Sphingosine kinase activity is required for sphingosine-mediated phospholipase D activation in C2C12 myoblasts

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Sphingosine (Sph) has been implicated as a modulator of membrane signal transduction systems and as a regulatory element of cardiac and skeletal muscle physiology, but little information is presently available on its precise mechanism of action. Recent studies have shown that sphingosine 1-phosphate (S1P), generated by the action of sphingosine kinase (SphK) on Sph, also possesses biological activity, acting as an intracellular messenger, as well as an extracellular ligand for specific membrane receptors. At present, however, it is not clear whether the biological effects elicited by Sph are attributable to its conversion into S1P. In the present study, we show that Sph significantly stimulated phospholipase D (PLD) activity in mouse C2C12 myoblasts via a previously unrecognized mechanism that requires the conversion of Sph into S1P and its subsequent action as extracellular ligand. Indeed, Sph-induced activation of PLD was inhibited by *N,N*-dimethyl-D-erythro-sphingosine (DMS), at concentrations cap-

able of specifically inhibiting SphK. Moreover, the crucial role of SphK-derived S1P in the activation of PLD by Sph was confirmed by the observed potentiated effect of Sph in myoblasts where SphK1 was overexpressed, and the attenuated response in cells transfected with the dominant negative form of SphK1. Notably, the measurement of S1P formation *in vivo* by employing labelled ATP revealed that cell-associated SphK activity in the extracellular compartment largely contributed to the transformation of Sph into S1P, with the amount of SphK released into the medium being negligible. It will be important to establish whether the mechanism of action identified in the present study is implicated in the multiple biological effects elicited by Sph in muscle cells.

Key words: myoblast C2C12 cell, phospholipase D, sphingosine, sphingosine kinase activity, sphingosine 1-phosphate.

INTRODUCTION

There is growing evidence that complex sphingolipids, as well as simple sphingoid molecules such as sphingosine (Sph), sphingosine 1-phosphate (S1P), ceramide and ceramide 1-phosphate, participate in the intracellular transfer of molecular signals involved in important cellular events, such as proliferation, differentiation, apoptosis and stress condition responses [1,2].

Although one of the primary effects of Sph on cell functions, such as the inhibition of cell growth and the induction of apoptosis [3], has long been considered to be due to the inhibition of protein kinase C (PKC) activity [4], many of the effects of Sph appear to be independent from PKC [5,6], involving other molecular mechanisms, such as stimulation of phosphatidylinositol turnover and calcium increase [7,8]. Endogenous Sph appears to be an important messenger in cardiac and skeletal muscle. Indeed, Sph is capable of modulating the function of key calcium channels, including the ryanodine receptor and voltage-dependent channels [9]. Moreover, it has been reported that a high turnover of neutral sphingomyelinase, localized at the junctional transverse tubule membrane, is responsible for the high levels of Sph observed in the T-tubules of skeletal and cardiac muscle, implying that Sph is a crucial regulatory element in malignant hyperthermia, as well as in the development of muscle fatigue [10]. Furthermore, Sph plays a modulatory role in the signal transduction triggered by β -adrenergic receptors, contributing to the reduction of heart rate in rats *in vivo*, as well as to the diminished contraction rate in spontaneously contracting cardiac cells [9,10].

In the last few years, the crucial biological role of S1P, a metabolite generated by the sphingosine kinase (SphK)-catalysed phosphorylation of Sph, has also been recognized. Notably, experimental evidence is in favour of a dual function of S1P, which is capable of acting as intracellular messenger, as well as an extracellular ligand [1,2]. Indeed, S1P plays a role as second messenger, being able to mobilize calcium from internal sources [11] and induce cell growth and survival [12]. Although the intracellular targets of S1P remain elusive, several studies reported an intracellular action of S1P produced by activated SphK in mammalian cells [13], as well as in yeast [14]. Moreover, it is well accepted that in higher eukaryotes S1P preferentially acts extracellularly by interacting with a family of highly specific G-protein-coupled receptors named EDG (endothelial differentiation gene)/S1P receptors [15]. The important physiological functions of S1P, ranging from the maturation of the vascular system to cell survival and proliferation, which are mediated by its specific receptors are well documented [1,2]. Moreover, other studies indicate that extracellular S1P can induce muscle hypertrophy via the EDG1/S1P1 receptor [16] and mediate calcium overload in rat myocytes [17]. Furthermore, in a different study, the negative inotropic effect of S1P in human atrial cardiomyocytes due to the activation of inwardly rectifying K^+ channels was described [18].

SphKs, responsible for the generation of S1P from Sph, are a newly discovered class of lipid kinases, expressed in humans, mice, yeasts and plants, with homologues in worms and flies [2]. In mammals two isoforms, SphK1 and SphK2, have been described and characterized. Although the two isoforms have different

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DMS, *N,N*-dimethyl-D-erythro-sphingosine; EDG, endothelial differentiation gene; ERK1/2, extracellular signal regulated kinase 1/2; FCS, fetal calf serum; HA, haemoagglutinin; PKC, protein kinase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEtOH, phosphatidylethanol; PTx, pertussis toxin; ODN, oligodeoxynucleotides; S1P, sphingosine 1-phosphate; Sph, sphingosine; SphK, sphingosine kinase; DNSphK1, dominant negative SphK1; TTBS, Tris-buffered saline containing 0.1% Tween; wtSphK1, wild-type SphK1.

