of this study is to determine the effects of ceramide, generated by different stimuli, specifically on PKC $\theta$  activation and translocation. Our results indicate that exogenous ceramide, bacterial sphingomyelinase, and Fas ligation bring about the accumulation of endogenous ceramide and subsequently inhibit PKC $\theta$  phosphorylation, activity, and translocation to lipid rafts. Implications of such findings may be valuable for therapeutic interventions in pathologies where suppression of PKC $\theta$  is the answer.

## Materials and methods

### Materials

Jurkat E6-1 leukemic T cells (a cell line that has been a useful model for the study of T cell activation) were obtained from ATCC, Rockville, MD. Phorbol 12-myristate 13-acetate (PMA; Sigma P-8139) was dissolved in dimethyl sulfoxide (DMSO). Mouse anti-human CD3 (cat. 555336) and CD28 (cat. 555725) monoclonal antibodies were purchased from BD Pharmingen, San Diego, CA. D-erythro-C6-ceramide, D-erythro-C6-dihydroceramide, sphingosine, and palmitate (Matreya, State College, PA) were dissolved in 100 % ethanol. Antibodies against human PKC $\theta$  (cat. 12206S) and phosphor-Thr538 (cat. 9377S) were purchased from Cell Signaling. Antibody against Flotillin 1 (ab41927) was purchased from Abcam, and PKC kinase activity kit was purchased from Enzo Life Sciences (ADI-EKS-420A). [ $\gamma$ - $^{32}$ P] ATP was from Izotop, institute of isotopes Co. Protease inhibitor cocktail tablets (Complete) were obtained from Roche Diagnostics, Mannheim, Germany. Anti-human Fas monoclonal antibody (SY-001, CH-11) was purchased from Medical and Biological Laboratories, Japan and Fumonisin B1 (BML-SL220) from Enzo Life Sciences and was diluted in sterile water. Bacterial sphingomyelinase was purchased from Enzo Life Sciences. Okadaic acid and calyculin A were purchased from Calbiochem. RPMI 1640 medium and FBS were purchased from Sigma.

### Cell culture and treatment

Jurkat E6-1 leukemic T cells were grown in RPMI 1640 medium (Sigma) supplemented with 10 % fetal bovine serum (FBS, Sigma). Cells were maintained at densities between 0.5 and 2 million cells/ml under standard incubator conditions (humidified atmosphere, 95 % air, 5 %

CO $_2$ , 37 °C). Jurkat T cells were seeded at a concentration of  $1 \times 10^6$  cells/ml 2 h prior to treatments. Cell counts were done using Trypan blue. All treatments were done in RPMI medium supplemented with 2 % FBS. To study the effects of ceramide, Jurkat cells were pretreated with 15  $\mu$ M C6-ceramide for 2 h, 0.3 U/ml bacterial sphingomyelinase for 3 h, or 150 ng/ml activating anti-Fas antibody for 7 h, followed by stimulation with 15 ng/ml PMA for 30 min. On the other hand, a higher concentration of C6-ceramide (30  $\mu$ M) was used for the 2-h pretreatment in cells that were to be activated with 4  $\mu$ g/ml of anti-CD3 and anti-CD28 antibodies for 30 min. To disrupt endogenous ceramide synthesis, cells were pretreated with 10  $\mu$ M fumonisin B1 for 2 h prior to the 7-h incubation with 150 ng/ml anti-Fas antibody, which was followed by stimulation with 15 ng/ml PMA for 30 min. To inhibit phosphatases, cells were pretreated for 2 h with 10 nM okadaic acid or 5 nM calyculin A followed by either 15  $\mu$ M or 30  $\mu$ M C6-ceramide for 2 h and then stimulated with either 15 ng/ml PMA or 4  $\mu$ g/ml of anti-CD3 and anti-CD28 antibodies for 30 min. Jurkat cells were also treated with 15  $\mu$ M of each dihydroceramide, palmitate, or sphingosine preceding induction with PMA.

### PKC $\theta$ kinase activity assay

Cell lysis and immunoprecipitation were performed using the Immunoprecipitation Kit (Protein A) from Roche, according to the manufacturer's instructions. Briefly, cells were lysed and homogenized by repeated strokes in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 % sodium deoxycholate, and 0.5 % Nonidet P40) containing a protease inhibitor cocktail (Roche). An amount of 500  $\mu$ g of the cell lysate was precleared with 50  $\mu$ l of protein A agarose beads and immunoprecipitated with mouse monoclonal antibody against nPKC $\theta$  (E-7, sc-1680) and protein A agarose beads overnight at 4 °C. The beads were then washed twice with each of wash buffer 1 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05 % sodium deoxycholate, and 0.1 % Nonidet P40) and wash buffer 2 (10 mM Tris-HCl, pH 7.5, 0.5 % sodium deoxycholate, and 0.1 % Nonidet P40). The beads were subsequently resuspended in kinase assay dilution buffer provided with the PKC kinase activity kit (Enzo, ADI-EKS-420A). The non-radioactive PKC kinase activity kit uses a specific synthetic peptide as a substrate for PKC and a polyclonal antibody that recognizes the phosphorylated form of the substrate. The assay was used on the immunoprecipitated and quantified PKC $\theta$  proteins. Briefly, samples from the resuspended PKC $\theta$  proteins were quantified using BioRad DC protein assay reagents. An ELISA procedure was

performed on the immunoprecipitated PKC $\theta$  according to the manufacturer's instructions. The color development was stopped with acid, and intensity of the color was measured in microplate reader at 450 nm. The relative kinase activity was computed using an equation provided by the manufacturer:

Relative kinase activity = (average absorbance of sample—average absorbance of blank)/quantity of purified kinase used per assay.

### Western blot

Protein expression levels were analyzed using 12 % acrylamide gel. Samples were prepared with a 1:1 volume ratio of proteins to loading buffer [Tris–HCl 0.25 M (pH 6.8), SDS 4 %, glycerol 20 %, 2 mg bromophenol blue, 5 %  $\beta$ -mercaptoethanol] and run using TGS 1X running buffer [TGS 10X: 30 g Tris (hydroxymethyl)-aminomethane, 144 g glycine, 10 g SDS, all supplied from Bio-Rad]. The migration was performed at 80 V for the stacking gel and 120 V for the resolving gel. Following migration, transfer to a polyvinylidene difluoride (PVDF) membrane was done in transfer buffer [TGS 1X with 20 % methanol] for 90 min at 100 V. Then, the membrane was blocked to prevent nonspecific binding using 5 % fat-free milk prepared in TBS 1X [TBS 10X: 12 g Tris (hydroxymethyl)-aminomethane, 87.8 g NaCl, pH 8] with 0.1 % Tween for 2 h. Following blocking, the membrane was incubated with 2 ml of specific primary antibody diluted as recommended by the supplier in 5 % milk-TBS 1X at 4 °C overnight. The membrane was then washed for 10 min with TBS 1X 0.1 % Tween for three cycles and incubated at room temperature for 1 h with 5 ml of the horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in 5 % milk-TBS 1X 0.1 % Tween. Finally, the bands were developed using ECL western blotting reagent (GE health care, UK). Densitometric quantification of western blots was performed with ImageJ software (NIH, USA), and band intensity was expressed as arbitrary unit (AU). Poncau-S staining was used as loading control in membranes.

### Membrane and cytosol fractionation of cells

Jurkat E6-1 T cells were harvested on ice, washed once with 1X cold PBS, and suspended in 0.5 ml of cold homogenization buffer [50 mM Tris/HCL pH 7.5 containing 0.3 % (w/v)  $\beta$ -mercaptoethanol, 5 mM EDTA, 10 mM EGTA, 50  $\mu$ g/ml phenylmethylsulfonyl fluoride]. Jurkat cells were lysed by four rounds of sonication on ice, 15 s each, vortexed, and then centrifuged at 500 $\times$ g for 5 min at 4 °C to remove unlysed cells and the nuclei (pellet). The lysate was centrifuged at 100,000 $\times$ g for 40 min at 4 °C. The

supernatant was collected as the cytosol preparation. The pellet was resuspended in 0.5 ml of homogenization buffer, homogenized well, and used as the membrane preparation (plasma membrane and endomembrane microsomes). For western blot application, the preparations were directly used. For ceramide measurement, lipid extraction was performed on these fractions as described below.

