

# Receptor-mediated activation of phospholipase D by sphingosine 1-phosphate in skeletal muscle C2C12 cells

## A role for protein kinase C

Elisabetta Meacci\*, Valeria Vasta, Chiara Donati, Marta Farnararo, Paola Bruni

*Dipartimento di Scienze Biochimiche, University of Florence, Viale G.B. Morgagni 50, 50134 Firenze, Italy*

Received 18 June 1999; received in revised form 22 July 1999

**Abstract** The present study showed that sphingosine 1-phosphate (SPP) induced rapid stimulation of phospholipase D (PLD) in skeletal muscle C2C12 cells. The effect was receptor-mediated since it was fully inhibited by pertussis toxin. All known SPP-specific receptors, Edg-1, Edg-3 and AGR16/H218, resulted to be expressed in C2C12 myoblasts, although at a different extent. SPP-induced PLD activation did not involve membrane translocation of PLD1 or PLD2 and appeared to be fully dependent on protein kinase C (PKC) catalytic activity. SPP increased membrane association of PKC $\alpha$ , PKC $\delta$  and PKC $\lambda$ , however, only PKC $\alpha$  and PKC $\delta$  played a role in PLD activation since low concentrations of GF109203X and rottlerin, a selective inhibitor of PKC $\delta$ , prevented PLD stimulation.

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**Key words:** Phospholipase D; Sphingosine 1-phosphate; Protein kinase C; C2C12 myoblast; Edg receptor

### 1. Introduction

Phospholipase D (PLD), a well-known enzyme responsible for the hydrolysis of phosphatidylcholine (PtdCho) and/or phosphatidylethanolamine to phosphatidic acid (PtdOH) and, respectively, choline or ethanolamine, is an important effector in cell signaling [1].

Two mammalian PLDs, PLD1 and PLD2, have recently been cloned, both widely expressed in many cell types and tissues [1]. PLD2, which has been less characterized than PLD1, appears to be constitutively active and under the control of physiological regulators not different from those of PLD1 [2,3]. Agonist-stimulated PLD activity absolutely requires phosphatidylinositol 4,5-bisphosphate as cofactor and appears to be highly regulated by a complex group of proteins, including protein kinase C (PKC), monomeric G proteins and protein-tyrosine kinases [1]. However, the exact order of events leading from receptor occupancy by ligand to PLD activation and their relative importance are not completely defined. Many studies generally support the involvement of PKC in the action of many G protein-linked agonists and growth factors on PLD in vivo, although negative or partial effects have been observed in some studies [1]. Among the known isoenzymes of PKC, the calcium-dependent forms are reported to play a major role in PLD regulation [1].

In skeletal muscle cells, several agonists including vasopressin, bradykinin (BK), thrombin and calcitriol can activate PLD [4–6], indicating that the enzyme is a key component of the membrane signaling machinery in this cell type.

Sphingosine 1-phosphate (SPP) is a member of a new class of lipid messengers important for the regulation of growth-related processes and cellular effector functions which are dependent on cytoskeletal responses [7]. SPP specifically binds to prototypical members of the orphan endothelial differentiation gene family Edg that includes until now Edg-1, Edg-3 and AGR16/H218 proteins. All known members of the Edg receptor family have been demonstrated to be coupled to heterotrimeric G proteins and to be responsible for specific downstream effects, including calcium mobilization, activation or inhibition of adenylate cyclase and mitogen-activated protein kinase activation [7].

SPP has been shown to stimulate PLD activity in a number of different cell types [8–10]. However, the exact signaling pathway involved has not been clarified. Moreover, in a recent report, the effect of SPP on PLD activation was found to be independent from Edg-1 receptor and suggested to be mediated by SPP internalization [10].

Here, we present evidence that SPP rapidly and transiently activates PLD in C2C12 mouse skeletal muscle cells. Both PLD isoforms appeared to be expressed in C2C12 myoblasts. SPP failed to increase membrane association of PLD1 or PLD2, indicating that SPP-induced enzyme activation did not require translocation of PLD from cytosol to the membrane. The activation of PLD is triggered by a receptor-mediated pertussis toxin (PTx)-sensitive G protein pathway and involves, besides PKC $\alpha$ , also the calcium-independent isoform PKC $\delta$ .