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kinetic properties, tissue distribution and temporal expression pattern during development, they share a high degree of homology in their catalytic domains [19]. However, despite the central role of SphK in mediating the effects attributed to S1P in the cell, the molecular mechanisms of activation and regulation of these enzymes have not been fully elucidated. In this regard, a PKC-dependent phosphorylation and activation of SphK1 by phorbol esters has been described [20]. In contrast, according to a recent study [21], SphK1 is activated by ERK1/2 (extracellular-signal-regulated kinase 1/2)-mediated phosphorylation *in vivo*. SphK activity has been shown to be localized predominantly in the cytosol, although a small fraction is associated with membrane compartments. Indeed, translocation to membrane is a common feature of SphK1 activation by phorbol esters [20], and it has been recently shown to be strictly dependent on its phosphorylation by ERK1/2 [21]. Interestingly, initial studies indicated that SphK1 is primarily cell associated [22], however, more recently, it has been reported that SphK1 is exported by endothelial cells [23], suggesting that S1P may be generated in the intracellular, as well as in the extracellular, environment.

In our previous studies [24–27], we demonstrated that in murine C2C12 myoblasts phospholipase D (PLD) is regulated by several agonists, including bradykinin, thrombin and S1P, and the signalling pathways involved were characterized. In the present study, we provide evidence that Sph is capable of activating PLD in C2C12 myoblasts through its phosphorylation to S1P, and that an intrinsic SphK activity, detected in the extracellular compartment, contributes to the observed biological activity.

EXPERIMENTAL

Materials

Biochemicals, cell culture reagents, DMEM (Dulbecco's modified Eagle's medium), FCS (fetal calf serum), horse serum, mouse monoclonal anti-HA (haemoagglutinin) antibodies, and rabbit polyclonal anti-FLAG antibodies were purchased from Sigma (Milan, Italy). Murine C2C12 cells were obtained from the A.T.C.C. (Manassas, VA, U.S.A.). Pertussis toxin (PTx) was from List Biological Laboratories (Campbell, CA, U.S.A.). S1P was obtained from Calbiochem (San Diego, CA, U.S.A.). *N,N*-Dimethyl-D-erythro-sphingosine (DMS) and antifade mounting medium (Mowiol) were from Alexis (Lausen, Switzerland). LipofectAMINE²⁰⁰⁰ and Lipofectin were from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). pcDNA3-hSphK1-FLAG and pcDNA3-hSphK1^{G82P}-FLAG plasmids were obtained as described previously [28], and were kindly given by Dr S. M. Pitson (Division of Human Immunology, Institute of Medical and Veterinary Science, Adelaide, Australia). DNA construct coding for EDG5/S1P2 was prepared as previously described [29] and was gift from Professor Y. Igarashi (Department of Biomembrane and Biofunctional Chemistry, Hokkaido University, Sapporo, Japan). Phosphothioate oligodeoxyribonucleotides (ODN), corresponding to the translation-initiation region of EDG5/S1P2 (5'-GTATAAGCCGCCCATGGTGGG-3'), and scrambled ODN were synthesized by MWG Biotech AG (Ebersberg, Germany). Rabbit polyclonal anti-Rab5 and anticalnexin antibodies were purchased from Stressgene Biotech (Victoria, BC, Canada). Rabbit polyclonal anti-PKC α antibodies, mouse monoclonal anti-caveolin-1 antibodies, goat polyclonal anti-actin antibodies, goat anti-EDG5/S1P2 antibodies, and anti-rabbit and anti-goat IgG1 conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rhodamine B- and Alexa 488-conjugated goat anti-mouse and anti-rabbit secondary antibodies were obtained from

Molecular Probes (Eugene, OR, U.S.A.). Enhanced chemiluminescence reagents were obtained from Amersham Bioscience (Uppsala, Sweden). [³H]Glycerol (30–60 Ci/mmol), [³²P]ATP (3000 Ci/mmol) and [³H]sphingosine (23 Ci/mmol) were purchased from NEN Life Science (Boston, MA, U.S.A.). Dye reagent for protein assays was from Bio-Rad (Hercules, CA, U.S.A.). The bioluminescence assay kit for ATP measurement was from Roche Applied Science (Monza, Italy).

Muscle cell culture

C2C12 cells were maintained in DMEM with 10 % FCS. Myoblasts were seeded on to 35- or 100-mm diameter dishes. Before being utilized for the experiments, cells were starved by replacing the medium with serum-free DMEM containing 0.1 % BSA for 24 h. Myogenic differentiation was obtained by transferring confluent myoblasts to DMEM supplemented with 2 % horse serum. Complete cell differentiation was observed after 5–6 days of incubation [29].

Cell treatment with sphingolipids

Serum-starved cells were added without or with various amounts of Sph (prepared from 1 mM stock solution in chloroform/methanol, which was dried and resuspended by brief sonication in DMEM containing 0.1 mg/ml BSA) or S1P (prepared by dilution of 2 mM stock solution in DMSO). In preliminary experiments it was verified that the employed amounts of Sph did not permeabilize cell membranes by measuring the eventual release into the medium of cellular ATP using a bioluminescence assay kit.