### Lipid extraction and phosphate assay

Cell pellets or cellular fractions were suspended in a ratio of 2:1 methanol to chloroform and stored at  $-80$  °C for 2–3 days [63]. Then the aqueous and lipid phases were separated using a mixture of methanol/chloroform/water ratio 2:2:1.7 and centrifuged at 2500 rpm for 10 min. The resulting lipid phase (lower phase) of each sample was then transferred into a new glass tube to be dried by speed vacuum. Finally, the invisible pellet was suspended in 1 ml chloroform, and 200  $\mu$ l was aliquoted to determine the phosphate concentration in the samples. After thawing at room temperature, the samples were dried by speed vacuum and then 150  $\mu$ l of perchloric acid (70 %) was added. The reaction was allowed to proceed for 1 h at 180 °C. Then, the samples were cooled and 830  $\mu$ l of water was added, followed by 170  $\mu$ l of ammonium molybdate (2.5 %) and 170  $\mu$ l of freshly prepared ascorbic acid (10 %). The mixture was incubated in a water bath at 50 °C for 15 min until color development. Absorbance was measured by a spectrophotometer at 820 nm. The concentration of lipid phosphate was determined using disodium hydrogen orthophosphate anhydrous (Na<sub>2</sub>HPO<sub>4</sub>) as standard.

### Ceramide assay

Ceramide assay was done according to a modified version of the diacylglycerol kinase (DGK) assay [64]. First, 200  $\mu$ l of lipid samples was dried, and then 20  $\mu$ l of micelles (made of octyl-  $\beta$ -D-glucoside/dioleoyl phosphatidylglycerol) was added per sample. The tubes were sonicated for 30 min in a water bath sonicator. A reaction mixture was prepared of 50  $\mu$ l 2X buffer (100 nM imidazole HCl pH 6.6, 100 mM LiCl, 25 mM MgCl<sub>2</sub>, 2 mM EGTA), 0.2  $\mu$ l of 1 M DTT (dithiothreitol), 5  $\mu$ g of diacylglycerol kinase enzyme (DGK, prepared from *E.coli*), and dilution buffer (10 mM imidazole of pH 6.6 and 1 mM diethylenetriaminepentacetic acid, pH = 7). Per sample, 70  $\mu$ l of the mixture was added, and then the reaction was initiated by adding 10  $\mu$ l of the ATP mixture made of 2.5 mM cold ATP (non-radioactive) and 1.3  $\mu$ Ci of the [ $\gamma$ -<sup>32</sup>P] ATP solution in water. The reaction was allowed to proceed for 30 min then stopped by the addition of 2 ml methanol, 1 ml chloroform, and 0.7 ml water. The tubes

were allowed to rest for 10 min, and lipids were extracted by adding 1 ml chloroform and 1 ml water. The lipid phase was transferred to a clean glass tube and dried by speed vacuum. Lipids were resuspended in 50  $\mu$ l of 9:1 ratio chloroform–methanol. Then 25  $\mu$ l of each sample was spotted on a lane of a silica gel thin-layer chromatography plate. The plates were placed in a previously prepared elution chamber, filled with elution mixture made of chloroform–acetone–methanol–glacial acetic acid–water in a ratio of 50:20:15:10:5. Samples were allowed to migrate for 2 h and then exposed to X-ray film overnight at  $-70$  °C. The ceramide phosphate bands (identified by comparing them to a known series of ceramide standards) were scrapped into scintillation vials, and 4 ml of scintillation fluid was added. The radioactivity was read using a liquid scintillation counter.

### Isolation and analysis of membrane rafts

The isolation of membrane rafts was performed according to the method of Phong et al. [65]. Jurkat cells ( $70 \times 10^6$ ) were lysed in 225  $\mu$ l of 25 mM Tris/HCl, pH 8.0, containing 1 % Triton X-100, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 % protease inhibitor cocktail, 1 mM sodium orthovanadate, and 5 mM sodium fluoride, and homogenized well. The lysate was mixed with 225  $\mu$ l of 85 % sucrose, overlaid with 2.7 ml of 35 % sucrose, followed by a final layer of 675  $\mu$ l of 5 % sucrose. The gradient was centrifuged for 18 h and 15 min at 44,000 rpm at 4 °C. Nine gradient fractions (415  $\mu$ l each) were harvested from the top to the bottom of the tube. The first three fractions collected from the top correspond to the membrane lipid raft fraction. The middle three fractions correspond to the interphase and the lower three fractions correspond to the non-raft membranes. On these fractions, either protein extraction or lipid extraction was performed. For lipid extraction, the procedure used was the same as the one done on cell pellets or cellular fractions above. For protein extraction, 4 volumes of methanol, 1 volume of chloroform and 3 volumes of water were added to the fractions, mixed well, and centrifuged at 3200 rpm for 20 min. The top aqueous layer was discarded, and 4 volumes of methanol were added again to the protein interface, and tubes were centrifuged at 3200 rpm for 20 min. The supernatant was discarded and the protein pellets were collected in sample loading buffer for use in western blot.

### Lipid raft labeling

Cells were stained with Vybrant<sup>®</sup> Alexa Fluor<sup>®</sup> 594 Lipid Raft Labeling Kit (V-34405) according to the manufacturer's protocol. The cells were incubated in fluorescent

cholera toxin subunit B (CT-B) conjugate for 10 min at 4 °C. After this incubation, cells were gently washed several times with chilled 1X PBS. The CT-B-labeled lipid rafts were cross-linked with the anti-CT-B antibody for 15 min at 4 °C. After this incubation, cells were gently washed several times with chilled 1X PBS, cytospinned on slides, fixed with 4 % paraformaldehyde, mounted with ProLong<sup>®</sup> Antifade Kit, P-7481, and then visualized by confocal microscope using a Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany) with a 63X oil-immersion objective and Zen software (Zeiss).

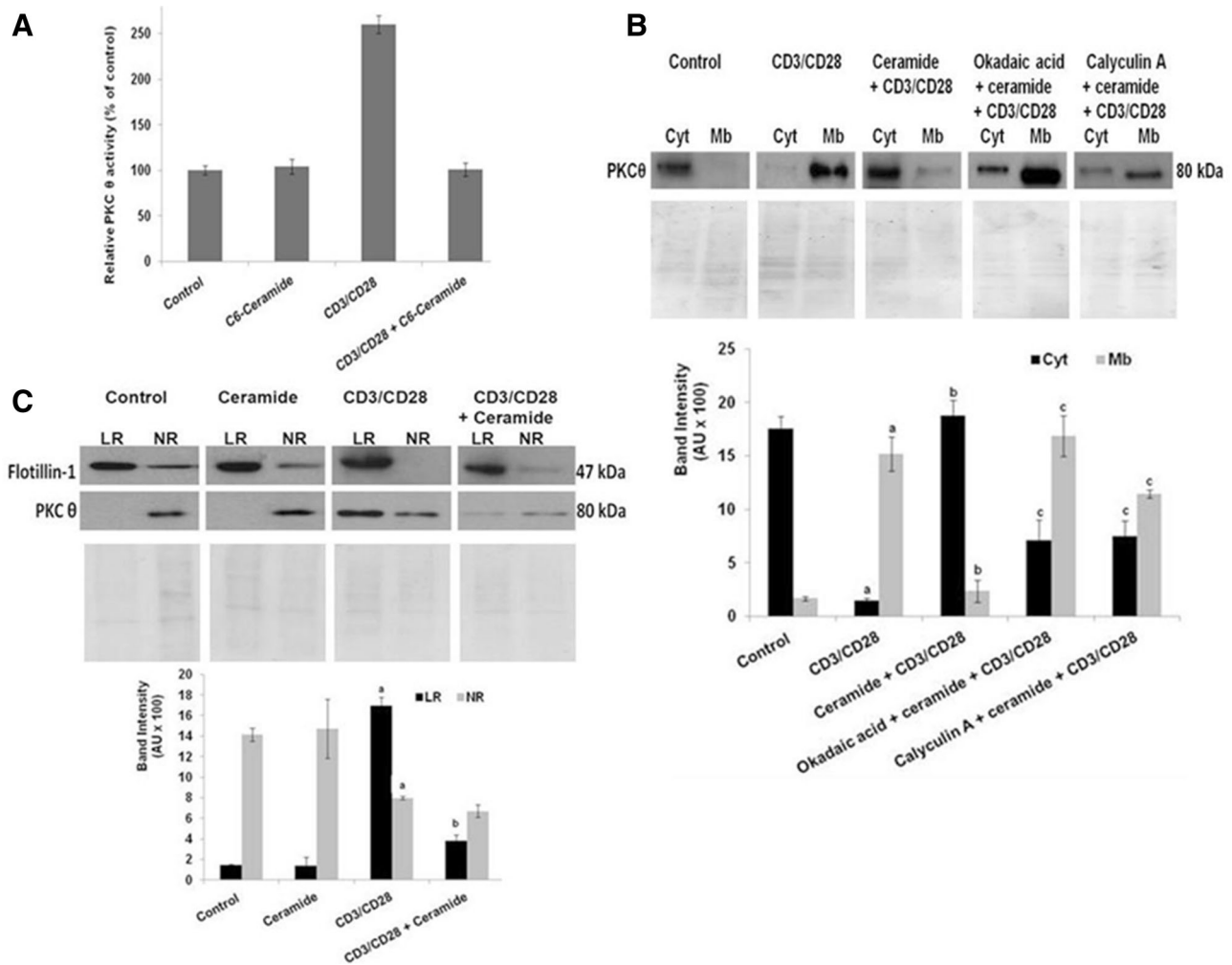
### Statistical analysis

Statistical significance of the data was determined by Student's *t* test. The differences presented correspond to  $P < 0.001$  and indicate statistically highly significant differences.

