Phospholipase D1 is threonine-phosphorylated in human-airway epithelial cells stimulated by sphingosine-1-phosphate by a mechanism involving Src tyrosine kinase and protein kinase Cδ

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The regulatory role of protein kinase C (PKC) δ isoform in the stimulation of phospholipase D (PLD) by sphingosine-1-phosphate (SPP) in a human-airway epithelial cell line (CFNPE9o$^-$) was revealed by using antisense oligodeoxynucleotide to PKCδ, in combination with the specific inhibitor rottlerin. Cell treatment with antisense oligodeoxynucleotide, but not with sense oligodeoxynucleotide, completely eliminated PKCδ expression and resulted in the strong inhibition of SPP-stimulated phosphatidic acid formation. Indeed, among the PKCα, β, δ, ϵ and ζ isoforms expressed in these cells, only PKCδ was activated on cell stimulation with SPP, as indicated by translocation into the membrane fraction. Furthermore, pertussis toxin and genistein eliminated both PKCδ translocation and PLD activation. In particular, a significant reduction in phosphatidylbutanol formation by SPP was observed in the presence of 4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine (PP1), an inhibitor of Src tyrosine kinase. Furthermore, the activity of Src kinase was slightly increased by SPP and inhibited by PP1. However, the level of PKCδ tyrosine phosphorylation was not increased in SPP-stimulated cells, suggesting that Src did not directly phosphorylate PKCδ. Finally, the level of serine phosphorylation of PLD1 and PLD2 isoforms was not changed, whereas the PLD1 isoform alone was threonine-phosphorylated in SPP-treated cells. PLD1 threonine phosphorylation was strongly inhibited by rottlerin, by anti-PKCδ oligodeoxynucleotide and by PP1. In conclusion, in CFNPE9o$^-$ cells, SPP interacts with a membrane receptor linked to a G_i type of G-protein, leading to activation of PLD, probably the PLD1 isoform, by a signalling pathway involving Src and PKCδ.

Key words: antisense oligodeoxynucleotide, phosphatidic acid, phosphatidylbutanol, protein phosphatase 1, rottlerin.

INTRODUCTION

Sphingosine-1-phosphate (SPP) belongs to a group of lipid mediators that regulate cell proliferation, differentiation and survival of cells (see for reviews [1–3]). SPP exhibits high affinity with the group B of the lysophospholipid (LP) subfamily of G-protein-coupled membrane receptors, which comprises LP_{B1}, LP_{B3}, LP_{B2}, LP_{B4} and LP_{C1} as described previously [4]; also called $S1P_{1/3/2/5/4}$ [5], formerly Edg-1, -3, -5, -8, -6 (Edg, endothelial differentiation gene) [1]. These receptors are coupled differentially via G_i, G_q and $G_{12/13}$ and Rho to multiple effector systems including adenylate cyclase, phospholipase C (PLC) and D (PLD), intracellular Ca^{2+} elevation, extracellular signal-regulated kinases, actin stress-fibres remodelling, etc. [see 1–4].

In a human-airway epithelial cell line (CFNPE9o$^-$), we have previously reported that SPP induced both elevation of cytosolic Ca^{2+} concentration and the activation of PLD by a pathway independent of PLC activation [6]. Both these effects were sensitive to pertussis toxin (Ptx) pretreatment, suggesting the involvement of a G_i type of G-protein [7].

PLD (EC 3.1.4.4) catalyses the hydrolysis of the most abundant membrane phospholipid, phosphatidylcholine, generating the potential second-messenger phosphatidic acid (PA) [8]. Two mammalian PLD enzymes PLD1 and PLD2 have been cloned and shown to have a broad tissue distribution [9]. Both enzymes prefer phosphatidylcholine as substrate and require phosphatidylinositol 4,5-bisphosphate for activity. PLD1 is activated by low-molecular-mass G-proteins either of the Rho family or ADP-ribosylation factor (ARF) [10,11] by protein kinase C (PKC) [11–14], and can also be tyrosine-phosphorylated [15]. In contrast, purified PLD2 is not stimulated by ARF, Rho and PKCα or PKCβ [16], but it can be tyrosine-phosphorylated [17]. Although it is known that PKC is involved in the activation of PLD in intact cells, the role of the different PKC isoforms is still poorly understood. In skeletal-muscle C2C12 cells, SPP-induced PLD activation has been shown to be dependent on PKCα and PKCδ isoforms [18]. Conversely, human PLD1 and PLD2 expressed in Sf9 cells were activated by co-expression with PKCα, whereas co-expression with the PKCδ isoform had no effect [14], in agreement with the results *in vitro* showing the lack of effect of this PKC isoform on PLD1 [19]. Furthermore, PKCα and PKCδ had antagonistic effects on PLD activation, exhibiting positive and negative regulation respectively, as reported by Hornia et al. [20].

We have recently reported that the monomeric GTPases Rho and ARF are not involved in the SPP-induced PLD activation in CFNPE9o$^-$ cells [21]. In the present study, we show that rottlerin, an inhibitor with high specificity for the PKCδ isoform [22], significantly inhibited SPP-stimulated PA formation. The finding that the effect of rottlerin was dependent on the preincubation time, in addition to its reported ability also to inhibit other kinases [23], prompted us to utilize the highly specific antisense RNA technology to demonstrate the role of PKCδ on PA production. We observed that among the various PKC isoforms, only the PKCδ isoenzyme became associated with membrane, with a time course similar to that of PA production. In addition,

Abbreviations used: ARF, ADP-ribosylation factor; BAPTA/AM, bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(acetoxymethyl ester); DMEM, Dulbecco's modified Eagle's medium; Edg, endothelial differentiation gene; FCS, foetal calf serum; MAP, mitogen-activated protein; LP, lysophospholipid; PA, phosphatidic acid; PEI, poly(ethyleneimine); PKC, protein kinase C; PLD, phospholipase D; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine; Ptx, pertussis toxin; Pyk, proline-rich tyrosine kinase-2; SPP, sphingosine-1-phosphate.