Measurement of PLD activity

PLD activity was determined by measuring [³H]phosphatidylethanol (PtdEtOH) produced via PLD-catalysed *trans*-phosphatidylolation in serum-starved cells labelled for 16 h with 5 μ Ci/ml [³H]glycerol. [³H]Glycerol-labelled cells were incubated for 2 min before sphingolipid addition in the presence of 2 % ethanol. The incubation was arrested after 5 min at 37 °C by removing the medium, washing the monolayers twice with ice-cold PBS and adding 1 ml of ice-cold methanol. Cells were collected by scraping, and dishes were washed with an additional 1 ml of ice-cold methanol. Lipids were extracted and separated by TLC on silica gel G60 plates, essentially as described previously [30]. Unlabelled PtdEtOH was added as a carrier during lipid extraction. Positions of lipids were determined by comparison with authentic standard after staining with iodine vapour. The lipid spots corresponding to [³H]PtdEtOH and [³H]phosphatidylcholine (PtdCho) were marked and scraped into scintillation vials, and 0.5 ml of methanol and liquid scintillation fluid was added before radioassay. Radioactivity associated with [³H]PtdEtOH and [³H]PtdCho was determined by liquid scintillation counting and quantified as described previously [30].

Cell fractionation and Western analysis

Serum-starved C2C12 cells were subjected to cell fractionation as described previously [26]. Briefly, the cells were scraped in 20 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM EDTA and 250 mM sucrose containing protease inhibitors [1 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride, 0.3 μ M aprotinin, 10 μ g/ml leupeptin and 10 μ g/ml pepstatin A], homogenized in a Dounce homogenizer (60 strokes) and then centrifuged for 7 min at 500 *g*. To obtain cytosolic and total particulate fractions, the supernatant was centrifuged at 200 000 *g* for 1 h. To prepare Golgi-enriched and endosome/plasma membranes, the pellet was dispersed in

the same buffer containing 1.4 M sucrose and transferred to the bottom of a centrifuge tube containing layers of 0.25, 0.85, 1.15 and 1.4 M sucrose. After centrifugation (19 h, 200 000 *g*), the subcellular fractions were collected and analysed for protein content with Coomassie Blue procedure using a commercially available kit. Fractions corresponding to Golgi-enriched membranes or endosome/plasma membrane were collected and used for Western analysis.

Proteins (10–30 μ g) from cell lysates or membrane fractions were separated by SDS/PAGE. Proteins were electrotransferred on to nitrocellulose membranes, which were incubated overnight in Tris-buffered saline containing 0.1 % Tween-20 (TTBS) and 1 % BSA. Membranes were subsequently incubated for 1 h with antibodies against PKC α , HA, FLAG peptide, actin, calnexin or Rab5. Hybridization with primary antibodies was followed by washing with TTBS and incubation with peroxidase-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence. Quantitative analysis of the bands corresponding to specific proteins was performed using Imaging and Analysis Software by Bio-Rad.

Measurement of labelled S1P formation

C2C12 cells at 80 % confluence were washed twice in serum-free DMEM and starved for 24 h. Before the beginning of the experiment (3 h), the medium was replaced with fresh serum-free DMEM, and 1 μ M [3 H]Sph (0.1 μ Ci) was added to the cells for 5 min. After the incubation, the cell medium was transferred to a test tube for lipid extraction, while the cells were washed twice with ice-cold PBS and scraped in methanol. In addition, immediately after the beginning of the incubation, 0.5 ml of conditioned medium was transferred to a test tube and the incubation continued for 5 min. Lipids were extracted from cells, cell medium and conditioned medium, essentially as described by Merrill et al. [31]. The organic phase was dried under a nitrogen stream and resuspended in chloroform/methanol (2:1, v/v). Lipids were resolved by TLC on silica gel G60 using 1-butanol/acetic acid/water (3:1:1, by vol.). Spots corresponding to S1P or Sph, identified by comparison with internal standards, were scraped from the plates and quantified in a scintillation counter.

The cell surface SphK activity assay was performed essentially as described by Ancellin et al. [23]. Briefly, C2C12 cells at 60 % confluence were processed as described above, except that the assay was initiated by adding to the medium 100 μ M [γ - 32 P]ATP (15 μ Ci) and 1 μ M cold Sph. After 5 min of incubation the cell medium was transferred to a test tube for lipid extraction, while the cells were washed twice with ice-cold PBS and scraped in methanol. In addition, immediately after the beginning of the incubation, 0.5 ml of conditioned medium was transferred to a test tube and the incubation continued for 5 min. Lipids were extracted from cells, cell medium and conditioned medium and analysed as described above.

Cell transfection

For transient expression of wild-type SphK1 (wtSphK1) or dominant negative SphK1^{G82D} (DNSphK1; where Gly⁸² $\rightarrow$ Asp), C2C12 myoblasts were plated on to 35- or 100-mm dishes and cultured for 24 h prior to transfection. Cells were transiently transfected using LipofectAMINE²⁰⁰⁰ reagent (1 mg/ml). Briefly, the cationic lipid was incubated according to the manufacturer's instructions with pcDNA3 plasmid coding for human wtSphK1 or DNSphK1 at 20 °C for 20 min. Successively, the lipid–DNA complexes were added with gentle agitation to C2C12 myoblasts and the cells were incubated in DMEM containing 10 % FCS.