## Results

### Ceramide inhibits CD3-/CD28-induced PKC $\theta$ activation and translocation to the plasma membrane and lipid rafts

Multiple studies have shown that ceramide differentially modulates various PKC isozymes [66–69]; in particular, a study suggests a prominent inhibitory effect of ceramide on PKC $\theta$  and PKC $\alpha$  [39]. To investigate the specific role of ceramide on PKC $\theta$  activation, Jurkat cells were pretreated with 30  $\mu$ M C6-ceramide for 2 h followed by co-stimulation with 4  $\mu$ g/ml of anti-CD3 and anti-CD28 antibodies for 30 min. Co-stimulation of CD3/CD28 in Jurkat cells significantly activated PKC $\theta$  and this was strongly inhibited by C6-ceramide (Fig. 1a). To investigate the role of ceramide-activated protein phosphatases (CAPP) in the regulation of PKC $\theta$  activity, cells were pretreated for 2 h with phosphatase inhibitors okadaic acid (10 nM) and calyculin A (5 nM) followed by 30  $\mu$ M C6-ceramide for 2 h and then stimulated with 4  $\mu$ g/ml of anti-CD3 and anti-CD28 antibodies for 30 min. Upon CD3/CD28 co-stimulation, PKC $\theta$  translocated from the cytosolic fraction to the membrane (Fig. 1B). Treatment with C6-ceramide largely reversed this translocation, whereas treatment with the CAPP inhibitors, okadaic acid, and calyculin A abrogated the inhibitory effect of ceramide. CD28 signaling involves the recruitment of PKC $\theta$  to CD28 receptors in the immunological synapse [19]. Lipid rafts were described to preferentially accumulate in the central zone of the immunological synapse, namely at the central supramolecular activation complex (C-SMAC)



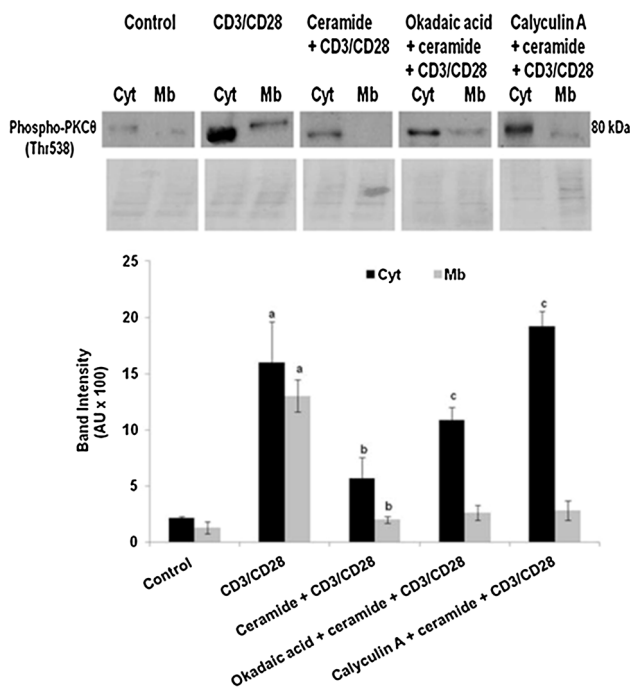
**Fig. 1** C6-Ceramide inhibits CD3/CD28-induced activation and translocation of PKC $\theta$ . Jurkat cells were pretreated with 30  $\mu$ M C6-ceramide for 2 h followed by co-stimulation with 4  $\mu$ g/ml of anti-CD3 and anti-CD28 antibodies for 30 min. In the conditions involving phosphatase inhibitors, cells were pretreated for 2 h with either okadaic acid (10 nM) or calyculin A (5 nM) followed by 30  $\mu$ M C6-ceramide for 2 h and then stimulated with 4  $\mu$ g/ml of anti-CD3 and anti-CD28 antibodies for 30 min. **a** The activity of PKC $\theta$  was assessed using a non-radioactive assay kit relative to the total amounts of immunoprecipitated PKC $\theta$  in each sample as detailed in the materials and methods. Data represent the average of triplicate measurements for each condition. *Error bars* represent the standard deviation. **b** Translocation of PKC $\theta$  to the membrane in response to

CD3/CD28 co-stimulation; the impact of ceramide on the translocation was assessed following cytosol (Cyt) versus membrane (Mb) fractionation. **c** Translocation of PKC $\theta$  to lipid rafts in response to CD3/CD28 co-stimulation; the impact of endogenous ceramide on the translocation was assessed following lipid raft isolation. The expression level of PKC $\theta$  in lipid raft (LR) and non-raft (NR) membranes was measured by western blot using flotillin-1 as a lipid raft marker. Each of the above figures is representative of three independent experiments. For western blots, band intensity data presented in the bar graphs is the average of three independent experiments with the standard deviation. Highly significant difference ( $P < 0.001$ ) with respect to <sup>(a)</sup> Control, <sup>(b)</sup> CD3/CD28 and <sup>(c)</sup> Ceramide + CD3/CD28. Ponceau-S staining was used as loading control in membranes

[70]. Within the membrane, we observed that PKC $\theta$  concentrates preferentially in the lipid rafts following CD3/CD28 co-stimulation of Jurkat cells (Fig. 1c). Ceramide also inhibited this translocation of PKC $\theta$  to lipid rafts. These findings indicated that ceramide inhibits PKC $\theta$  activity, its translocation from cytosol to membrane, and its distribution to lipid rafts in response to CD3/CD28 stimulation. Ceramide inhibitory effects on PKC $\theta$  translocation from cytosol to membrane were dependent on CAPP.

### Ceramide inhibits CD3-/CD28-induced phosphorylation of PKC $\theta$

Thr538, Ser696, and Ser695 are autophosphorylation sites within the catalytic domain of PKC $\theta$  [35, 71]. Thr538 is a critical site that regulates PKC $\theta$  kinase activity and T cell activation [71]. CD3-/CD28-induced activation of PKC $\theta$  was achieved by stimulation with 4  $\mu$ g/ml of anti-CD3 and anti-CD28 antibodies for 30 min and was found to correlate with phosphorylation of Thr538 residue (Fig. 2). Such



**Fig. 2** Effect of exogenous ceramide on the phosphorylation of PKC $\theta$  at threonine 538. Western blot analysis of the expression level of phospho-PKC $\theta$  (thr-538) was assayed on cytosolic (Cyt) and membrane (Mb) fractions. The blots are representative of three independent experiments. Band intensity data presented in the bar graphs are the average of the three experiments with the standard deviation. Highly significant difference ( $P < 0.001$ ) with respect to <sup>(a)</sup> Control, <sup>(b)</sup> CD3/CD28 and <sup>(c)</sup> Ceramide + CD3/CD28. Ponceau-S staining was used as loading control in membranes

phosphorylation and the associated membrane translocation were significantly decreased upon pre-treatment with 30  $\mu$ M exogenous ceramide for 2 h (Fig. 2). Inhibition of CAPP by adding the inhibitors okadaic acid or calyculin A partially restored the phosphorylation of PKC $\theta$  at Thr538 (Fig. 2). This indicated that the ceramide-induced dephosphorylation of PKC $\theta$  following its phosphorylation by CD3/CD28 is dependent on CAPP.