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the Src tyrosine kinase has been shown to be activated by the SPP treatment and involved in the PLD activation. Finally, analysis of serine and threonine phosphorylation of the endogenous PLD1/PLD2 isoforms revealed that the PLD1 isoform alone was threonine-phosphorylated after SPP stimulation.

MATERIALS AND METHODS

Materials

Ptx, genistein and poly(ethyleneimine) (PEI) were purchased from Sigma, rottlerin and SPP were from Calbiochem (La Jolla, CA, U.S.A.) and [^{14}C]palmitic acid from NEN Products (Stevenage, Herts, U.K.). 4-Amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine (PP1) was purchased from Alexis Biochemicals (San Diego, CA, U.S.A.) and bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM) from Molecular probes (Eugene, OR, U.S.A.). PKC isoforms polyclonal antibodies and phosphospecific antibodies anti-P-serine and P-threonine were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.); phosphospecific antibody anti-P-tyrosine was from New England Biolabs (MA, U.S.A.). Phosphorylated form of Src was detected with rabbit polyclonal anti-P-Tyr^{418} antibody and pan antiserum purchased from BioSource Europe (Nivelles, Belgium). PLD1 and PLD2 antibodies were a gift from Dr S. Bourgoin (Centre de Recherche en Rhumatologie et Immunologie, Centre de Recherche de l'Hotel-Dieu-de-Qubec, Faculté de Médecine, Université Laval, Quebec, Canada).

Cells

The CFNPE9o$^-$ cell line was derived from a nasal polyp [24]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) foetal calf serum (FCS), 4 mM L-glutamine and antibiotics.

Oligonucleotide treatment of cells

Phosphodiester oligodeoxynucleotides were purchased from MWG-Biotech (Ebersberg, Germany). Antisense oligodeoxynucleotide (5′-AGGGTGCCATGATGGA-3′) was complementary to the translation-initiation region (nucleotides 6–10) of mRNA-specific mouse PKCδ [25], previously shown to be effective also in human cells [26]. Sense (5′-TCGATCATGGCACCCT-3′) oligodeoxynucleotide was used as a control [25,26]. Cells were seeded in 6-well plates or Petri dishes, grown to confluence and incubated in DMEM containing 10 % (v/v) FCS, in the absence or presence of 1 mM PEI plus oligodeoxynucleotides (1.7 μg/ml) for 24 h. The culture medium was then replaced with fresh medium without FCS, containing all the additions and incubated for 24 h.

Measurement of PA and phosphatidylbutanol

Cells grown in 6-well plates were serum-starved for 24 h and labelled for 16 h with 1 μCi/ml of [1-^{14}C]palmitic acid. The medium was aspirated and cells were rinsed and stimulated for 2 min with the indicated agents, in the absence or presence of 0.5 % butan-1-ol. Cells were preincubated for 10 min with butan-1-ol before stimulation. Extraction and separation of lipids by TLC were performed as described previously [7].

Subcellular fractionation and immunoblotting

Cells were grown in 10 mm diameter Petri dishes and maintained without FCS for 24 h (five Petri dishes for each experimental condition). Cells were washed with PBS and stimulated at 37 °C, as described in the Figure legends. Cells were washed again with PBS and scraped in 1 ml of 250 mM sucrose, 2 mM EGTA, 1 mM EDTA, 1 mM PMSF and 20 mM K-Hepes (pH 7.4). Homogenization with 30 strokes of a Dounce homogenizer was performed at 4 °C. Homogenates were centrifuged at 500 ***g*** for 10 min at 4 °C, and the resulting supernatant was centrifuged at 110000 ***g*** for 30 min. The supernatant (cytosolic fraction) was stored at −80 °C until use. In some experiments, the cytosolic fractions were immunoprecipitated as described in the following section. The resulting pellet was resuspended in 0.1 ml of the same buffer containing 1 % (v/v) Triton X-100 and 100 μl/ml protease inhibitors cocktail and centrifuged at 110000 ***g*** for 50 min. The supernatant (Triton X-100-soluble membrane fraction) was stored at −80 °C. The protein content in the fractions was determined as in [27]. The protein (30–50 μg) was dissolved in Laemmli buffer, separated by SDS/PAGE (10 % gel) and transferred on to a nitrocellulose membrane (Bio-Rad Laboratories, Glattbrugg, Switzerland). The anti-PKC isoform primary antibodies were diluted in the ratio 1 : 500. Antigen–antibody complexes were revealed by using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminesence detection kit (Amersham Pharmacia Biotech). The densities of the bands were measured using the Fluo-2 MAX Multimager system (Bio-Rad Laboratories).

Src kinase assay

Cell lysates were immunoblotted with anti-P-Tyr^{418} Src or antibodies raised against Src. Src kinase activity was quantified as an increase in P-Tyr^{418} Src phosphorylation, normalized for the amount of total Src.