Figure 1 Dose-dependent effect of Sph on PLD activation in C2C12 myoblasts

Serum-starved [3 H]glycerol-labelled C2C12 myoblasts were incubated with increasing doses of Sph in the presence of 2 % ethanol for 5 min. Successively, the cells were scraped in methanol and lipids were extracted, separated by TLC and the formation of [3 H]PtdEtOH was measured, as described in the Experimental section. PLD activity is reported as percentage of [3 H]PtdEtOH formed over [3 H]PtdCho. The results are expressed as the means $\pm$ S.E.M. for three separate experiments performed in triplicate; **P* < 0.05.

Transfection efficiency was approx. 50 %. Thirty-six hours after the beginning of transfection the cells were collected and processed for further analysis. Transient expression of EDG5/S1P2 and ODN administration were performed as described previously [29].

Confocal microscopy

C2C12 cells were grown on coverslips, fixed in 0.5 % buffered paraformaldehyde for 10 min, then washed with PBS and placed in blocking buffer (PBS containing 10 % goat serum and 0.1 % BSA) for 20 min. To detect the over-expressed SphK1 or endogenous caveolin-1, the cells were stained using rabbit polyclonal anti-FLAG or mouse monoclonal anti-caveolin-1 respectively for 1 h, followed by Alexa 488- or Rhodamine-conjugated goat anti-rabbit or anti-mouse secondary antibody for 1 h at 20 °C. Negative controls were obtained by substituting primary antibodies with blocking solution followed by secondary antibodies treatment. Coverslips containing the immunolabelled cells were then mounted with an antifade mounting medium and observed under a Bio-Rad MCR 1024 ES confocal laser scanning microscope (Bio-Rad, Hercules, CA, U.S.A.) equipped with a Kr/Ar laser source (15 mW) for fluorescence measurements. Series of optical sections (512 pixels $\times$ 512 pixels) were then taken through the depth of the cells with a thickness of 1 μ m at intervals of 0.8 μ m. Twelve optical sections for each sample examined were projected as a single composite image by superimposition.

Statistical analysis

The results of multiple observations are presented as the means $\pm$ S.E.M. for at least three separate experiments, unless otherwise stated. Student's *t* test was applied for the analysis of the results; differences at *P* < 0.05 were considered statistically significant.

RESULTS

Effect of Sph on PLD activity in C2C12 cells

To characterize the effect of Sph on PLD activity, [3 H]glycerol-labelled C2C12 myoblasts were incubated with increasing concentrations of Sph (0.01–1 μ M). As shown in Figure 1, Sph

Table 1 Effect of Sph and S1P on PLD activity in C2C12 myoblasts and myotubes

Confluent C2C12 cells were serum-starved and labelled with [3 H]glycerol for 16 h. Sph or S1P was added to the medium in the presence of 2% ethanol for 5 min. Successively, the lipids were extracted, separated by TLC, and the [3 H]PtdEtOH formed was quantified as described in the Experimental section. The results are expressed as the means $\pm$ S.E.M. for four separate experiments performed in triplicate; * $P < 0.05$.

	PLD activity (% PtdEtOH/PtdCho)	
	Myoblasts	Myotubes
Control	0.46 $\pm$ 0.02	0.41 $\pm$ 0.01
Sph (1 μ M)	0.66 $\pm$ 0.05*	0.49 $\pm$ 0.05
S1P (1 μ M)	0.85 $\pm$ 0.09*	0.44 $\pm$ 0.02

induced a dose-dependent increase of PLD activity, which was measured as [3 H]PtdEtOH formed in the *trans*-phosphatidyl reaction catalysed by the enzyme in the presence of ethanol for 5 min. Sph significantly activated PLD at concentrations above 50 nM.

Previously, we found that S1P was capable of activating PLD in C2C12 myoblasts, whereas it failed to increase the enzymic activity in C2C12 myotubes [29]. Therefore, to examine whether Sph shared the same signalling pathway to PLD activation with S1P, we compared the ability of 1 μ M Sph and 1 μ M S1P to stimulate PLD activity in undifferentiated and differentiated cells. Table 1 shows that in myoblasts the increase of PLD activity promoted by Sph was approximately half of that induced by S1P. Of note, Sph, as well as S1P, was unable to increase PLD activity in myotubes. This finding demonstrates that Sph, similar to S1P, may act through a signalling pathway that is impaired during myogenic differentiation.

Next, the molecular mechanism involved in Sph action on PLD activity was further investigated, taking into account that in C2C12 myoblasts S1P activates PLD in a PTx-sensitive manner [25], mainly through EDG5/S1P2 receptor [29]. Results presented in Figure 2(A) clearly show that inhibition of G_i protein by treatment with 200 ng/ml PTx for 16 h prior to the addition of 1 μ M Sph fully prevented the activation of PLD by Sph. Moreover, ectopic expression of EDG5/S1P2 that resulted in an elevated content of the receptor protein (Figure 2B, inset), was responsible for an appreciably higher Sph-induced PLD activity in comparison with vector control cells (Figure 2B). Furthermore, the loading of myoblasts with antisense ODN to EDG5/S1P2, which significantly down-regulated EDG5/S1P2 (Figure 2C, inset), provoked a marked reduction of Sph-mediated PLD activity (Figure 2C). No significant change was observed in cells treated with scrambled ODN. These results favour the hypothesis that the conversion of Sph into S1P followed by ligation to a specific receptor is required for PLD activation by Sph.

