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A role for calcium in sphingosine 1-phosphate-induced phospholipase D activity in C2C12 myoblasts

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Abstract Receptor-regulated phospholipase D (PLD) is a key signaling pathway implicated in the control of fundamental biological processes. Here evidence is presented that in addition to protein kinase C (PKC) and Rho GTPases, Ca^{2+} response evoked by sphingosine 1-phosphate (S1P) also participates to the enzyme regulation. Ca^{2+} was found critical for PKCα-mediated PLD activation. Moreover, S1P-induced PLD activity resulted diminished by calmodulin inhibitors such as W-7 and CGS9343B implicating its involvement in the process. A plausible candidate for Ca^{2+}-dependent PLD regulation by S1P was represented by calcineurin, in view of the observed reduction of the stimulatory effect by cyclosporin A. In contrast, monomeric GTP-binding protein Ral was translocated to membranes by S1P in a Ca^{2+}-independent manner, ruling out its possible role in agonist-mediated regulation of PLD. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Sphingosine 1-phosphate; Phospholipase D; C2C12 myoblast; Calcium; Calmodulin; Calcineurin

1. Introduction

Phospholipase D (PLD), which hydrolyzes phosphatidylcholine (PtdCho) to phosphatidic acid and choline, represents a key enzyme in membrane cell signaling. A plethora of extracellular ligands including hormones, neurotransmitters and cytokines are indeed able to activate the enzymatic activity upon interaction with their receptors [1–4]. Activation of PLD appears to be involved in the regulation of various fundamental biological events including cell proliferation and differentiation, progression through cell cycle, vesicular trafficking, cell secretion [1–4] and, more recently, receptor endocytosis [5]. Studies performed over the last decade have clarified that two distinct isoforms, PLD1 and PLD2, account for PLD activity in mammalian cells. Both proteins appear to be complexly regulated, in many instances in an agonist- and cell-specific manner, but the molecular mechanisms involved have not been fully elucidated.

Our previous studies have shown that both PLD1 and PLD2 are expressed in C2C12 satellite muscle cells and that PLD activity is significantly increased by sphingosine 1-phosphate (S1P) [6], a lysophospholipid known to exert pleiotropic effects in a variety of cell systems [7,8]. Activation of PLD by the bioactive lipid was totally abrogated by pertussis toxin, supporting the view that the process was dependent on the binding of S1P to its specific receptors [6]. As regards the signaling pathways downstream of receptor coupling specifically involved in PLD regulation, a major role was found for protein kinase C (PKC). Indeed, Ca^{2+}-dependent PKC, exclusively represented in C2C12 cells by the alpha isoform, as well as PKCδ, a member of the novel, Ca^{2+}-independent PKC subfamily, were identified as major regulators of S1P-induced PLD activity [6]. Moreover, monomeric GTP-binding proteins belonging to the Rho family were also identified as modulators of myoblast PLD activity by S1P: RhoA translocation/activation elicited by the bioactive lipid, which resulted downstream of PKCα [9], was found involved in activation of PLD specifically localized in Golgi-enriched fractions [10]. Our findings on PLD regulation by S1P in C2C12 myoblasts are in keeping with other studies in which agonist-induced PLD activity resulted to be concomitantly under the control of multiple inputs such as PKC and monomeric Rho GTPase family proteins [1–3]. However, several examples are reported in the literature for participation of Ca^{2+} in the regulation of PLD activity, although the effector molecules involved have not been fully characterized so far [11–14].

Given that in our recent work evidence was provided for a significant effect of S1P on intracellular Ca^{2+} levels in C2C12 myoblasts [15,16], we decided to address the question whether the Ca^{2+} rise elicited by the bioactive lipid plays a role in the mechanism by which S1P regulates PLD activity in skeletal muscle cells. Here, we report that Ca^{2+} mobilization is implicated in PLD regulation by S1P and that multiple Ca^{2+} effectors are potentially involved in the process.