PLD1/PLD2 and PKCδ immunoprecipitation and phosphorylation

Cells were incubated as above and harvested in lysis buffer containing 20 mM Tris/HCl (pH 8.0), 100 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 % (v/v) Nonidet P40, 1 mM benzamidine, 5 mM NaF, 1 mM PMSF, 10 mM *p*-nitrophenylphosphate, 1 mM dithiothreitol, 10 mM β-glycerophosphate and the protease inhibitors. Lysates were clarified, incubated with antisera raised against PKCδ or PLD1 (antiserum 3) or PLD2 (antiserum 26) for 2 h and then with Protein A–agarose beads (Amersham Pharmacia Biotech, Cologno Monzese, Italy) for 1 h. The immunocomplex was centrifuged, washed three times with lysis buffer and dissolved in Laemmli buffer. Immunoprecipitated proteins were separated in SDS/PAGE (10 % gel), transferred on to the nitrocellulose membranes and probed with anti-P-serine/anti-P-threonine antibodies (Sigma Aldrich) and anti-P-tyrosine (Biolabs). Immunoreactivity was detected with an ECL® system.

Statistical analysis

Statistical analysis was performed using Student's *t* test.

RESULTS

PKCδ isoform is required for SPP-induced PLD activity

We have previously shown that SPP induced a time- and dose-dependent stimulation of PA formation, which was due to activation of PLD. Indeed, PA production, which was maximal after 2 min incubation with SPP, was almost completely inhibited

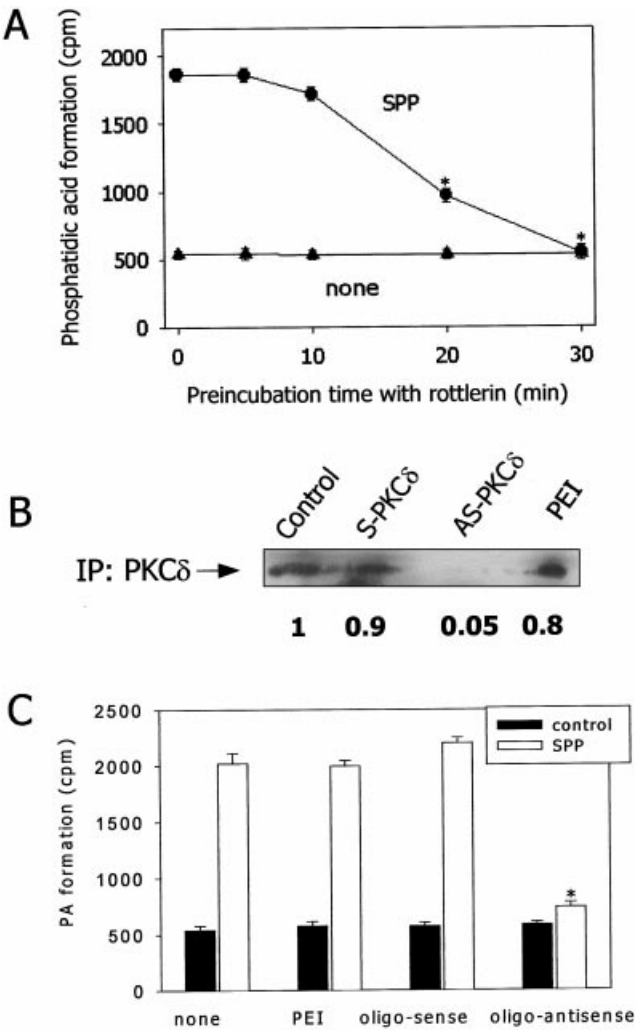

Figure 1 Effect of the PKCδ inhibitor rottlerin and antisense oligodeoxynucleotide to PKCδ on PA formation by SPP

(**A**) CFNPE9o$^-$ cells, serum-starved for 24 h, were labelled with [^{14}C]palmitic acid for 16 h, incubated for the indicated time with 10 μM rottlerin, then stimulated for 2 min in the absence (none) or presence of 1 μM SPP. PA was determined as described in the Materials and methods section. Values are means $\pm$ S.D. for four determinations. *$P < 0.01$, significantly different from SPP-treated cells without rottlerin. (**B**) Cells were incubated in DMEM (control), in the presence of PEI alone or with antisense oligodeoxynucleotide to PKCδ (AS-PKCδ) or sense oligodeoxynucleotide to PKCδ (S-PKCδ) for 48 h. The level of PKCδ isoform in cell lysates was determined after immunoprecipitation and Western blotting; the density of bands was quantified as described in the Materials and methods section, and expressed as fold increase or decrease over the value measured in untreated cells, denoted as 1. The plot is representative of three similar experiments, obtained from three different immunoprecipitated lysates. IP, immunoprecipitation. (**C**) Cells were preincubated in the presence of PEI alone or antisense oligodeoxynucleotide to PKCδ or sense oligodeoxynucleotide to PKCδ for 48 h, serum-starved for 8 h and, after addition of [^{14}C]palmitic acid, incubated for 16 h. Cells were stimulated without (none) or with 1 μM SPP for 2 min and PA was determined. Values are means $\pm$ S.D. for three determinations. *$P < 0.01$, significantly different from SPP-treated cells.