Effect of DMS on Sph- or S1P-induced PLD activation

To prove the hypothesis that SphK-catalysed formation of S1P has a major role in Sph-induced PLD activity, C2C12 cells were treated with the N-methyl derivative of Sph, DMS, reported to inhibit SphK activity *in vivo* and *in vitro* [32]. However, since DMS has been shown to exert unspecific effects, i.e. inhibition of PKC [33,34], to determine the concentration of DMS capable to inhibit specifically SphK, the effect of different amounts of DMS on PKC α translocation induced by S1P was evaluated in preliminary experiments, given that in this cell system membrane association of PKC α reflects the activation state of the enzyme

Figure 2 Effect of PTx (A), over-expression (B) or down-regulation (C) of EDG5/S1P2 receptor on PLD activation induced by Sph in C2C12 myoblasts

(A) C2C12 myoblasts were serum-starved, labelled with 5 μ Ci/ml [3 H]glycerol and incubated with 200 ng/ml PTx for 16 h. Sph (1 μ M) was added to the medium in the presence of 2% ethanol for 5 min. [3 H]PtdEtOH formation was quantified as described in the Experimental section. [3 H]PtdEtOH was normalized to [3 H]PtdCho. The results are expressed as the means $\pm$ S.E.M. for three independent experiments performed in duplicate. The effect of PTx was statistically significant; * $P < 0.05$. (B) C2C12 myoblasts were transiently transfected with either empty pcDNA3 vector (vector) or pcDNA3 containing EDG5/S1P2 receptor cDNA (HA-EDG5/S1P2). After transfection (24 h) myoblasts were incubated in DMEM containing 0.1 mg/ml BSA and 5 μ Ci/ml [3 H]glycerol for further 16 h. [3 H]Glycerol-labelled cells were incubated without and with Sph (1 μ M) in the presence of 2% ethanol for 5 min, and processed for the quantification of [3 H]PtdEtOH. PLD activity is reported as the percentage of [3 H]PtdEtOH formed over [3 H]PtdCho. The results are expressed as the means $\pm$ S.E.M. for three separate experiments performed in triplicate; * $P < 0.05$. Inset: Western analysis of EDG5/S1P2 receptor expression in HA-EDG5/S1P2-transfected myoblasts. Samples (25 μ g) of total membrane fraction, obtained from vector alone- or HA-EDG5/S1P2-overexpressing C2C12 cells, were separated by SDS/PAGE, transferred on to nitrocellulose and the recombinant protein expressed as chimera with HA, detected using specific monoclonal anti-HA antibodies. The blot shown is representative of three. (C) C2C12 myoblasts were transiently transfected with scrambled ODN or antisense ODN against EDG5/S1P2. Cells were serum starved and labelled with [3 H]glycerol for 16 h. [3 H]Glycerol-labelled myoblasts were incubated without or with Sph (1 μ M) in the presence of 2% ethanol for 5 min, and [3 H]PtdEtOH formation was quantified as described in the

Figure 3 Effect of DMS on S1P-induced association to membrane of PKC α (A) and on Sph- and S1P-induced PLD activation in C2C12 myoblasts (B)

(A) Serum-starved C2C12 myoblasts were incubated for 30 min with the indicated amount of DMS and then stimulated (+) or not (–) with 1 μ M S1P for 30 s. Western analysis was performed as described in the Experimental section. Proteins from the membrane fraction (20 μ g) were separated by SDS/PAGE and electrotransferred on to nitrocellulose. Specific bands were detected using anti-PKC α antibodies. Actin, chosen as protein for loading control, was detected using specific goat anti-actin antibodies. Band intensity is reported as percentage relative to control of three separate experiments with similar results (no addition, 100%; S.E.M. was less than 15%). A representative blot is shown. (B) Serum-starved C2C12 myoblasts were labelled with [3 H]glycerol for 16 h and incubated with increasing amounts of DMS prior the addition of 1 μ M Sph or 1 μ M S1P in the presence of 2% ethanol for 5 min. [3 H]PtdEtOH formation was quantified as described in the Experimental section. PLD activity is reported as the percentage of [3 H]PtdEtOH formed over [3 H]PtdCho. The results are expressed as the means $\pm$ S.E.M. for three separate experiments performed in duplicate. Statistical significance by Student's *t* test of the DMS effect, **P* < 0.05.

[26]. As shown in Figure 3(A), 5 μ M, but not 0.5 μ M, DMS significantly reduced the positive effect of S1P on PKC α association to membrane, indicating that at this relatively high concentration DMS acts as a PKC inhibitor and is not suitable to selectively block SphK. Therefore, in the subsequent experiments concentrations of DMS below 5 μ M were employed to investigate the role of SphK in the activation of PLD elicited by Sph. As shown in Figure 3(B), a significant reduction of Sph-induced PLD activation was observed in the presence of 0.5 and 1 μ M DMS, whereas the same amounts of inhibitor did not affect the enzyme activation by S1P. Instead, higher concentrations of DMS markedly reduced PLD activation induced by S1P. In

view of the important role of PKC in the activation of PLD by S1P in myoblasts [25], this finding further proves that DMS at concentration above 2.5 μ M reduces PKC functionality. Overall, these results strongly support the hypothesis that Sph action on PLD activity requires SphK-mediated generation of S1P.