### 2. Materials and methods

#### 2.1. Materials

[ $^3$ H]glycerol (30–60 Ci/mmol) was purchased from Du Pont NEN (Boston, MA, USA), standard lipids were from Avanti Polar Lipids (Alabaster, AL, USA), solvents and silica gel 60 plates for thin-layer chromatography (TLC) from Merck (Darmstadt, Germany), BK, RedTaq polymerase, TriReagent and other chemicals were from Sigma (St. Louis, MO, USA). Sphingosine 1-phosphate was from Calbiochem (San Diego, CA, USA), pertussis toxin was from List Biological Labs (Campbell, CA, USA). Mouse monoclonal antibodies against the specific isoforms of PKC were from Transduction Laboratories (Lexington, KY, USA). PLD1 and PLD2 anti-peptide antibodies were a generous gift of Dr S. Bourgoin (Ste-Foy, Que., Canada) and were generated as described ([11], Houle, M.G. and Bourgoin, S., personal communication). Superscript II Reverse transcriptase was from Life Technologies (Eggenstein, Germany). Secondary antibodies (goat anti-mouse or anti-rabbit immunoglobulin G1 conjugated) were from Santa Cruz (Santa Cruz, CA, USA).

\*Corresponding author. Fax: (39) (0554) 222725.  
E-mail: biocell@scibio.unifi.it

## 2.2. Muscle cell culture

Mouse skeletal C2C12 myoblasts [12] were obtained from ATCC (Manassas, VA, USA) and maintained as previously described [5].

## 2.3. Measurement of PLD activity and [ $^3\text{H}$ ]PtdOH levels

PLD activity was determined by measuring [ $^3\text{H}$ ]phosphatidylethanol ([ $^3\text{H}$ ]PtdEtOH) produced via PLD-catalyzed transphosphatidylation in serum-starved cells labelled for 24 h with 5  $\mu\text{Ci}/\text{ml}$  [ $^3\text{H}$ ]glycerol and stimulated in the presence of 2% ethanol. Cells were collected by scraping, lipids were extracted and [ $^3\text{H}$ ]PtdEtOH was measured after TLC separation essentially as described by Meacci et al. [13]. [ $^3\text{H}$ ]PtdOH levels were measured as described by Vasta et al. [5].

## 2.4. Expression of Edg receptors in C2C12 cells by reverse transcription (RT)-PCR

Total cellular RNA used to prepare cDNA was isolated using TriReagent. The RT reaction was performed at 42°C for 1 h in 20  $\mu\text{l}$  of 50 mM KCl, 2.5 mM Tris-HCl (pH 8.4), 2 mM dNTPs (0.5 mM each), 200 ng of random hexamers as primers, 200 ng oligo(dT), 10 mM DTT, 5  $\mu\text{g}$  of total cellular RNA using 200 U of Superscript II RT. To terminate the reaction, samples were incubated at 70°C for 15 min. The resulting cDNA was used directly for PCR. Mouse Edg-1-specific PCR primers (CCTATCATGGGCTGGA-CTGCATC and CCCAGAAGGACGATGGGGACAACCCA), mouse Edg-3-specific PCR primers (GCAACCACGCATGCGCAG-GGCCACCAGG and GGAAAAACAATAATTTTCAACCCG-C), AGR16/H218-specific PCR primers (GGCAGCGACAAGA-GTGCCGCATG and CTGGAGGGCAACACGGTGGTCTGA) were synthesized by Pharmacia Biotech (Uppsala, Sweden). PCR was carried out in 50  $\mu\text{l}$  of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 0.8 mM dNTPs (200  $\mu\text{M}$  of each), 0.5  $\mu\text{M}$  of each primer using 2  $\mu\text{l}$  of RedTaq DNA polymerase. cDNA (50 ng) was added as PCR template. 30 Cycles of amplification were carried out as follows: at 92°C for 40 s, at 57°C for 40 s and at 72°C for 90 s. PCR products (8  $\mu\text{l}$ ) were analyzed by electrophoresis in a 1.8% agarose gel.