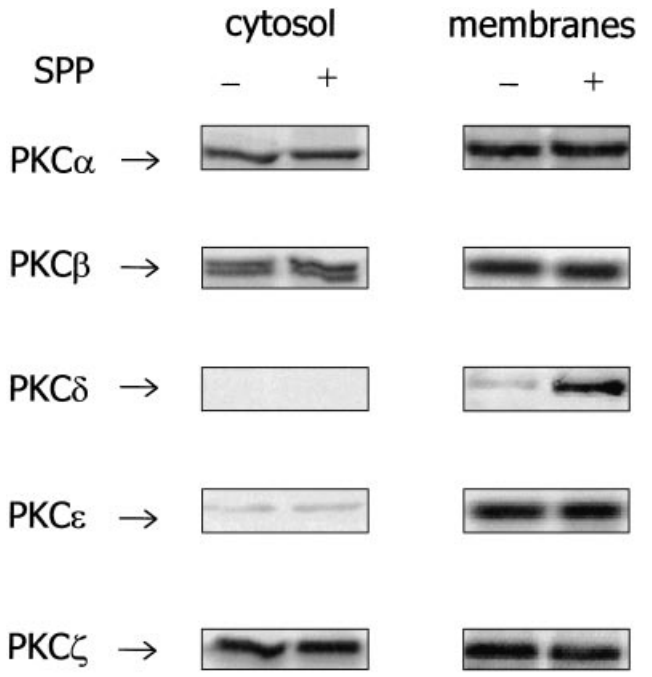

Figure 2 Effect of SPP on PKC isoforms distribution between cytosol and membrane fractions

Serum-starved CFNPE9o$^-$ cells were incubated in the absence or presence of 1 μM SPP for 2 min. The cytosolic fractions and the 1% (v/v) Triton X-100-soluble membrane fractions (30–50 μg), obtained as described in the Materials and methods section, were separated by SDS/PAGE and Western-blot analysis. Results are representative of three similar experiments.

by preincubation with butan-1-ol, with concomitant production of phosphatidylbutanol, whereas it was insensitive to butan-2-ol [7,21]. Figure 1(A) shows that rottlerin (10 μM), a PKC inhibitor with high selectivity for the PKCδ isoform [22], caused a time-dependent inhibition of PA formation by SPP, with a preincubation time of 30 min required to achieve complete inhibition. Formation of phosphatidylbutanol by SPP was markedly reduced by rottlerin preincubation [332 ± 18 ($n = 3$) and 338 ± 22 ($n = 3$) c.p.m.: control cells in the absence and presence of rottlerin; 1508 ± 33 ($n = 3$) and 358 ± 22 ($n = 3$) c.p.m. ($P < 0.01$): SPP-treated cells in the absence and presence of rottlerin], indicating that SPP-induced PA formation was generated by PLD activation.

To pinpoint the role of PKCδ isoform in SPP-induced PLD activation, we used the antisense RNA technology. CFNPE9o$^-$ cells were cultured in the presence of antisense oligodeoxynucleotide to PKCδ for 48 h. PEI was used to increase the potential of antisense oligodeoxynucleotide, reducing the concentration of oligodeoxynucleotide necessary to achieve the maximal effect. PKCδ expression of cells treated with antisense oligodeoxynucleotide to PKCδ, and as a control with sense oligodeoxynucleotide to PKCδ, was assessed after immunoprecipitation of cell lysates with anti-PKCδ antibody. Figure 1(B) illustrates that treatment with antisense oligodeoxynucleotide to PKCδ completely eliminated PKCδ expression, whereas the sense oligodeoxynucleotide or PEI alone had no effect. The specificity of antisense oligodeoxynucleotide to PKCδ was assessed from immunoblot analysis for other PKC isoforms: PKCα, β and ϵ levels were not altered in cells treated with the antisense to PKCδ (results not shown). Antisense oligodeoxynucleotide to PKCδ caused complete inhibition of PA formation by SPP, whereas PEI alone and sense oligodeoxynucleotide to PKCδ were ineffective, as shown in Figure 1(C). Taken together, these results suggest that PKCδ is required for SPP-induced PLD activation.

The PKC family consists of multiple isoenzymes that differ in their regulatory domain, and their dependence on Ca^{2+} and diacylglycerol [28]. By means of a Western-blot analysis of