Effect of over-expression of SphK1 on Sph-induced PLD activation

To further demonstrate the involvement of SphK activity in Sph-induced activation of PLD, FLAG-tagged wtSphK1 or DNSphK1 were over-expressed in C2C12 myoblasts. As shown in Figure 4(A), both proteins were detected, in the cytosolic fraction, in Golgi-enriched and endosome/plasma membrane fraction of transfected cells. Moreover, the over-expressed SphK1 was localized at plasma membrane, as well as at internal membranes, and seen by confocal immunofluorescence microscopy (Figure 4B, panel E). Furthermore, as shown in the merged field (Figure 4B, panel F), SphK1 partially colocalized with caveolin-1, known to be an integral plasma membrane protein.

Next, we determined whether Sph-induced PLD activation was affected by over-expression of wtSphK1 or DNSphK1. Indeed, as shown in Figure 4(C), PLD activation by Sph was strongly potentiated in cells over-expressing SphK1, in which the enzymic activity was almost doubled. Moreover, enforced expression of DNSphK1 significantly reduced the effect of Sph on PLD. These results support the notion that the observed stimulation of PLD by Sph depends on SphK activity.

Formation of S1P in C2C12 myoblasts

To localize the site of S1P generation by SphK upon Sph administration, we initially performed experiments by incubating C2C12 cells with 1 μ M [3 H]Sph for 5 min and monitoring [3 H]S1P formation in medium and cells. In these experimental conditions approx. 60% of the added radioactive sphingoid base was recovered into the cellular fraction. [3 H]S1P was found mainly in the cell medium, whereas only a small percentage of the labelled bioactive lipid was present in the cells (less than 5% of the total [3 H]S1P) (Figure 5A). This finding shows that in C2C12 myoblasts a significant amount of exogenous Sph can be rapidly transformed into S1P. Interestingly, from Figure 5 it can be observed that almost all the [3 H]S1P formed was recovered in the cell medium, but it was undetectable when the incubation was carried out using the conditioned medium, suggesting that SphK activity is not released at significant amount into the medium by C2C12 myoblasts. As [3 H]S1P detected in the cell medium could be produced extracellularly by a cell-associated SphK with a catalytic site freely accessible by exogenous Sph, but it could also be generated intracellularly and then transported out of the cell, further investigation was performed using the cell surface SphK assay, which, by employing labelled ATP, allows the products of extracellular phosphorylation reactions to be measured exclusively [23]. Cells were incubated in the presence of 100 μ M [32 P]ATP and 1 μ M Sph for 5 min and processed for the evaluation of [32 P]S1P formation. Interestingly, as shown in Figure 5(B), also carried out in these experimental conditions, the prevailing amount of the labelled S1P was found in the cell medium, although a small fraction of the total bioactive lipid formed was measured in the cells. This finding supports the notion that extracellular S1P can be generated in myoblasts via phosphorylation of exogenous Sph catalysed by cell-associated SphK that is active in the extracellular compartment. Cell treatment with 0.5 μ M DMS, as expected, significantly reduced the amount of labelled S1P formed (results not shown). Finally, Figure 5(C) shows that in myoblasts transiently over-expressing

Experimental section. PLD activity is reported as the percentage of [3 H]PtdEtOH formed over [3 H]PtdCho. The results are expressed as the means $\pm$ S.E.M. for three independent experiments performed in triplicate; **P* < 0.05. Inset: Western analysis of the effect of antisense ODN against EDG5/S1P2 on EDG5/S1P2 receptor in C2C12 myoblasts. Samples (30 μ g) of the total membrane fraction, obtained from C2C12 cells transfected with scrambled or EDG5/S1P2-specific antisense ODN, were separated by SDS/PAGE, electrotransferred on to nitrocellulose and immunodetected with specific anti-EDG5/S1P2 antibodies. The experiment shown is representative of three.

Figure 4 Expression of wtSphK1 or DNSphK1 in C2C12 myoblasts and its effect on Sph-mediated PLD activation