## 2.5. Cellular fractionation

Serum-starved C2C12 cells were treated or not with 1  $\mu\text{M}$  SPP for 30 s. The medium was removed and the cells washed twice with ice-cold PBS, scraped and collected by centrifugation at 1000 $\times g$ . The cell pellet was resuspended in buffer A (20 mM HEPES, pH 7.4, 2 mM EGTA, 1 mM EDTA, 250 mM sucrose, 5 mM  $\text{NaN}_3$ ) containing protease inhibitors (1 mM AEBSF, 0.3  $\mu\text{M}$  aprotinin, 10  $\mu\text{g}/\text{ml}$  leupeptin and 10  $\mu\text{g}/\text{ml}$  pepstatin) and homogenized in a Dounce (60 strokes).

Lysates were centrifuged for 7 min at 500 $\times g$  to separate nuclei. To prepare cytosolic and total particulate fractions, the supernatant was centrifuged at 200 000 $\times g$  for 1 h. The pellet was resuspended in the same buffer, containing 1% Triton X-100, incubated in ice for 30 min and successively centrifuged at 200 000 $\times g$  for 50 min to prepare Triton X-100-soluble and -insoluble membrane fractions.

## 2.6. Western blot analysis

Proteins (30  $\mu\text{g}$ ) from cellular fractions of unstimulated and agonist-stimulated myoblasts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was performed as described [11].

## 2.7. Presentation of data and statistical analysis

The data were analyzed by Student's *t*-test and  $P < 0.05$  was considered significant. All data are presented as the mean  $\pm$  S.E.M. of at least three separated experiments performed in duplicate. In immunoblot experiments, a blot representative of at least three similar experiments is presented.

## 3. Results

### 3.1. SPP induces PLD activation in myoblasts

Incubation of intact [ $^3\text{H}$ ]glycerol-labelled C2C12 myoblasts with SPP led to a significant increase in PLD activity. PLD activation was very rapid reaching a maximum at 1 min and was still detectable after 5 min (Fig. 1A). In the presence of 2% ethanol, SPP failed to increase [ $^3\text{H}$ ]PtdOH significantly ( $2.0 \pm 0.15$  and  $5.5 \pm 0.7\%$  PtdOH/PtdCho in the absence of ethanol in unstimulated and SPP-treated cells, respectively ( $P < 0.05$ ,  $n = 3$ );  $2.05 \pm 0.16$  and  $2.42 \pm 0.25\%$  PtdOH/PtdCho in the presence of ethanol in unstimulated and SPP-treated cells, respectively ( $n = 3$ )), indicating that stimulation of PLD played a major role in the transient rise of PtdOH levels induced by SPP. PLD activation by SPP was dose-dependent with a half-maximal effect with approximately 50 nM SPP (Fig. 1B).

### 3.2. PLD activation by SPP is sensitive to PTx

SPP-induced PLD activation was totally prevented in cells previously treated with 200 ng/ml PTx for 16 h, indicating that the bioactive lipid acts through a Gi or Go protein-coupled receptor (Table 1). In parallel experiments, BK, a pro-inflammatory peptide able to stimulate PLD activity in these cells [5], was not affected by PTx, in agreement with a signaling pathway triggered through Gq-coupled receptors.

### 3.3. Expression of Edg receptors in C2C12 myoblasts

The expression of Edg-1, Edg-3 and AGR16/H218 was analyzed in C2C12 myoblasts by RT-PCR using total cellular RNA. Fig. 2 shows the agarose gel in which RT-PCR products were electrophoretically separated. cDNAs corresponding