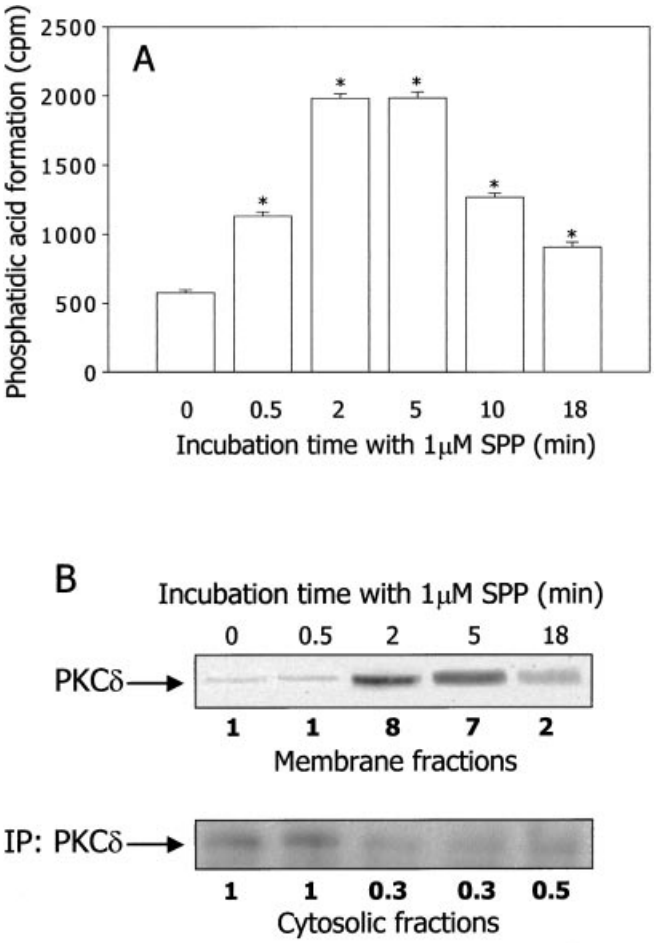

Figure 3 Time course of SPP-induced PA production and PKCδ membrane association

(**A**) Serum-starved CFNPE9o$^-$ cells were labelled for 16 h with [^{14}C]palmitic acid and then incubated in the presence of 1 μM SPP for the times indicated. Values are means $\pm$ S.D. for three determinations. *$P < 0.01$, significantly different from untreated cells at time 0. (**B**) Serum-starved cells were stimulated for the indicated times in the presence of 1 μM SPP, harvested and the cytosolic and membrane fractions were separated as described in the Materials and methods section. The cytosolic fractions were immunoprecipitated with anti-PKCδ antibody. The amount of PKCδ in both fractions was determined by Western blotting. The boldface numbers under each band of the blots are the quantitative values of the band density, expressed as fold increase or decrease over the value measured in untreated cells at time 0, denoted as 1. The results are the mean values from three different experiments. IP, immunoprecipitation.

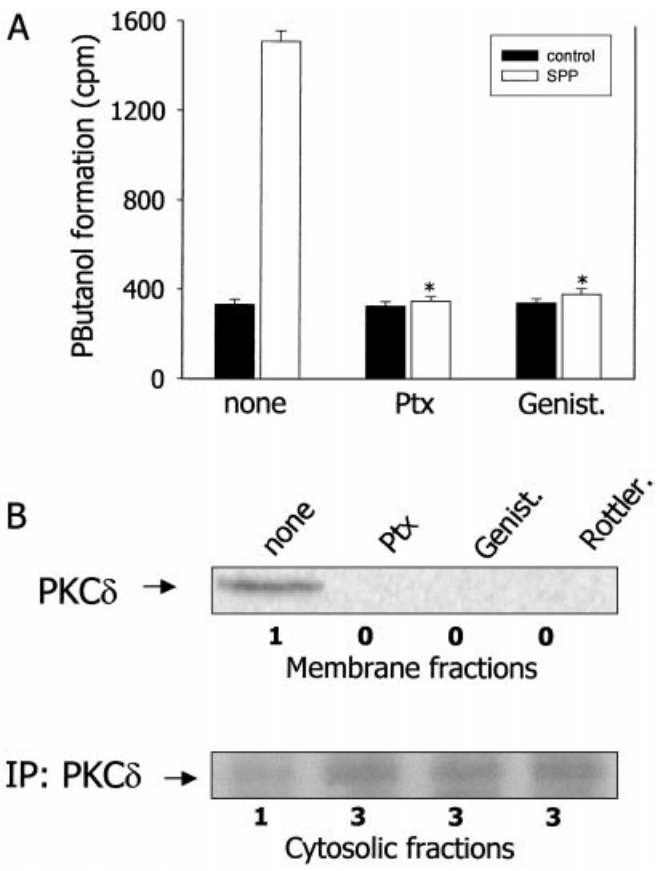

Figure 4 Inhibition of SPP-induced phosphatidylbutanol production and PKCδ membrane association by Ptx and genistein

(**A**) Serum-starved CFNPE9o$^-$ cells were labelled for 16 h with [^{14}C]palmitate, preincubated for 4 h with 400 ng/ml Ptx or for 30 min with 100 μm genistein (Genist.), incubated for 10 min with 0.5% butan-1-ol, and then stimulated for 2 min without (control) or with 1 μM SPP. Phosphatidylbutanol (PButanol) was determined as described in the Materials and methods section. Values are means $\pm$ S.D. for at least three determinations. *$P < 0.01$, significantly different from SPP-treated cells. (**B**) Serum-starved CFNPE9o$^-$ cells were preincubated for 4 h with 400 ng/ml Ptx or for 30 min with 100 μm genistein (Genist.) or with 10 μM rottlerin (Rottler.), and then stimulated for 2 min with 1 μM SPP. Membrane and immunoprecipitated cytosolic fractions were obtained as described in Figure 3(B). The amount of PKCδ in the fractions was determined and quantified as described in Figure 3(B). Results are representative of three similar experiments. IP, immunoprecipitation.

whole-cell extracts, we established that five PKC isoforms are expressed in CFNPE9o$^-$ cells (α, β, δ, ϵ and ζ; results not shown). To assess the activation of PKC isoforms, we took advantage of the fact that translocation to a membrane fraction is widely recognized as an index of activation for many PKC isoforms [29]. In the cytosolic fractions, the PKCδ isoform level was undetectable and also the ϵ isoform was barely detectable in comparison with the α, β and ζ isoforms (Figure 2). The level of all isoforms in this fraction did not change after incubation of cells with 1 μM of SPP for 2 min. Only the amount of PKCδ associated with the Triton X-100-soluble membrane fraction was increased, whereas the PKC isoenzymes α, β, ϵ and ζ were not translocated on to the membrane on SPP treatment.

Figure 3 illustrates that the time courses of both PA formation and PKCδ membrane association by SPP were similar. The maximal stimulation of PA production and PKCδ translocation was apparent between 2 and 5 min and then decreased to return to the basal level after 18 min. Accordingly, the amount of PKCδ in the cytosolic fractions, determined after immunoprecipitation decreased, after 2–5 min. These results strongly support the suggestion that activation of PLD is dependent on PKCδ stimulation.