#### PMA-induced translocation of PKC $\theta$ to the plasma membrane and lipid rafts is decreased by ceramide-activated protein phosphatases

Phorbol esters were described to substitute for the second messenger diacylglycerol in stimulating the downstream signaling pathway of many members of the PKC family [72]. Phorbol-12-myristate-13-acetate (PMA) is a potent non-physiological PKC $\theta$  agonist that mimics the action of DAG by binding it with a higher affinity than DAG [67]. We used PMA to dissect the stimulatory effects of CD3/CD28 on PKC $\theta$ . We previously reported that, in Jurkat cells, C6-ceramide inhibited the activity of PKC $\theta$  after stimulation with PMA for 20 min [39]. Upon stimulation with 15 ng/ml PMA

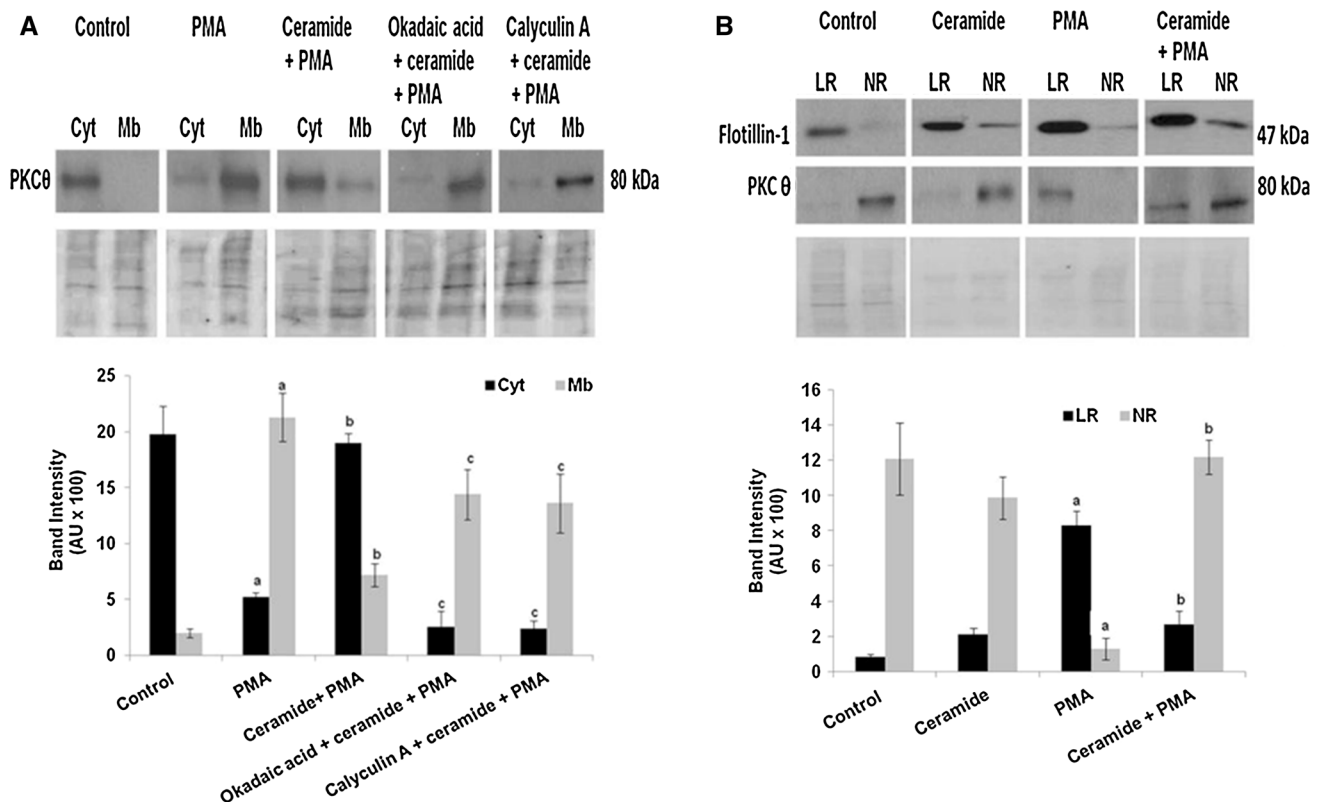
for 30 min, PKC $\theta$  translocates from the cytosol to the plasma membrane as shown in Fig. 3a. To study the effects of ceramide, Jurkat cells were pretreated with 15  $\mu$ M C6-ceramide for 2 h, followed by stimulation with 15 ng/ml PMA for 30 min. Exogenous C6-ceramide substantially decreased the translocation from cytosol to plasma membrane (Fig. 3a). On the other hand, pre-treatment for 2 h with the ceramide-activated phosphatase inhibitors okadaic acid (10 nM) and calyculin A (5 nM) followed by 15  $\mu$ M C6-ceramide for 2 h, then 15 ng/ml PMA for 30 min, reversed the inhibitory effect of ceramide on the PMA-induced membrane translocation of PKC $\theta$  (Fig. 3a). Moreover, it was found that upon its PMA-induced translocation to the plasma membrane, PKC $\theta$  localized preferentially to the lipid rafts in Jurkat cells (Fig. 3b). In this case also, ceramide interfered with the ability of PKC $\theta$  to concentrate in lipid rafts (Fig. 3b). Therefore, as in CD3/CD28 stimulation, ceramide inhibited PMA-induced membrane translocation of PKC $\theta$  in a CAPP-dependent manner.

#### Ceramide inhibits PMA-induced phosphorylation of PKC $\theta$

As illustrated earlier, Thr538 is a critical site that regulates PKC $\theta$  kinase activity and T cell activation [71]. We investigated the effect of 30 min PMA treatment and ceramide-activated phosphatase inhibitors on PKC $\theta$  phosphorylation as described previously. PMA-induced activation and membrane translocation of PKC $\theta$  were found to correlate with its phosphorylation at Thr538 (Fig. 4), and such phosphorylation and translocation were significantly decreased upon the addition of exogenous ceramide. Treatment with the CAPP inhibitors okadaic acid or calyculin A reversed the inhibitory effect of ceramide on Thr538 phosphorylation and PKC $\theta$  membrane translocation. These experiments demonstrate a correlation between Thr538 dephosphorylation and the inhibitory effects of ceramide on PKC $\theta$  activation and translocation.

#### Ceramide generated by bacterial sphingomyelinase and Fas stimulation inhibits PMA-induced activation of PKC $\theta$ and its translocation to lipid rafts

When Jurkat cells are exposed to exogenous C6-ceramide, endogenous ceramide accumulates significantly due to the deacylation and reacylation reaction of the absorbed synthetic ceramide as described previously [73] and as shown in Fig. 5a. Fas ligation was shown to induce ceramide accumulation through de novo synthesis [74]. Bacterial sphingomyelinase, on the other hand, generates ceramide at the outer membrane leaflet and only part of it gets absorbed into the cell. To study the effects of ceramide generated by



**Fig. 3** C6-Ceramide inhibits PMA-induced translocation of PKC $\theta$  to the plasma membrane and lipid rafts. Jurkat cells were pretreated with 15  $\mu$ M C6-Ceramide for 2 h followed by stimulation with 15 ng/ml PMA for 30 min. In the conditions involving phosphatase inhibitors, cells were pretreated for 2 h with either okadaic acid (10 nM) or calyculin A (5 nM) followed by 15  $\mu$ M C6-ceramide for 2 h and then stimulated with 15 ng/ml PMA for 30 min. **a** The impact of ceramide on the translocation of PKC $\theta$  to the plasma membrane was assessed following cytosol (Cyt) versus membrane (Mb) fractionation. **b** The impact of ceramide on the translocation of PKC $\theta$  to lipid rafts was

assessed following lipid raft isolation. The expression level of PKC $\theta$  in lipid raft (LR) and non-raft (NR) membrane fractions was measured by western blot using flotillin-1 as a lipid raft marker. The blots are representative of three independent experiments. Band intensity data presented in the bar graphs are the average of the three experiments with the standard deviation. Highly significant difference ( $P < 0.001$ ) with respect to <sup>(a)</sup> Control, <sup>(b)</sup> PMA and <sup>(c)</sup> Ceramide + PMA. Ponceau-S staining was used as loading control in membranes