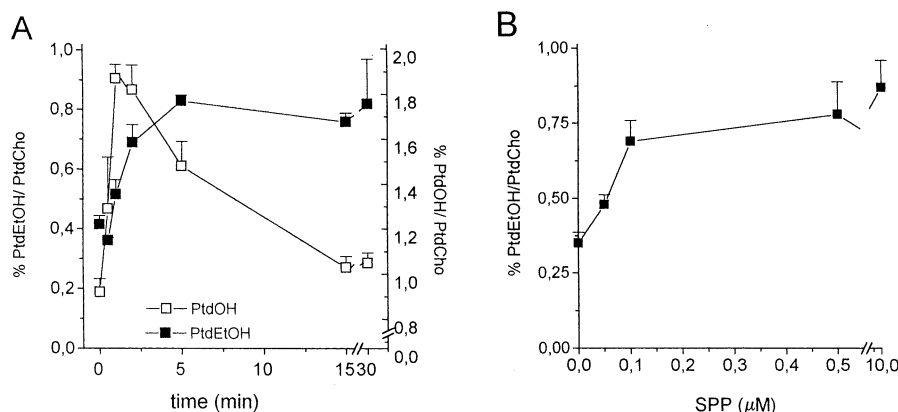


Fig. 1. Time-course and dose-dependence of the effect of SPP on PLD activation. [ $^3\text{H}$ ]glycerol-labelled C2C12 cells were stimulated with 1  $\mu\text{M}$  SPP in the presence or absence of 2% ethanol for the indicated time (A) or with different concentrations of SPP for 10 min (B). Lipids were then extracted and [ $^3\text{H}$ ]PtdEtOH, [ $^3\text{H}$ ]PtdOH and [ $^3\text{H}$ ]PtdCho were determined as described in Section 2.

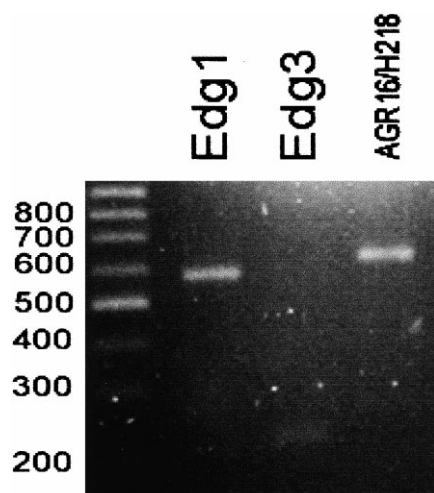


Fig. 2. Expression of Edg receptor isoforms in C2C12 myoblasts as assessed by RT-PCR. An ethidium bromide-stained 1.8% agarose gel is shown after separation of RT-PCR products. The experiment was repeated three times with similar results. Lane 1 shows DNA molecular markers (GeneRuler DNA Ladder Mix).

to the expected length of Edg-1 (563 bp), Edg-3 (225 bp) and AGR16/H218 (621 bp) were detected. No PCR product was visible in control experiments in the absence of template DNA (data not shown).

### 3.4. Expression of PLD isoforms and the effect of SPP treatment on the protein subcellular distribution

PLD1-specific antibodies recognized a protein band of approximately 120 kDa and two others of 85 and 50 kDa (Fig. 3). The two higher bands were not recognized by peptide-neutralized PLD1 antibodies (data not shown). The 50 kDa band might be a contaminant protein.

Immunoblot analysis of PLD2 showed a single band at approximately 90 kDa in cytosol and two bands around 100 and 90 kDa in a Triton X-100-insoluble membrane (Fig. 3). Peptide-neutralized antibodies did not detect any band (data not shown).

PLD1 and PLD2 resulted to be differently localized: both isoforms were present in soluble and particulate fractions, however, PLD1 was mainly associated with the Triton-soluble membrane fraction, while PLD2 was mostly detected in the Triton-insoluble membrane fraction.

Treatment with 1  $\mu$ M SPP for 30 s did not alter the intracellular distribution of both PLD isoforms (Fig. 3).