Effect of tyrosine phosphorylation inhibitors on SPP-induced phosphatidylbutanol formation and PKCδ translocation

In agreement with previously reported results [6,7], preincubation with Ptx blocked SPP-induced PLD activation in CFNPE9o$^-$ cells (Figure 4A). Furthermore, Ptx eliminated PKCδ membrane translocation, which was almost undetectable after rottlerin treatment (Figure 4B). Preincubation with the protein tyrosine kinase inhibitor genistein significantly inhibited both SPP-induced phosphatidylbutanol formation and PKCδ membrane

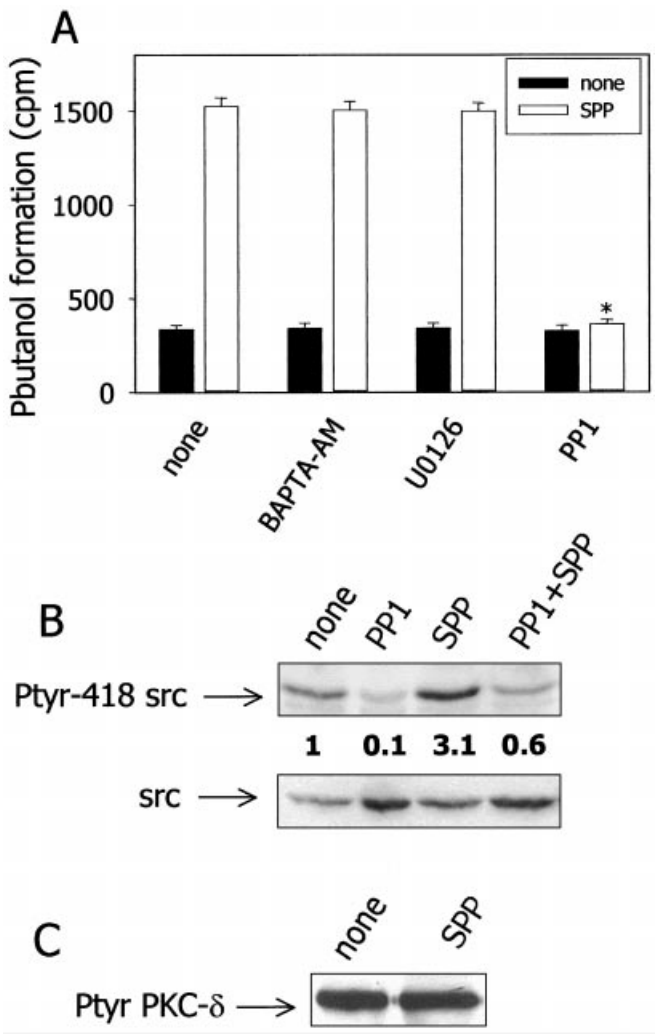

Figure 5 Src kinase is involved in SPP-induced phosphatidylbutanol formation

(**A**) Serum-starved CFNPE9o$^-$ cells were labelled for 16 h with [^{14}C]palmitic acid, preincubated for 30 min with 10 μM BAPTA/AM or with 10 μM U0126 or 10 μM PP1, for 10 min with 0.5% butan-1-ol, and then stimulated for 2 min without (none) or with 1 μM SPP. Phosphatidylbutanol formation was determined as described in the Materials and methods section. Values are means $\pm$ S.D. for four determinations. *$P < 0.01$, values significantly different from SPP-treated cells. (**B**) Serum-starved cells were preincubated for 30 min without or with 10 μM PP1, and then stimulated for 2 min in the absence or presence of 1 μM SPP. Cells were scraped and cell lysates were immunoblotted with antibody against P-Tyr^{418} Src and then with antibody raised against Src. Src kinase activity was quantified as an increase in P-Tyr^{418} Src phosphorylation, normalized to Src content, and expressed as fold increase over untreated cells, denoted as 1 (boldface numbers under each band of the blot). Results are representative of three similar experiments. (**C**) Serum-starved cells were incubated for 2 min in the absence or presence of 1 μM SPP. Cells were scraped and cell lysates were subjected to immunoprecipitation with anti-PKCδ antibody and immunoblotting with anti-phosphotyrosine and anti-PKCδ antibodies. Tyrosine phosphorylation was quantified from the band density and normalized to the amount of PKCδ in the immunoprecipitates. No significant difference in the normalized band density was determined in three independent experiments.

translocation (Figures 4A and 4B), suggesting that a tyrosine phosphorylation step must occur between the SPP-receptor activation and PLD. As a control, determination of PKCδ in the immunoprecipitated cytosolic fractions showed an increase in the PKCδ level, under all these three conditions, in comparison with untreated cells (Figure 4B).