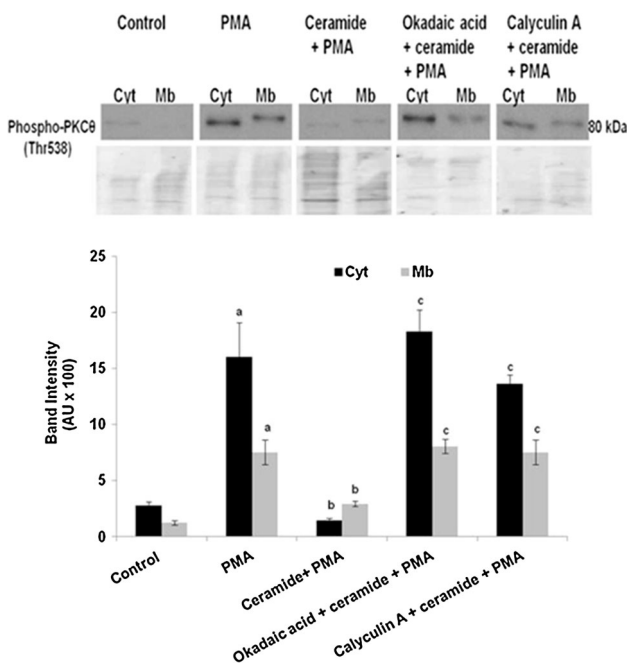
bacterial sphingomyelinase and Fas stimulation, Jurkat cells were pretreated, respectively, with 0.3 U/ml bacterial sphingomyelinase for 3 h or 150 ng/ml activating anti-Fas antibody for 7 h, followed by stimulation with 15 ng/ml PMA for 30 min. Similar to exogenous C6-ceramide, both bacterial sphingomyelinase and Fas stimulation induced ceramide buildup in Jurkat cells (Fig. 5a). The application of the ceramide synthase inhibitor fumonisins B1 (FB1) caused a substantial decrease in ceramide levels in response to Fas stimulation. As demonstrated in Fig. 5b, ceramide generated by the three different conditions considerably reduced the PMA-induced kinase activity of PKC $\theta$ . When de novo synthesis was inhibited by FB1 in Fas-stimulated cells, the kinase activity of PKC $\theta$  induced by PMA was largely restored in Jurkat cells (Fig. 5b).

It was previously shown that physiological T cell activation results in translocation of PKC $\theta$  to the membrane, in particular to lipid rafts, where it localizes to the T cell synapse to carry out its function [6]. Therefore, we treated

Jurkat cells with either sphingomyelinase or activating anti-Fas antibody, followed by PMA, in order to assess the effect of ceramide accumulation on the translocation of PKC $\theta$  to lipid rafts. By examining the expression levels of PKC $\theta$  in raft versus non-raft membrane fractions, we observed that the ceramide generated by either bacterial sphingomyelinase or Fas activation significantly reduced the translocation of PKC $\theta$  to lipid rafts (Fig. 5c). These results indicate that ceramide generated by sphingomyelinase, Fas ligation, or exogenous ceramide inhibits both the activity and the lipid raft translocation of PKC $\theta$  induced by PMA.

#### Endogenous ceramide but not dihydroceramide, palmitate, or sphingosine reduces the translocation of PKC $\theta$ to lipid rafts

In order to determine whether the impact of ceramide on PKC $\theta$ 's translocation is specific, we treated Jurkat cells



**Fig. 4** Effect of exogenous ceramide on the phosphorylation of PKCθ at threonine 538. Western blot analysis of the expression level of phospho-PKCθ (thr-538) was assayed on cytosolic (Cyt) and membrane (Mb) fractions of cells treated with 15 ng/ml PMA either alone or with 15 μM C6-ceramide. The blots are representative of three independent experiments. Band intensity data presented in the bar graphs is the average of the three experiments with the standard deviation. Highly significant difference ( $P < 0.001$ ) with respect to (a) Control, (b) PMA and (c) Ceramide + PMA. Ponceau-S staining was used as loading control in membranes

with 15 μM of dihydroceramide, palmitate, or sphingosine 30 min before induction with PMA. None of these closely related lipids inhibited the translocation of PKCθ to lipid rafts when compared to ceramide (Fig. 6a).

Next, we aimed to establish whether endogenous ceramide was regulating PKCθ translocation by directly sequestering the kinase or displacing it from the membrane or lipid rafts through differential distribution within membrane fractions. Therefore, we examined the relative amounts of long-chain ceramides in raft and non-raft lipid fractions in response to various stimuli (Fig. 6b). All the treatments were as described previously. CD3/CD28 signaling localized endogenous long-chain ceramide, with or without the addition of exogenous C6-ceramide, to the lipid rafts by almost fivefold compared to control. In contrast, PMA had no effect on ceramide displacement to lipid rafts (Fig. 6b). Ceramide generated by the activity of sphingomyelinase increased significantly but fractionated equally in raft and non-raft fractions (Fig. 6b). Similarly, de novo synthesized ceramide by Fas ligation significantly increased with a slight preference for lipid rafts (Fig. 6b). In comparing the effects on ceramide distribution of the three PKCθ-inhibiting treatments, C6-ceramide,

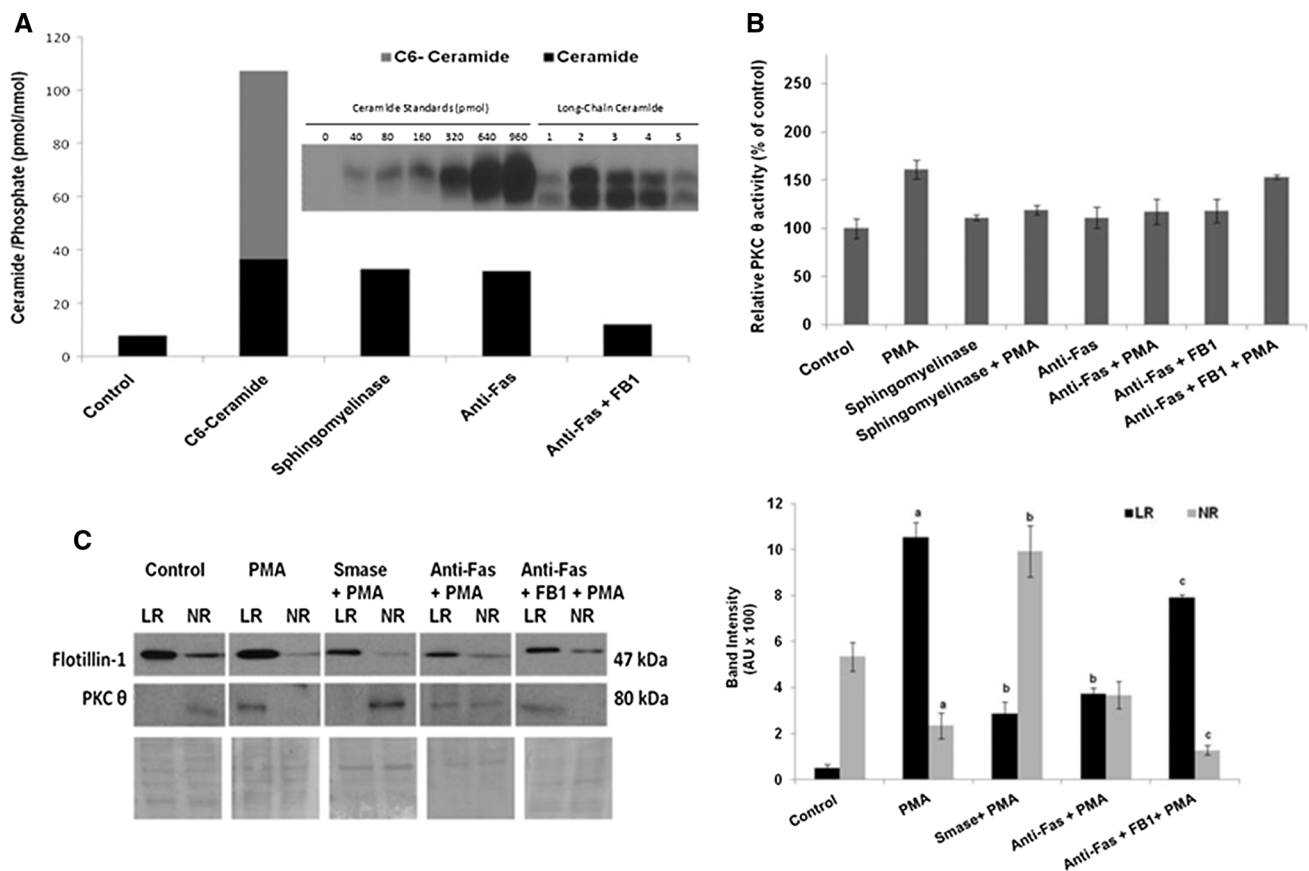
sphingomyelinase, and anti-Fas, no specific pattern emerged relating the lipid raft distribution of the generated ceramide with the inhibitory effects. Therefore, it is unlikely that the accumulation of ceramide in the lipid raft fraction, per se, plays a critical role in the inhibition of PKCθ activity.