(A) C2C12 myoblasts were transiently transfected with either empty pcDNA3 vector (vector) or pcDNA3 containing FLAG-tagged human wtSphK1 (wtSphK1) or DNSphK1 cDNA (DNSphK1). After transfection (36 h) C2C12 cells were scraped in lysis buffer and centrifuged to remove nuclei. Cytosolic, Golgi-enriched and endosome/plasma membrane fractions were prepared from vector-, wtSphK1- or DNSphK1-transfected cells as described in the Experimental section. Proteins (10 μ g) from cytosol (C), Golgi-enriched (G) and endosome/plasma membrane (E/PM) fractions were separated by SDS/PAGE and electrotransferred on to nitrocellulose. Hybridization with rabbit polyclonal anti-FLAG antibodies was performed to immunodetect FLAG-tagged protein. Actin, calnexin and Rab5, chosen as protein loading controls for cytosol, Golgi-enriched and endosome/plasma membrane fraction respectively, were detected using specific anti-actin, anti-calnexin or anti-Rab5 antibodies. A representative blot of three with similar results is shown. (B) C2C12 myoblasts transiently transfected with empty vector (panels A and B) or pcDNA3 containing FLAG-tagged human wtSphK1 cDNA (panels C–F) were grown on coverslips and fixed with 0.5% paraformaldehyde. The fixed cells were double labelled with mouse monoclonal anti-caveolin-1 antibodies (panel D) and rabbit polyclonal anti-FLAG antibodies (panel E), which were immunodetected using anti-mouse rhodamine B- or anti-rabbit Alexa-488-tagged secondary antibodies. The two images were superimposed (panel F). The negative control is shown in panel B. The coverslips were mounted on Mowiol and examined with a Bio-Rad confocal laser scanning microscope. An image from a representative experiment of three independently performed experiments with similar results is shown. A differential interference contrast image of myoblasts transfected with vector alone (panel A) or with wtSphK1 (panel C) is also shown. (C) C2C12 myoblasts transiently transfected with empty vector (vector) or pcDNA3 containing wtSphK1 (wtSphK1) or DNSphK1 (DNSphK1) cDNA were serum-starved and labelled with [3 H]glycerol for 16 h. Cells were incubated without (open bar) or with Sph (1 μ M) (closed bar) for 5 min in the presence of 2% ethanol and processed for the quantification of [3 H]PtdEtOH as described in the Experimental section. PLD activity is reported as percentage of [3 H]PtdEtOH over [3 H]PtdCho. The results are expressed as the means $\pm$ S.E.M. for triplicate samples and are representative of three independent experiments. Over-expression of wtSphK1 or DNSphK1 did not significantly affect basal PLD activity. Stimulation of PLD by Sph in wtSphK1-transfected cells and reduction of the Sph-induced PLD activation in DNSphK1-transfected cells versus Sph-induced PLD activation in vector-transfected cells were both statistically significant; * $P < 0.05$.

wtSphK1 the amount of [32 P]S1P formed in the cell medium after 5 min of incubation with Sph and labelled ATP was 2.5-fold higher than that measured in vector control cells, further supporting a key role for SphK1 in the extracellular formation of S1P.

DISCUSSION

In the present study we investigated the effect of Sph on PLD activity in C2C12 myoblasts and the molecular mechanism involved in this action. Sph was found to stimulate PLD activity in myoblasts and, notably, it was clearly demonstrated that Sph-induced PLD activation occurs via a novel mechanism that requires SphK activity, as well as the extracellular action of the newly generated S1P. The finding that Sph can stimulate PLD activity is in agreement with previous studies [35] performed

over the last decade in different cell systems in which, however, the molecular mechanism of Sph action was not addressed. In those studies the possible conversion of Sph into S1P was postulated, but the biological action of S1P was essentially attributed to its action as intracellular mediator. More recently, following the identification of several members of the S1P-specific receptor family, major scientific interest was focused on the role of S1P as extracellular ligand which was also found to activate PLD. Indeed, although PLD was initially found to be stimulated by S1P in a receptor-independent manner in HEK293 cells and NIH-3T3 cells over-expressing EDG1/S1P1 receptor [12], several reports have subsequently demonstrated that PLD stimulation by S1P is a receptor-mediated event in different cell types, such as C2C12 myoblasts, human airway epithelial cells and C6 glioma cells [25,36,37]. These latter observations hampered the attribution of the Sph effect on PLD to its metabolic transformation into S1P. However, the recent

Figure 5 Formation of labelled S1P in extracellular compartments of native (A and B) and wtSphK1-overexpressing (C) C2C12 myoblasts

(A) Serum-starved C2C12 cells were incubated with [³H]Sph (1 μ M) for 5 min. Immediately after the beginning of the incubation reaction, an aliquot of conditioned medium was transferred to a test tube and incubation continued for 5 min at 37 °C. Lipids were extracted from cell medium (open bars), cells (closed bars) and conditioned medium (hatched bars). [³H]Sph and [³H]S1P were identified by TLC and quantified by scintillation counting as described in the Experimental section. The results are expressed as percentage of [³H]sphingoid base over total extracted labelled lipids. The results are expressed as the means $\pm$ S.E.M. for triplicate samples and are representative of two independent experiments. (B) Serum-starved C2C12 myoblasts were incubated in DMEM with [³²P]ATP (100 μ M) in the presence of 1 μ M Sph. [³²P]S1P from cell medium (open bar), cells (closed bar) and conditioned medium (hatched bar) was extracted and quantified as described in (A). Results are reported as pmol of [³²P]S1P/min per 10⁶ cells (means $\pm$ S.E.M., n = 4). (C) C2C12 myoblasts transiently transfected with empty expression vector (vector) or pcDNA3 containing wtSphK1 cDNA (wtSphK1) were incubated in DMEM with [³²P]ATP (100 μ M) in the presence of 1 μ M Sph. [³²P]S1P from cell medium was extracted and quantified as described in (A). Results are reported as pmol of [³²P]S1P/min per 10⁶ cells. The results are expressed as the means $\pm$ S.E.M. of a representative experiment performed in triplicate and repeated three times with analogous results.

findings that SphK1 can be exported by human umbilical vein endothelial cells and HEK293 cells [23] and S1P-specific receptors can be activated by endogenously generated S1P [38] led us to investigate the possible involvement of SphK-derived S1P in the Sph action on PLD activity.