2. Materials and methods

2.1. Materials

C2C12 cells were obtained from ATCC (Manassas, VA, USA). S1P was purchased from Avanti Polar Lipids (Alabaster, AL, USA). BAPTA/acetoxymethyl ester (AM), cyclosporin A, and Gö6976 were obtained from Calbiochem (La Jolla, CA, USA). Bradykinin (BK), A23187, W-7, KN-62 were from Sigma-Aldrich (Milan, Italy). CGS9343B (Zaldaride) was the generous gift of Dr. Jacob A. Zijlstra, Novartis Consumer Health, Nyon, Switzerland. [^{3}H]Glycerol (16.5 Ci/

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Abbreviations: S1P, sphingosine 1-phosphate; PLD, phospholipase D; PtdCho, phosphatidylcholine; BK, bradykinin; PKC, protein kinase C; PtdEtOH, phosphatidylethanol; AM, acetoxymethyl ester; CaM, calmodulin

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mmol) was purchased from Du Pont NEN (Boston, MA, USA). Fluo-3/AM and Pluronic F-127 were from Molecular Probes (Eugene, OR, USA). Mouse monoclonal antibodies against Ral were from Transduction Laboratories (Lexington, KY, USA). Rabbit polyclonal anti-PKCα antibodies and secondary antibodies (goat anti-mouse or anti-rabbit immunoglobulin G1 conjugated to horseradish peroxidase) for Western analysis were from Santa Cruz (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

2.2. Cell culture

C2C12 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2/95% air. For the experiments cells were seeded into 35-mm or 100-mm diameter dishes and utilized when 90% confluent. The day before the experiment the cells were shifted to serum-free medium and in some circumstances labeled overnight with [^{3}H]glycerol.

2.3. Ca^{2+} imaging

Imaging of intracellular Ca^{2+} was obtained using a Bio-Rad MRC 1024ES (Bio-Rad, Hercules, CA, USA) confocal scanning head coupled to a Nikon TE300 inverted microscope. Cells plated on a coverslip were loaded with 10 μM Fluo-3/AM in the presence of 0.1% anhydrous dimethylsulfoxide (DMSO) and 0.01% (w/v) Pluronic F-127 as dispersing agents for 10 min at room temperature and subjected to laser scanning imaging essentially as described [15,16]. Fluo-3-loaded cells were excited at 488 nm and emitted fluorescence was collected by a Nikon Plan Apo 60× oil immersion objective through a 510-nm long-wave pass-filter. Analysis of images was obtained using the free software Confocal Assistant (Bio-Rad).

2.4. PLD in vivo assay

PLD activity was determined by measuring [^{3}H]phosphatidylethanol (PtdEtOH) produced via PLD-catalyzed transphosphatidylation in the presence of ethanol, as previously described [17]. Briefly, C2C12 cells were metabolically labeled by incubation with 5 μCi/ml [^{3}H]glycerol for 24 h. After washing with ice-cold phosphate-buffered saline (PBS) to remove unincorporated [^{3}H]glycerol, labeled cells were treated or not with the agonist in the presence of 2% ethanol. Incubations were performed at 37°C and terminated by washing the monolayers twice with ice-cold PBS followed immediately by addition of 1 ml of ice-cold methanol. Cells were collected by scraping; lipids were then extracted essentially as described by Bligh and Dyer [18] except that water was replaced with 1 M NaCl. [^{3}H]PtdEtOH and [^{3}H]PtdCho were separated by thin-layer chromatography and quantified as described [17]. [^{3}H]PtdEtOH formed was reported as the percentage of [^{3}H]PtdCho. In all the experiments PLD assay was performed in duplicate and the variation coefficient was always below 8%.