**The presence of ceramide in lipid rafts does not disrupt their integrity**

Another possibility to explain the effects of ceramide on PKCθ activity and translocation was that the elevated levels of ceramide were disrupting the integrity of lipid rafts or contributing to their reorganization, thus interfering with signals necessary for PKCθ activation. To investigate this possibility, we used ganglioside GM1 and cholera toxin B to label the rafts before examining the cells with fluorescent microscopy. Exposure of cells to bacterial sphingomyelinase depletes sphingomyelin from the outer leaflet [75], which probably explains the significant decrease in lipid rafts observed in Jurkat cells following sphingomyelinase treatment in Fig. 7. However, upon ceramide generation either by addition of exogenous ceramide or Fas stimulation, we observed a redistribution of lipid rafts in the plasma membrane comparable to the control (Fig. 7). This indicates that although sphingomyelinase can disrupt lipid rafts, this is not essential in the observed effects of exogenous ceramide or Fas ligation.

**Evidence of the role of ceramide-activated protein phosphatases in decreasing the activity of PKCθ**

Ceramide generated by Jurkat cells in response to exogenous C6-ceramide seems to be more localized to the cytosol than the membrane, although the exogenous C6-ceramide was equally distributed in cytosol and membrane fractions (Fig. 8a, b). These observations support our hypothesis that the ceramide fraction responsible for the regulation of PKCθ is more likely cytosolic. This does not come as a surprise, since ceramide is known to have several downstream targets in the cytosol including kinases and phosphatases. Hence, we tested the effects of okadaic acid and calyculin A, two known inhibitors of the CAPPs PP2A and PP1, respectively. We found that both inhibitors actually reversed the effect of C6-ceramide on the PMA-induced activation of PKCθ (Fig. 8c). Moreover, treatment in the presence of fumonisins B1, which inhibits the production of endogenous ceramide in response to C6-ceramide by virtue of its inhibition of ceramide synthase, reversed the effect of C6-ceramide on PMA-induced PKCθ activation (Fig. 8c). This indicated that it is the endogenous ceramide rather than the exogenous C6-ceramide that is responsible for the inhibition of PKCθ.



**Fig. 5** Ceramide generated by bacterial sphingomyelinase and Fas stimulation inhibits PMA-induced activation of PKC $\theta$  and translocation to lipid rafts. Jurkat cells were treated with 15  $\mu$ M C6-ceramide for 2 h (2), 0.3 U/ml bacterial sphingomyelinase for 3 h (3), anti-Fas ligation (150 ng/ml) for 7 h (4) or 10  $\mu$ M FB1 for 2 h followed by anti-Fas (150 ng/ml) treatment for 7 h (5). **a** Endogenous ceramide accumulation in Jurkat cells is measured in the aforementioned conditions and compared to the control cells (1). Ceramide levels in cell lipid extracts were normalized to lipid phosphate. The bands observed in the film represent ceramide phosphate bands that are compared to a known series of ceramide standards. **b** Ceramide produced by sphingomyelinase or by anti-Fas ligation inhibits PMA-

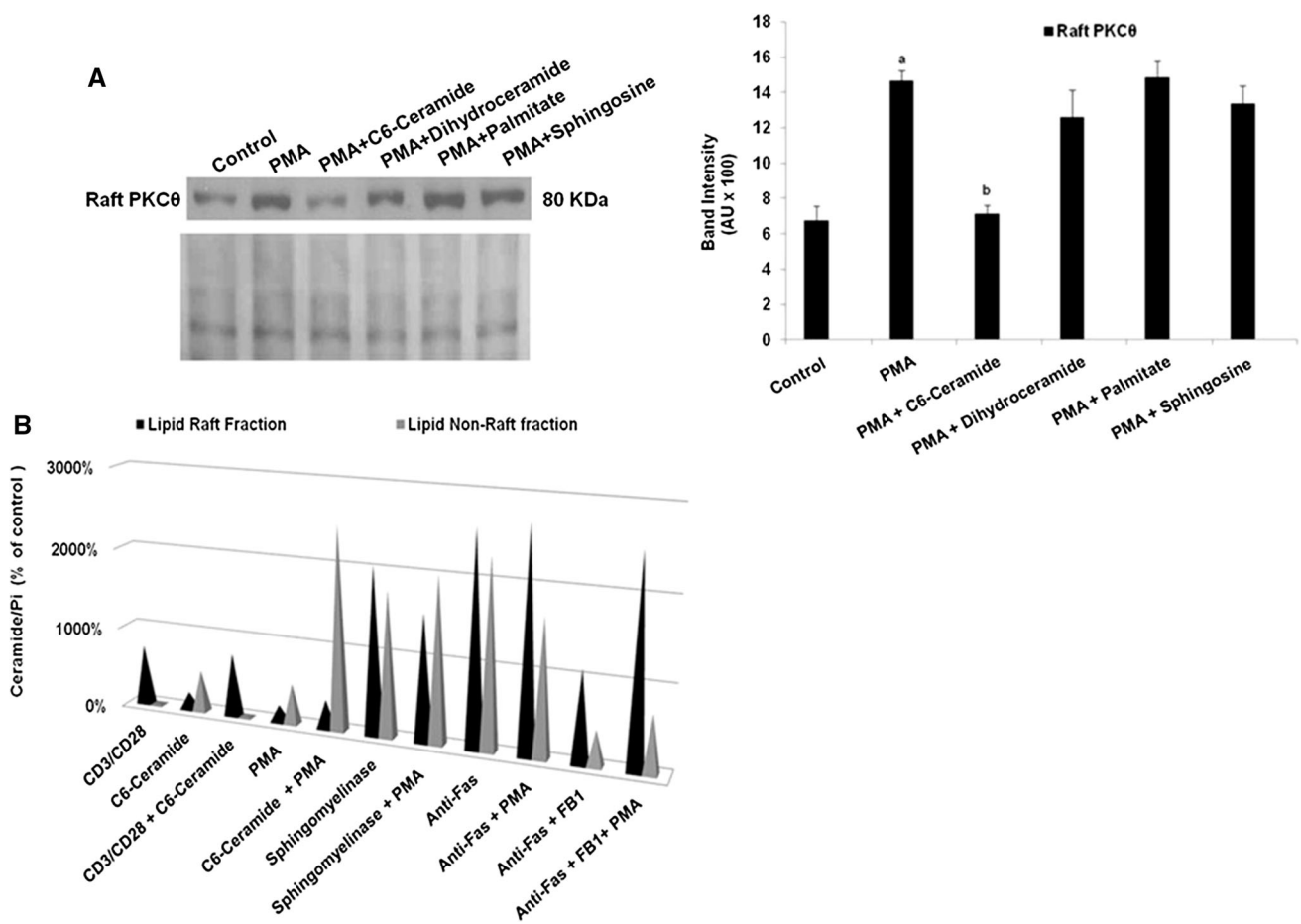
induced activation of PKC $\theta$ . The activity was normalized to the amount of immunoprecipitated PKC $\theta$  as detailed in materials and methods. Data represents the average of triplicate measurements for each condition. *Error bars* represent the standard deviation. **c** Effect of ceramide generated by bacterial sphingomyelinase or Fas ligation on the translocation of PKC $\theta$  to lipid rafts in response to PMA. The blots (*left*) are representative of three independent experiments. Band intensity data presented in the *bar graphs* (*right*) are the average of the three experiments with the standard deviation. Highly significant difference ( $P < 0.001$ ) with respect to <sup>(a)</sup> Control, <sup>(b)</sup> PMA and <sup>(c)</sup> Anti-Fas + PMA. Ponceau-S staining was used as loading control in membranes

## Discussion

Lipid rafts are specialized membrane microdomains rich in cholesterol and sphingolipids, especially sphingomyelin, in addition to an array of membrane proteins [76]. These membrane rafts are the “effector” sites in T cell signaling [77]. Upon stimulation, they cluster into a IS/SMAC which harbors key proteins including TCR, CD3, CD28, Lck, PLC $\gamma$ , and ZAP-70 as well as adaptor proteins like LAT and SLP-76 [8, 78]. Ceramide is a sphingolipid that is known to influence cell fate by participating in various signaling pathways either through modulation of lipid rafts or through direct interaction with downstream effectors. We took interest in studying how this versatile sphingolipid may

affect T cell proliferation, which involves PKC $\theta$  as a key player. The antagonistic relationship between the PKC pathway and ceramide has long been suggested [49, 79]. For instance, the phorbol ester PMA, which is commonly used as an inducer of the PKC $\theta$  pathway, has been shown to oppose the effects of ceramide and vice versa [49, 80, 81].