Several lines of evidence obtained in the present study indicate that the effect of Sph on PLD activity is due to its conversion into

S1P and subsequent action as extracellular ligand. Indeed DMS, at concentrations that inhibit SphK, but not PKC, considerably reduced the action of Sph on PLD activity in C2C12 cells. Moreover, in myoblasts over-expressing SphK1, the Sph-induced PLD activation was largely potentiated, whereas the response to the sphingoid molecule was attenuated in cells over-expressing the dominant negative form of SphK1. Consistent with a role for SphK-derived S1P as an extracellular ligand in the action of Sph, the stimulation of PLD activity by Sph was fully prevented by the treatment with PTx, similar to the previous observation of S1P-induced PLD activity in C2C12 myoblasts [25]. Furthermore, the observation that Sph failed to activate PLD in myotubes further supports the hypothesis that the action of Sph is mediated by S1P, since in a previous study we showed that in myotubes S1P is uncoupled from the PLD pathway as a consequence of the selective down-regulation of EDG5/S1P2 receptor [29]. In this regard, the crucial role of EDG5/S1P2 in Sph-induced PLD activity was further proved by the enhanced response observed in myoblasts over-expressing the receptor protein, as well as by the diminished ability of Sph to stimulate PLD in cells transfected with a specific antisense ODN designed to down-regulate EDG5/S1P2. The results in the present study provide strong support for the hypothesis that Sph action on PLD activity is due to its conversion into S1P and the subsequent binding to EDG5/S1P2. In favour of the Sph action via its transformation into S1P is also the finding that Sph, when administered at the same concentration as S1P, activated PLD to a significantly smaller extent than S1P. Interestingly, however, given that the sub-micromolar concentration of the Sph effective on PLD was 50-fold less than that efficacious in other cell systems, the C2C12 myoblasts employed in this study were highly responsive to Sph [35]. On the basis of these observations, it can be hypothesized that the here identified mechanism of activation of PLD by Sph does exist in other cell types, the higher responsiveness of C2C12 myoblasts to PLD activation by Sph being ascribed to a more efficient conversion of Sph into S1P and/or to an elevated availability of SphK-derived S1P in the extracellular compartment. In addition, it is possible to speculate that in the cell systems in which Sph was found unable to activate PLD [39], a different cellular distribution of SphK activity occurs, making it unavailable at exogenous Sph, or, in the case of an intracellular conversion of Sph to S1P, a poor export of the sphingoid molecule takes place.

Another interesting finding of the present study is that in C2C12 myoblasts S1P is generated extracellularly by cell-associated SphK. In this regard our results rule out the possibility that SphK activity released into the medium significantly participates in S1P generation from exogenous Sph, although SphK activity was detectable in 20-fold concentrated conditioned medium (E. Meacci, F. Cencetti, C. Donati and P. Bruni, unpublished work). Since previously SphK1 was found to be released into the medium by HEK293 cells, human umbilical vein endothelial cells and airway smooth muscle cells [23,40], but not by murine endothelial H-end cells [41], it is clear that extracellular export of the enzyme may be highly cell specific.

The cell surface assay, employed in the present study to measure S1P formation, allowed it to be clearly established that the conversion of Sph into S1P occurs in the extracellular compartment of C2C12 cells, although at the present stage, it cannot be excluded that intracellular formation of S1P and its subsequent transport outside the cell takes place and participates in the biological action of Sph. Remarkably, the generation of S1P via phosphorylation of exogenous Sph occurred in the absence of extracellular cues and was due to an enzymically active form of SphK. Previous studies showed that in other cell types SphK1 was activated by phosphorylation-dependent membrane

translocation [20,21]. Intriguingly, in this study over-expressed SphK1 was found instead to be appreciably associated to membranes and co-localized with caveolin-1 at plasma membrane in unchallenged C2C12 myoblasts. This finding further supports the notion that in these cells the enzyme is at least in part intrinsically active as consequence of constitutive processes of phosphorylation/translocation. Notably, in the present study we provide compelling evidence that in unchallenged myoblasts SphK1 can function as ecto-enzyme, able to phosphorylate the Sph available in the extracellular environment. Indeed, in myoblasts over-expressing wtSphK1, in agreement with the action as ecto-enzyme of membrane-associated SphK1, the formation of S1P in the extracellular compartment was found to be markedly potentiated. At present it is not known, although it will be worth future investigation, whether membrane-bound SphK1 in C2C12 cells can also employ intracellular Sph to generate extracellular S1P, subsequently able to act in an autocrine/paracrine fashion, or whether exclusively extracellularly formed Sph can be employed by SphK1 for extracellular accumulation of S1P.

The results of the present study are in line with recent studies in which exogenous Sph-mediated prostaglandin E2 production [42] and p42/p44 mitogen-activated protein kinase activation [40] were due to its conversion into S1P. However, interestingly, in the present study Sph-induced PLD activation can be ascribed to the action of cell-associated SphK active in the extracellular compartment. Intriguingly, here, for the first time a biological effect mediated by Sph via its conversion into S1P in a native cell system has been identified where the expression of enzymes responsible for S1P metabolism was not manipulated.

In the light of the molecular mechanism of Sph action on PLD reported in the present study, it will be worth investigating in the future whether it represents a more general mechanism of action of Sph in skeletal and cardiac muscle, implicated in the multiple biological effects elicited by the sphingoid molecule in these tissues.

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