2.5. Cell fractionation

Serum-starved C2C12 cells were treated or not with S1P for the indicated times. The medium was removed and the cells washed twice with ice-cold PBS, scraped in 20 mM HEPES, pH 7.4, 2 mM EGTA, 1 mM EDTA, 250 mM sucrose, 5 mM NaN_3 containing protease inhibitors (1 mM AEBSF, 0.3 μM aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin) and homogenized in a Dounce homogenizer (60 strokes). Lysates were centrifuged for 7 min at 750×g to separate nuclei. To prepare cytosolic and total particulate fractions, the supernatant was centrifuged at 200 000×g for 1 h. Protein content was quantified according to the Coomassie blue procedure using a commercially available kit (Bio-Rad).

2.6. Immunodetection

Proteins (15–25 μg) from cellular fractions of control or stimulated myoblasts were separated by SDS–PAGE. Proteins were electrotransferred to nitrocellulose membranes, which were incubated overnight in Tris-buffered saline containing 0.1% Tween-20 (TTBS) and 1% bovine serum albumin. Membranes were subsequently incubated for 1 h with antibodies against Ral or PKCα. Hybridization with primary antibodies was followed by washing with TTBS and incubation with peroxidase-conjugated goat anti-mouse or anti-rabbit IgG1. Proteins were detected by ECL. Quantitative analysis of the bands was performed using Imaging and Analysis Software by Bio-Rad.

3. Results

To examine the sensitivity to Ca^{2+} of myoblast PLD, the enzymatic activity was initially assayed in C2C12 cells incubated for 10 min in the presence of the Ca^{2+} ionophore A23187. As shown in Fig. 1, the addition of 3 μM A23187 provoked a significant increase of PLD activity, indicating that Ca^{2+} can regulate PLD in skeletal muscle cells. Results presented in the same figure illustrate that the positive effect exerted by Ca^{2+} on PLD activity was reduced in cells treated with the conventional PKC inhibitor Gö6976 (1 μM), suggesting that PKCα, the sole Ca^{2+}-dependent isoform expressed in these cells [6], could represent a downstream effector of Ca^{2+} in the signaling to PLD. It was then evaluated whether intracellular Ca^{2+} increase participates in the agonist-mediated regulation of PLD in C2C12 cells. To this end PLD activity was measured in myoblasts loaded for 1 h with 15 μM BAPTA/AM, intracellular chelator of Ca^{2+}. In this experimental condition agonist-induced rise of cytosolic Ca^{2+} was fully prevented, as proved by the monitoring with a confocal laser scanning fluorescent microscope of Ca^{2+} signals evoked by 1 μM S1P in Fluo-3-loaded myoblasts (Fig. 2A). Remarkably, the presence of BAPTA reduced significantly the activation of PLD by 1 μM S1P and 1 μM BK, indicating that intracellular Ca^{2+} plays also a critical role in the regulation of agonist-induced PLD activity (Fig. 2B). The possible mechanisms by which intracellular Ca^{2+} regulates S1P-stimulated PLD activity were then investigated. As illustrated in Fig. 3, calmodulin (CaM) inhibitors W-7 and CGS9343B both reduced at a significant extent activation of PLD by S1P, clearly demonstrating that Ca^{2+}–CaM complex was implicated in the S1P-evoked Ca^{2+} signaling leading to PLD activation; the insensitivity to the specific inhibitor KN-62 [19] suggested however that CaM-dependent protein kinase II was not involved in the process.

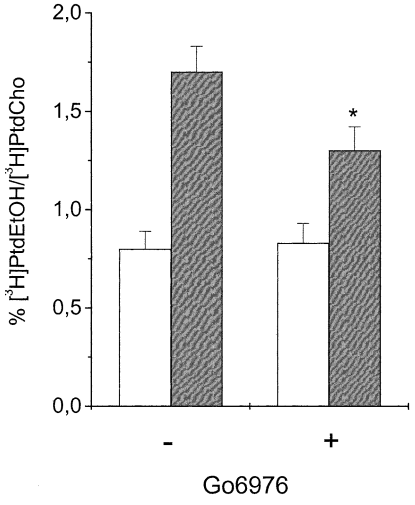

Fig. 1. Effect of Ca^{2+} on PLD activity in C2C12 cells. Serum-starved myoblasts were labeled with [^{3}H]glycerol and incubated without (−) or with (+) Gö6976 (1 μM) 30 min prior to the addition of A23187 (3 μM) (filled bar) for 10 min in the presence of 2% ethanol. Lipid extraction and [^{3}H]PtdEtOH measurement were performed as described in Section 2. Data are means ± S.E.M. of four independent experiments performed in duplicate. The effect of inhibition was statistically significant (Student's t-test, $^*P < 0.05$).