We used either PMA or anti-CD3 plus anti-CD28 antibodies to activate PKC $\theta$ . The translocation and activation of PKC $\theta$  induced by CD3/CD28 co-stimulation were somewhat stronger than by PMA only. This is not surprising, since PMA alone induces low proliferative responses in T cells [82]. Its effect is enhanced when combined with an ionophore such as ionomycin or even by co-stimulation with anti-CD28 antibody, whereas CD3/



**Fig. 6** Impact of related lipids on PKCθ translocation to lipid rafts and distribution of ceramide in lipid rafts. **a** The impact of exogenous ceramide and other closely related lipids on the translocation of PKCθ to lipid rafts was assessed by measuring the expression level of PKCθ in lipid rafts under each of these conditions. The blot (left) is representative of three independent experiments. Band intensity data presented in the bar graphs (right) are the average of the three experiments with the standard deviations. Highly significant difference ( $P < 0.001$ ) with respect to <sup>(a)</sup> Control and <sup>(b)</sup> PMA. Ponceau-S

staining was used as loading control in membranes. **b** Long-chain ceramide content in lipid raft and non-raft membrane fractions is presented as percentage of the control. Jurkat cells were treated for 30 min with 4 μg/ml anti-CD3 plus 4 μg/ml anti-CD28 or 15 ng/ml PMA either alone or combined with one of the following: 15 μM C6-ceramide for 2 h, 0.3 U/ml bacterial sphingomyelinase for 3 h, anti-Fas ligation (150 ng/ml) for 7 h, and 10 μM FB1 for 2 h followed by anti-Fas (150 ng/ml) treatment for 7 h. The above figures are representative of three independent experiments

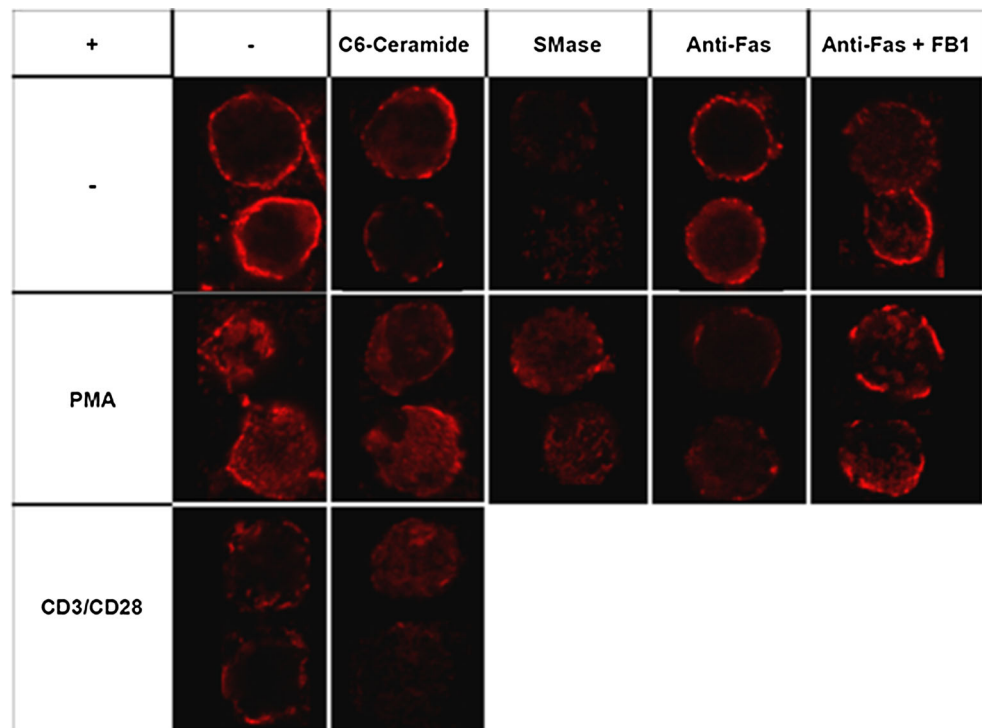
CD28 co-stimulation provides stronger activation of T cells in addition to recruitment of PKCθ to the immunological synapse [19, 83].

Ceramide is said to displace cholesterol from lipid rafts by competing with the sterol, given the similarity in sizes of their polar head groups [84, 85]. When Jurkat cells are exposed to bacterial sphingomyelinase, ceramide is generated at the outer leaflet of the plasma membrane as well as endogenously [86]. Fas ligation triggers ceramide synthesis through activation of both the sphingomyelinase and de novo pathways [74, 87–89]. Such a step is a prerequisite to downstream apoptotic effects [90–92]. The fungal toxin, fumonisins B1 (FB1), has been commonly used to inhibit de novo ceramide synthesis through its inhibition of ceramide

synthase [93–95]. In our experiments, treatment of Jurkat cells with the activating anti-Fas monoclonal antibody reduced PKCθ activity and translocation to lipid rafts. When we pretreated cells with FB1, the inhibitory effects of anti-Fas and C6-ceramide on PKCθ activity and translocation were reversed, indicating that de novo synthesized ceramide is likely a major player in the inhibition of PKCθ.

Additionally, Fas ligation causes intracellular acid sphingomyelinase to translocate to membrane rafts where it generates ceramide from sphingomyelin [96–98]. Ceramide associates with lipid rafts and mediates their re-organization into large membrane platforms where receptor molecules cluster [96, 97, 99]. This process increases

**Fig. 7** Ceramide does not disrupt lipid rafts. Cells were subjected to the previously described conditions then incubated for 1 h at 37 °C in the presence of Alexa Fluor 594-labeled cholera toxin B subunit that specifically binds ganglioside GM1 in lipid rafts



receptor density and facilitates signal transduction partly by stabilizing association of the receptors with their corresponding ligands or intracellular signaling molecules [97, 100]. In our current study, the fact that the levels of flotillin-1 are comparable in the presence and absence of ceramide as well as the gross integrity of lipid rafts, as visualized in Fig. 7, negates the idea that ceramide is disrupting the lipid raft (Fig. 3) or that such mechanism is behind its inhibition of PKC $\theta$ . Moreover, based on our results (Figs. 5, 7), we can say that irrespective of lipid raft distribution, ceramide from different sources decreases PKC $\theta$  activity and translocation to lipid rafts.

In our experiments, ceramide appears to exert its inhibitory effect on PKC $\theta$  activity by regulating its phosphorylation, particularly at Thr538. We suggest that the endogenously generated cytosolic ceramide is responsible. Both long-chain and short-chain forms of ceramide have been shown to activate protein phosphatases, including two major Ser/Thr phosphatases PP2A and PP1 [101–104]. Notably, PP2A inhibits AMPK, which is identified as an activator of PKC $\theta$  [105–108]; it also inhibits the other enzyme shown to phosphorylate PKC $\theta$ , namely GLK/MAP4K3 [109, 110]. When we pretreated Jurkat cells—prior to ceramide addition—with two CAPP inhibitors, okadaic acid and calyculin A, the membrane translocation, phosphorylation, and activity of PKC $\theta$  were restored. These results support the involvement of a CAPP in the dephosphorylation of PKC $\theta$ —either directly by acting on