Among the cytosolic protein tyrosine kinases, proline-rich tyrosine kinase-2 (Pyk2, also called RAFTK) has been shown to be activated by a variety of signals that elevate intracellular Ca^{2+} concentration [30], including SPP [31]. A role for Pyk2 has been ruled out by the finding that preincubation with the intracellular Ca^{2+} chelator BAPTA/AM failed to inhibit SPP-induced PLD activation, as shown in Figure 5(A). Recently [32,33], the tyrosine kinase Src also has been reported to act as an intermediate in the SPP-activated intracellular signalling pathway. Figure 5(A) shows that the Src inhibitor PP1 (10 μM), but not the mitogen-activated protein (MAP) kinase/extracellular-signal-regulated kinase (ERK) inhibitor U0126 (10 μM), markedly inhibited phosphatidylbutanol formation by SPP. Src kinase activation in response to SPP has been directly determined by detection of Tyr^{418} phosphorylation. Src kinase activity was slightly increased (approx. 3-fold) by SPP stimulation and significantly reduced by preincubation with PP1 in both untreated and SPP-stimulated cells (Figure 5B). Then, we tested whether the tyrosine phosphorylation level of PKCδ was increased by SPP stimulation. Lysates from control and SPP-treated cells were immunoprecipitated with PKCδ antibody and probed with an anti-phosphotyrosine antibody. Figure 5(C) shows that the remarkable level of tyrosine phosphorylation, already apparent in untreated cells, was not increased even after incubation with SPP.

PLD1 isoform is threonine-phosphorylated in response to SPP

The results shown in Figures 1–4 suggest that PKCδ is required for SPP-induced stimulation of PLD. We have therefore examined the serine and threonine phosphorylation status of both the PLD isoforms expressed in CFNPE9o$^-$ cells [21]. Cell lysates were immunoprecipitated with PLD1 or PLD2 antibody, immunoblotted with anti-phosphoserine and anti-phosphothreonine antibodies and then probed with anti-PLD1 and -PLD2 antisera. The level of PLD2 serine and threonine phosphorylation was not altered in cells treated with SPP, whereas the level of PLD1 threonine, but not of serine phosphorylation, was increased 2.5-fold (Figures 6A and 6B). In addition, preincubating for 30 min with rottlerin or PP1 or 48 h treatment with antisense oligodeoxynucleotide to PKCδ remarkably reduced SPP-induced PLD1 threonine phosphorylation (Figures 6C and 6D).

To assess whether PKCδ and PLD1 interact directly, experiments of co-immunoprecipitation have been performed. Cell lysates from untreated and SPP-treated cells have been immunoprecipitated with PKCδ antibodies and probed with anti-PLD1 antibodies or immunoprecipitated with PLD1 antibodies and probed for PKCδ antibodies. Under both conditions, the two proteins, extracted either from control or SPP-stimulated cells, did not co-immunoprecipitate (results not shown).

DISCUSSION

PKC has been considered as one of the major activators of PLD in most cells, but the mechanism of activation *in vivo* is not known in detail. One of the reasons for this uncertainty is that some pharmacological inhibitors, historically employed to inhibit PKC, have complex effects on cellular responses, leading to incorrect conclusions. The antisense RNA technique, in combination with the use of the isotype-specific inhibitor rottlerin, allowed us to reveal the crucial role for the PKCδ isoform in the PLD activation by SPP in CFNPE9o$^-$ cells. The antisense oligodeoxynucleotides to the translation-initiation region of mRNA for PKCδ were previously shown to be effective particu-

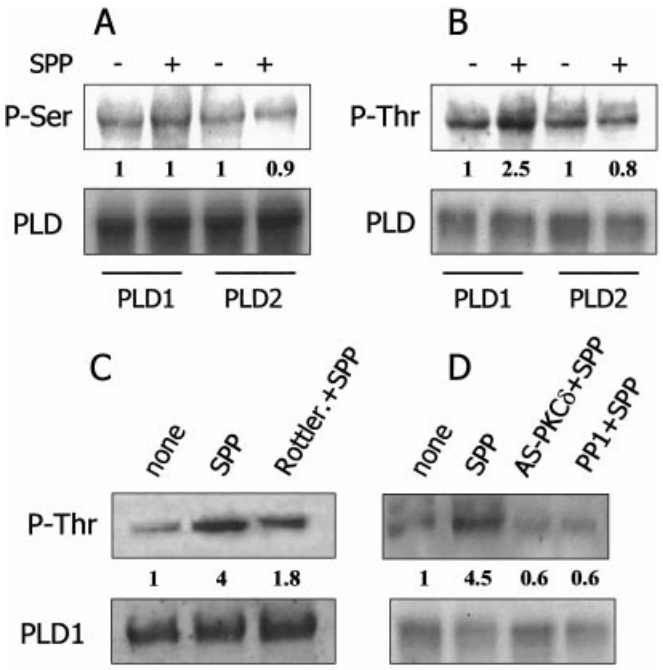

Figure 6 Serine and threonine phosphorylation of PLD1 and PLD2 in SPP-stimulated cells: effect of rottlerin, antisense oligodeoxynucelotide to PKCδ and PP1

Serum-starved CFNPE9o$^-$ cells were incubated in the absence or presence of 1 μM SPP for 2 min. (**A**) PLD1 and PLD2 were immunoprecipitated from cell lysates and immunoblotted with anti-phosphoserine or (**B**) anti-phosphothreonine antibodies and then reprobed with PLD1/PLD2 antibodies. (**C**) Where indicated, cells were preincubated for 30 min with 10 μM rottlerin or (**D**) with 10 μM PP1 or for 48 h in the presence of PEI and antisense oligodeoxynucelotide to PKCδ and then stimulated for 2 min with 1 μM SPP. (**C**, **D**) PLD1 was immunoprecipitated from the cell lysates, immunoblotted with anti-phospho-threonine antibody and reprobed with anti-PLD1 antibody. The densities of phosphorylated bands, normalized to PLD1/PLD2 levels in the immunoprecipitates, quantified as described in the Materials and methods section are expressed as fold increase over untreated cells, denoted as 1 (boldface numbers under each band of the blots). Data are representative of three consistent experiments.

larly in blocking mRNA processing, transport or translation [25,26]. Treatment of cells with antisense oligodeoxynucleotide to PKCδ for 48 h blocked the SPP-stimulated PLD activity. Under this condition, the amount of PKCδ in immunoprecipitated cell lysates was almost undetectable. The involvement of PKCδ was further confirmed by the finding that among the various PKC isoforms analysed, only PKCδ translocated into the Triton X-100-soluble membrane fraction on cell stimulation with SPP, with a time course similar to that of PA generation. It should be noted that no activation of the PKCα isoform was observed, in contrast with the results reported previously [16,18,34].