Table 1  
Effect of Ptx treatment on SPP-induced PLD activation

| PTx<br>(200 ng/ml) | SPP<br>(1 $\mu$ M) | BK<br>(1 $\mu$ M) | PLD activity<br>(%PtdEtOH/PtdCho) |
|--------------------|--------------------|-------------------|-----------------------------------|
| –                  | –                  | –                 | $0.42 \pm 0.05$                   |
| –                  | +                  | –                 | $0.80 \pm 0.06^*$                 |
| –                  | –                  | +                 | $0.97 \pm 0.10^*$                 |
| +                  | –                  | –                 | $0.52 \pm 0.06$                   |
| +                  | +                  | –                 | $0.53 \pm 0.03$                   |
| +                  | –                  | +                 | $1.18 \pm 0.11^*$                 |

Confluent myoblasts were labelled with [ $^3$ H]glycerol for 16 h in the presence or absence of 200 ng/ml of PTx before stimulation with 1  $\mu$ M SPP or 1  $\mu$ M BK for 10 min in the presence of 2% ethanol. [ $^3$ H]PtdEtOH formation was quantified as described in Section 2.

\*Statistical significant ( $P < 0.05$ ).

### 3.5. SPP-induced PLD activation is dependent on PKC activation

Immunoblot analysis showed that the following PKCs could be dominantly detected in unstimulated C2C12 myoblasts: PKC $\alpha$ , PKC $\delta$ , PKC $\lambda$  and PKC $\mu$ . PKC $\beta$ ,  $\epsilon$  and  $\zeta$  were not detected (data not shown). Since it is known that the PKC content in a particulate fraction can reflect PKC activity, the effect of SPP on the pool of PKC associated with the membrane was examined to investigate its role in PLD activation. Treatment with 1  $\mu$ M SPP for 30 s induced a rapid translocation of PKC $\alpha$  to the Triton X-100-soluble membrane fraction (Fig. 4). The activation was indeed transient, at 10 min of stimulation, membrane-associated PKC $\alpha$  appeared significantly decreased (data not shown). Moreover, SPP could also induce membrane translocation of the calcium-independent forms, PKC $\delta$  and PKC $\lambda$ , but did not affect the pool of PKC $\mu$  (Fig. 4). To examine the role of PKC catalytic activity in the SPP action on PLD, GF109203X, which has been reported to be a stronger inhibitor of classical PKC than novel PKC [14], and rottlerin, a selective inhibitor of PKC $\delta$  [15], were used. Treatment of C2C12 myoblasts with GF109203X diminished SPP-induced PLD activation approximately by 50% at 5  $\mu$ M and blocked the stimulation at 10  $\mu$ M (Fig. 5A). Similar results were achieved with Ro 31-8220 and

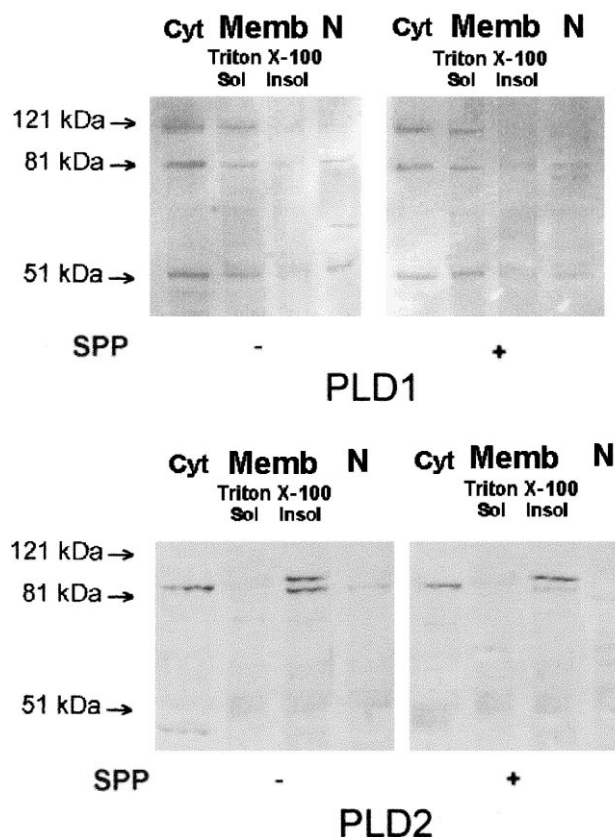


Fig. 3. Intracellular distribution of PLD1 and PLD2. Proteins (30  $\mu$ g) from the cytosol (Cyt), 1% Triton X-100-soluble (Memb sol), 1% Triton X-100-insoluble membrane fraction (Memb insol) or nuclei (N) prepared from myoblasts treated or not with 1  $\mu$ M SPP for 30 s were separated by SDS-PAGE (8%) and Western analysis performed as described in Section 2. PLD1 and PLD2 were detected using polyclonal anti-rabbit antibodies kindly provided by Dr S. Bourgoign.