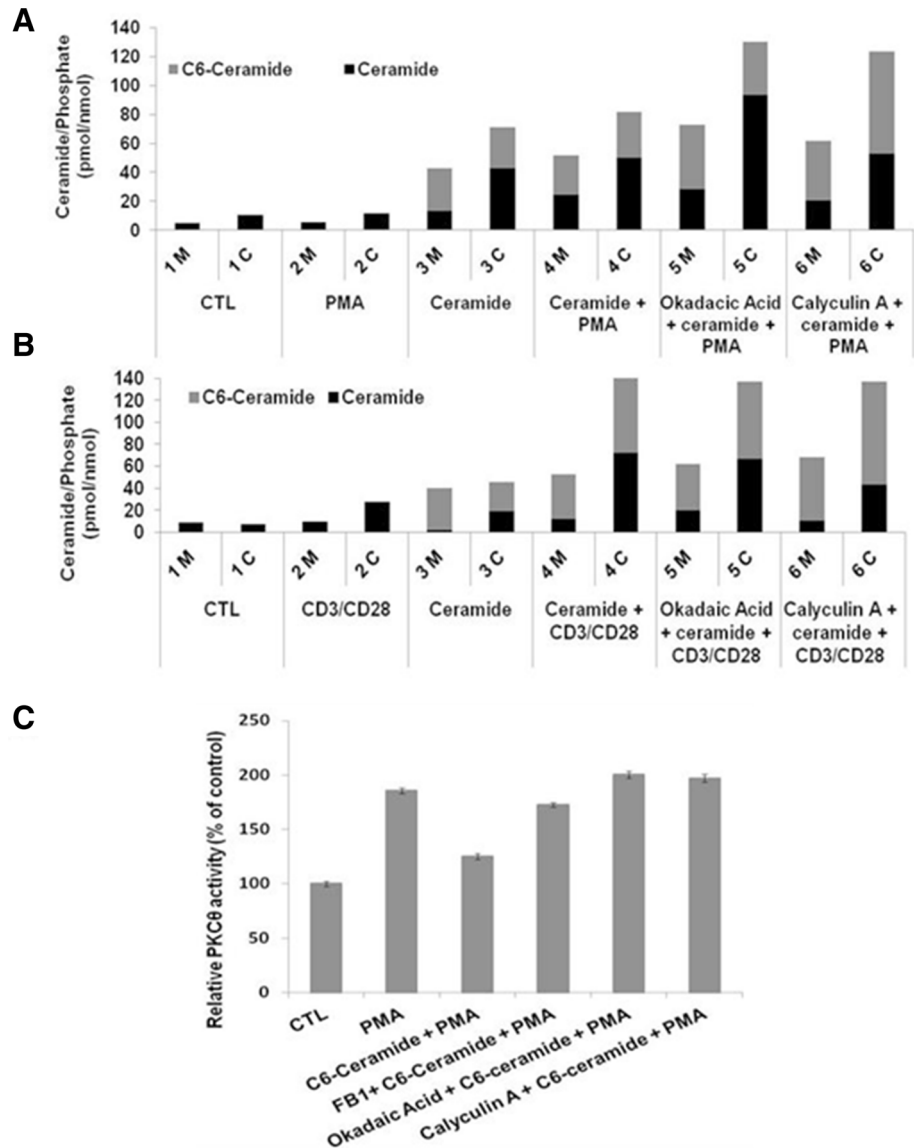
PKC $\theta$  or indirectly by targeting a kinase that activates PKC, such as GLK or AMPK. Similarly, ceramide was shown to inhibit phosphorylation and translocation of PKC $\alpha$  and PKC $\beta$ II particularly through the action of protein phosphatase 1 (PP1) [67, 69, 111, 112].

An interesting model in which ceramide plays double roles to specifically regulate a survival pathway, without disrupting lipid rafts, is its inhibition of the PI3-K/Akt pathway [113]. Ceramide was shown to recruit PTEN, an inhibitor of PDK1, to lipid rafts where PDK1 is supposed to activate Akt [113, 114]. On the other hand, the CAPP PP2A dephosphorylates and inactivates Akt [115]. Furthermore, ceramide was shown to recruit the atypical PKC isoform PKC $\zeta$  to membrane rafts, where PKC $\zeta$  phosphorylates the PH domain of Akt and blocks its ability to interact with 39-phosphoinositides [116, 117].

Additionally, the negative effect of ceramide on the translocation of PKC $\theta$  to lipid rafts may also be amplified by ceramide's inhibition of the PI3-K pathway. As mentioned earlier, activation of PI3-K initiates the recruitment of PKC $\theta$  to the T cell SMAC (10). Hence, besides ceramide's regulation of PDK1, the sphingolipid's regulation of PKC $\theta$  is probably yet another mechanism by which ceramide blocks activation of IKK $\beta$ , resulting in inhibition of NF- $\kappa$ B.

In this study, we examined the phosphorylation of the Thr538 residue because it has been considered a critical activation site in the activation loop, which itself

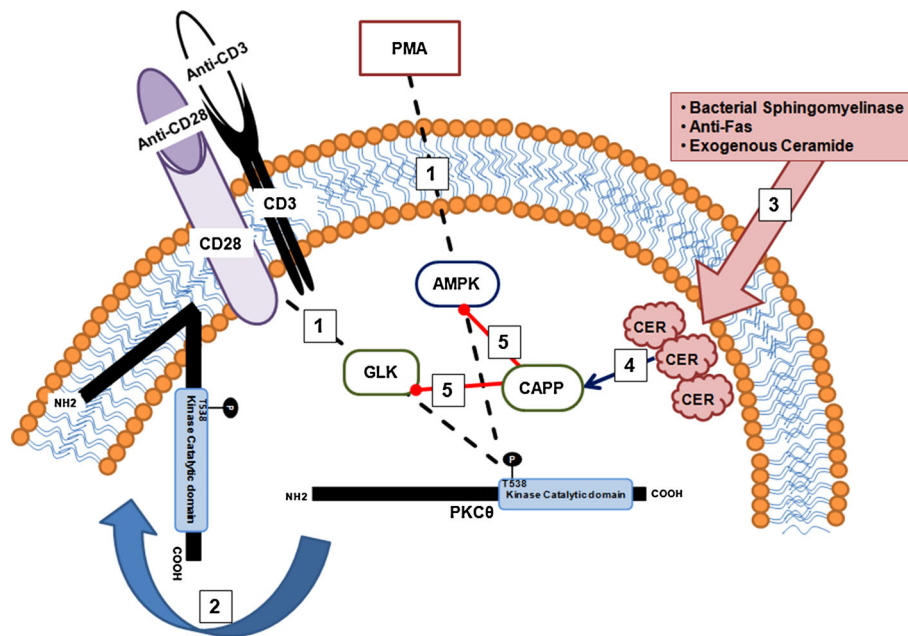
**Fig. 8** Evidence of the role of ceramide-activated protein phosphatases in decreasing the activity of PKC $\theta$ . The levels of endogenous ceramide (Black) and C6-ceramide (Gray) are measured in the cytosol (C) and membrane (M) fractions of Jurkat cells activated for 30 min with **a** PMA or **b** CD3/CD28—in the presence of ceramide (15  $\mu$ M) and the phosphatase inhibitors okadaic acid (10  $\mu$ M) and calyculin A (5 nM). **c** The activity of PKC $\theta$  in Jurkat cells in response to PMA and exogenous ceramide was assessed in the presence of ceramide as well as the phosphatase inhibitors okadaic acid and calyculin A. Data represent the average of triplicate measurements for each condition. Error bars represent the standard deviation. Each of the above figures is representative of three independent experiments



contributes to the binding properties of this kinase [32, 35]. Upon treatment with ceramide, we observed a reduction in Thr538 phosphorylation triggered by PMA or CD3/CD28 co-stimulation. Moreover, the PKC $\theta$  phosphorylation sites are interdependent: Lack of phosphorylation of the activation loop Thr538 residue causes PKC $\theta$  to be in the “closed/inactive” state that is unfavorable for substrate binding and for phosphorylation at other sites [32, 35], including autophosphorylation at S676 and S695, which actually become more susceptible to dephosphorylation by phosphatases [71]. This probably results in the reduction in PKC $\theta$  activity and its translocation to lipid rafts as we had observed upon ceramide accumulation. Furthermore, such

conformation may well affect interactions of PKC $\theta$  with other molecules, including Lck and GLK for instance. As mentioned earlier, Lck directly phosphorylates PKC $\theta$  at tyrosine 90 (Y90), which positively regulates NF-AT and NF- $\kappa$ B activation in T cells [6, 33]. In addition, Y90 phosphorylation regulates the membrane translocation of PKC $\theta$ , although the mechanism is still unclear [6, 33]. In other studies, it was suggested that Lck regulates membrane translocation by participating in the formation of the PKC $\theta$ /Lck/CD28 complex [12, 118, 119].

In summary, we propose a role for cytosolic ceramide in regulating T cell activation through inhibiting PKC $\theta$ 's phosphorylation and translocation to lipid rafts (Fig. 9).



**Fig. 9** Schematic illustration of the proposed model for ceramide's regulation of PKC $\theta$  phosphorylation and translocation to lipid rafts in Jurkat cells. (1) CD3/CD28 co-stimulation and PMA trigger the phosphorylation of PKC $\theta$  at its threonine 538 residue in the catalytic domain in pathways involving GLK and AMPK, respectively. (2) Subsequently, the open/active conformation of PKC $\theta$  allows its translocation to the lipid rafts. (3) Upon addition of bacterial

sphingomyelinase, anti-Fas, or exogenous ceramide, endogenous ceramide (CER) is generated in the cytosol. (4) Ceramide activates one or more ceramide-activated protein phosphatases (CAPP). (5) The activated CAPP dephosphorylates GLK and AMPK, inactivating them. This therefore prevents the phosphorylation and subsequent activation of PKC $\theta$

Our results point to a mechanism involving a CAPP, which either directly or indirectly disrupts activation of PKC $\theta$ . Therefore, ceramide may well serve as a therapeutic tool in research involving auto-immune and inflammatory diseases, where excessive activity of PKC $\theta$  is detected.

#### Compliance with ethical standards

**Conflict of interest** The authors declare no conflict of interest.

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