PKCδ is a novel PKC isoform, which is activated by diacylglycerol [28]. We have previously shown that in CFNPE9o$^-$ cells, the receptor-mediated elevation of cytosolic Ca^{2+} concentration caused by SPP was independent of PLC activation, since no production of total inositol phosphates was detected [6]. Accordingly, the level of the other PLC product diacylglycerol did not change up to 8 min of incubation with SPP, whereas a significant increase was observed after 10 min [7]. The increase in diacylglycerol formation occurred simultaneously with the decrease in PA and was eliminated by butan-1-ol, suggesting that the late diacylglycerol production occurred via a sequential PLD/PA phosphohydrolase-linked pathway [7]. It is apparent that at the time of maximal SPP-induced PKCδ membrane translocation (2–5 min), the level of the PKCδ activator diacylglycerol was not increased, and therefore an alternative mechanism for PKCδ stimulation must occur. Like many other protein kinases, PKCδ is functionally modulated by tyrosine phosphorylation [35]. Accordingly, both PKCδ membrane translocation and phosphatidylbutanol formation induced by SPP were inhibited by the tyrosine kinase inhibitor genistein, suggesting the involvement of a tyrosine protein kinase. It has been reported that both the non-receptor tyrosine kinases Pyk2 [31] and Src [32,33] are activated in the signalling cascade triggered by receptor-mediated SPP binding. The possible role of Pyk2 in PLD activation, which is well known to be stimulated by a variety of agents that increase intracellular Ca^{2+} concentrations, has been ruled out by the finding that preincubation of CFNPE9o$^-$ cells with the intracellular Ca^{2+} chelator BAPTA/AM failed to inhibit SPP-induced PLD activation. The non-receptor tyrosine kinase Src has been previously shown to be required for SPP-induced p42/p44 MAP kinase pathway activation via a Ptx-sensitive mechanism [32]. In the present study, we show in CFNPE9o$^-$ cells that Src kinase activity was also slightly increased in response to SPP and it was also involved in SPP-induced PLD stimulation. However, the MAP kinase pathway did not play any role, in disagreement with results recently reported for (norepinephrine) noradrenaline-induced PLD2 stimulation in vascular smooth-muscle cells [17]. However, we were unable to detect any increase in the level of PKCδ tyrosine phosphorylation after stimulation with SPP. Owing to the fact that the level of tyrosine phosphorylation of PKCδ in non-stimulated cells was very high, we cannot rule out the possibility that a low tyrosine phosphorylation by SPP might indeed occur. It is also possible that another protein might be tyrosine-phosphorylated by Src upstream of PKCδ or, alternatively, stimulus-dependent association of active Src and PKCδ might induce the activation of PKC, since it has been reported that tyrosine phosphorylation of PKCδ is not essential for kinase activity [36].

In the present study, we show that the PLD1 isoform alone becomes threonine-phosphorylated in SPP-stimulated cells. In contrast, the level of phosphorylated serine as well as tyrosine (results not shown) of both PLD1 and PLD2 isoforms was not changed after SPP treatment. This result is in agreement with recent reports [13,37] showing that PLD1, transiently overexpressed in COS-7 cells, was activated by the phorbol ester PMA and phosphorylated in three residues: Ser^{2}, Thr^{147} and Ser^{561}. One site in particular, amino acid 147, was significantly phosphorylated and suggested to affect the activation of PLD *in vivo* [37].

It is remarkable that the increased threonine phosphorylation of PLD1 isoform, reported in the present study, was significantly reduced by incubation with either rottlerin or oligodeoxynucleotides anti-PKCδ or PP1, which blocked SPP-stimulated PA/phosphatidylbutanol formation. Although these results do not directly demonstrate the involvement of threonine phosphorylation in the activation of PLD, similar inhibitions caused by the three compounds indirectly support this assumption.

In this respect, it is interesting to note that both PKCδ and PLD1 were detected in the Triton X-100-soluble membrane fraction (see Figure 2 and [20] respectively), although a direct interaction between the two proteins could not be determined. It is also noteworthy that the increased threonine phosphorylation of PLD1, shown in the present study, has been determined after activation of an endogenous receptor and without overexpression of the phospholipase and of the PKC isoform.

Finally, it is noteworthy that both PLD activation and PKCδ membrane translocation were completely eliminated by Ptx, a G_i-mediated signal, in agreement with the recent hypothesis

that receptors which signal via G_i alone activate PKC, but do not activate Rho effectively [38]. We have previously shown that the high-affinity SPP receptors LP_{B1} ($S1P_1$/Edg-1), LP_{B3} ($S1P_3$/Edg-3) and LP_{B2} ($S1P_2$/Edg-5) are expressed in CFNPE9o$^-$ cells [7]. The sensitivity to Ptx does not allow us to discriminate among these three receptors, as all of them can be coupled with the G_i type of G-proteins. Identification of the SPP receptor involved in PKCδ-dependent PLD activation remains a challenge for further studies.

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