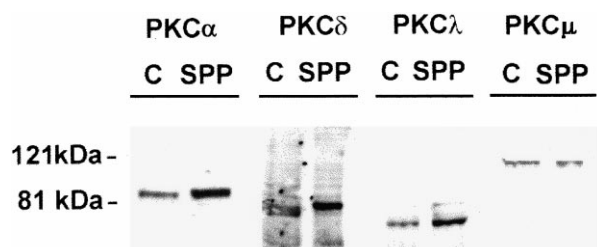


Fig. 4. Effect of SPP on PKC isoform membrane association. Serum-starved myoblasts were incubated in the absence or in the presence of 1  $\mu$ M SPP for 30 s. Proteins (30  $\mu$ g) from the 1% Triton X-100-soluble membrane fraction of control (C) or SPP-stimulated cells (SPP) were separated by SDS-PAGE (8%) and Western analysis performed as described in Section 2.

down-regulation of PKC by prolonged treatment (20 h) with 800 nM PMA (data not shown). Interestingly, the stimulation of PLD activity by SPP was also inhibited in a dose-dependent manner by rottlerin (Fig. 5B), indicating a crucial role for PKC $\delta$  in the SPP-induced PLD activation.

#### 4. Discussion

We have demonstrated here that SPP stimulates PLD activity, which accounts for the SPP-induced increase of PtdOH levels, in a receptor-mediated manner. The finding that SPP-induced activation of PLD was abolished by treatment with PTx clearly indicates that in C2C12 muscle cells, SPP acts as ligand for one or more Gi-coupled receptor(s) and provides evidence for a fully Gi-dependent signaling pathway directly involved in PLD regulation. In agreement, the concentrations of SPP capable to increase PLD activity were in the same range as the  $K_d$  values reported for the SPP receptors [7]. Analysis by RT-PCR showed that all known SPP-specific Edg receptors were expressed at the mRNA level, although at a different extent. This is the first evidence for mRNA expression of all described Edg receptors in skeletal muscle cells. Because Edg-1 and AGR16/H218 can be coupled to Gi proteins [16,17] and were both expressed in myoblasts, it is not possible to attribute the observed SPP-induced signaling pathway to a distinct receptor.

Determination of PLD1 and PLD2 expression in various tissues indicates that skeletal muscle has relatively low levels of mRNA for both isoforms [18]. Here, we reported for the first time that PLD1 and PLD2 proteins are both expressed in C2C12 myoblasts and are differently distributed in Triton X-100-soluble and -insoluble membrane fractions, suggesting that the two isoforms are associated to different membrane microdomains. Translocation to the cytoskeleton of PLD1 has been reported after treatment with opsonized zymosan in U937 cells [19] and also PLD2 redistribution upon serum stimulation has been observed [2]. However, in our cell system, SPP failed to increase membrane association either of PLD1 or PLD2, excluding that enzyme translocation from cytosol to the membrane is a step in agonist-induced PLD activation. This is in agreement with a previous observation in PC-3, human prostate cancer cells [20].

Studies in L6 myoblasts demonstrated the presence of several PKC isoforms [21], but, so far, a full characterization of PKC isoforms expressed in mouse skeletal muscle C2C12 cells has not been reported. Here, evidence is provided that PKC $\alpha$ ,

PKC $\delta$ , PKC $\lambda$  and PKC $\mu$  are dominantly detected in C2C12 myoblasts and that SPP can translocate PKC $\alpha$ , PKC $\delta$  and PKC $\lambda$  to the membrane fraction.

Regulation of PLD activities by PKC is complex and phosphorylation-dependent and -independent mechanisms for PKC-dependent PLD activation have been reported [1]. Remarkably, in the present study, SPP-induced PLD activation was totally prevented by inhibition of PKC. Since the PKC inhibitors used, GF109203X and rottlerin, compete for binding of ATP to PKCs, our results imply that phosphorylation is required for PLD stimulation, as previously proposed [22,23]. However, the present literature can not lead to exclude that phosphorylation-dependent and -independent mechanisms concur to regulate PLD activity.

An interesting finding is that PKC $\delta$  catalytic activity is involved in SPP stimulation of PLD as demonstrated by the efficacy of rottlerin, reported to be specific for PKC $\delta$  [15]. So far, a role for PKC $\delta$  as PLD activator has been reported in *in vitro* experiments [24]. The involvement of PKC $\alpha$  in the regulation of PLD activity has been widely investigated *in vivo* and *in vitro* [1]. Here, evidence is provided for a role of PKC $\alpha$  in the SPP signaling pathway in C2C12 cells. PKC $\alpha$  is likely involved in the agonist-induced stimulation of PLD in view of the observed reduction of enzyme activa-

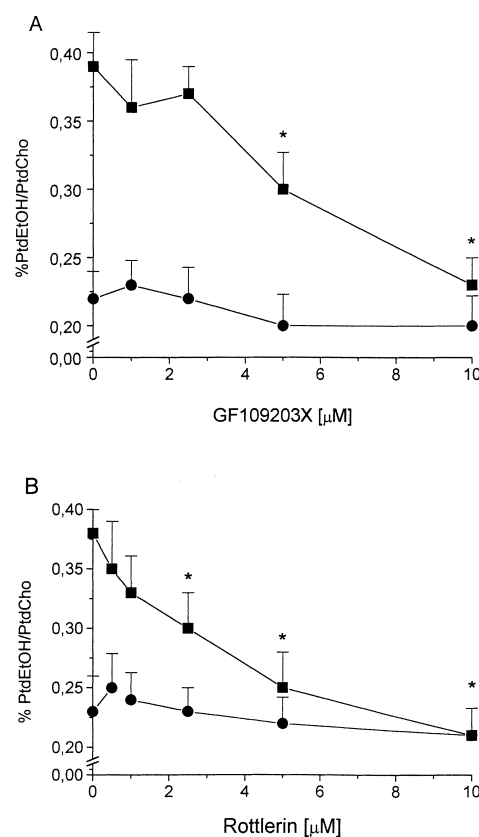


Fig. 5. Effect of PKC inhibitors on SPP-induced PLD activation. Confluent myoblasts were labelled with [ $^3$ H]glycerol and incubated without or with the indicated concentrations of GF109203X (A) or rottlerin (B) for 30 min prior to incubation with vehicle (0.05% DMSO) or 1  $\mu$ M SPP for 10 min in the presence of 2% ethanol (● control cells, ■ SPP-treated cells). [ $^3$ H]PtdEtOH formation was quantified as described in Section 2. \*Statistical significance of PKC inhibition of SPP-induced PLD activation ( $P < 0.05$ ).

tion at concentrations of GF109203X which are not effective on calcium-independent PKC [14].

Although SPP induced also PKC $\lambda$  translocation, it appeared not to be required for PLD activation, as suggested by the observed GF109203X-independent membrane association of this isoform at a high concentration of inhibitor (data not shown).

The here reported results are consistent with a model in which SPP-dependent PLD activation is fully mediated by PTx-sensitive G protein-coupled receptor(s) and mainly requires PKC $\alpha$  and PKC $\delta$  activation. Further studies are required to clarify in more details the SPP receptor-mediated events upstream of PKC activation.

**Acknowledgements:** We thank Dr S. Bourgoin (Centre de Recherche du CHUL Rhumatologie et Immunologie, Ste-Foy, Que., Canada) for kindly providing PLD1 and PLD2 antisera and Dr J. Moss (Pulmonary-Critical Care Medicine Branch, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA) for critical reading of the manuscript. This work was supported by the Italian Telethon Foundation (Grant n.1086 to P.B.).

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