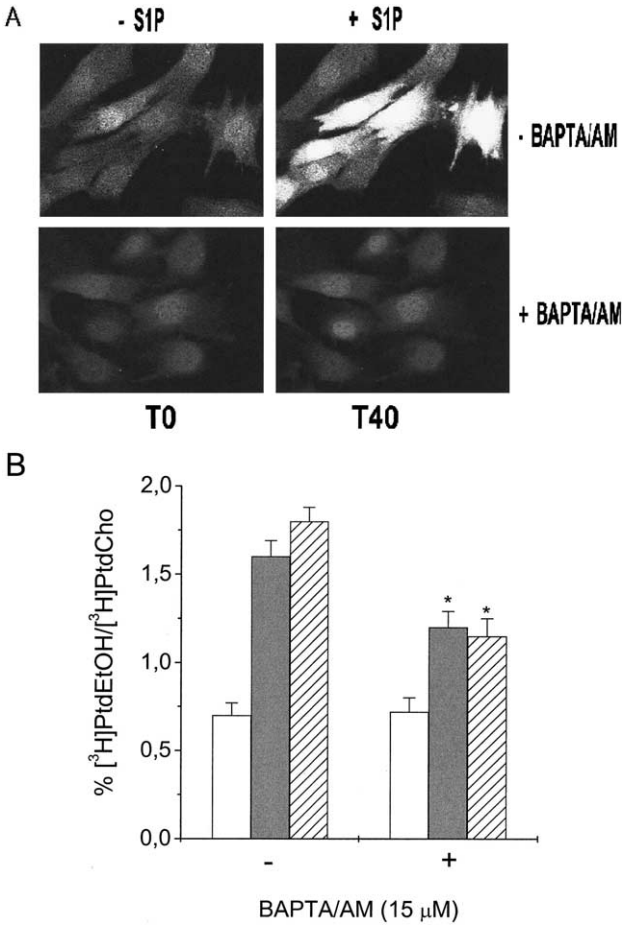

Fig. 2. Effect of BAPTA/AM on Ca^{2+} increase and S1P-induced PLD activation in C2C12 cells. A: Images represent the fluorescence emission of Fluo-3 related to the Ca^{2+} level changes in cells treated without or with 1 μM S1P for 40 s (T40). For the experiments myoblasts were plated on the coverslip, starved in serum-free medium and incubated with or without BAPTA/AM (15 μM) for 1 h. Successively, the cells were loaded with Fluo-3/AM (10 μM) for 10 min, mounted on the stage of the confocal laser scanning microscope and treated or not with the agonist. B: Serum-starved myoblasts labeled with [^{3}H]glycerol were incubated with (+) or without (−) BAPTA/AM (15 μM) 1 h prior to the stimulation with S1P (1 μM) (filled bar) or BK (1 μM) (hatched bar) for 10 min in the presence of 2% ethanol. Lipid extraction and [^{3}H]PtdEtOH measurement were performed as described in Section 2. Data are means ± S.E.M. of at least three independent experiments performed in duplicate. The effect of inhibition was statistically significant (Student's *t*-test, $^*P < 0.05$).

Given that the monomeric GTP-binding protein Ral participates in some instances to PLD regulation [20,21] and is stimulated in vitro by Ca^{2+}–CaM [22] and in vivo by Ca^{2+} [23], next we examined whether Ral was activated by S1P. Indeed, 30 s incubation in the presence of the bioactive lipid provoked a specific translocation of Ral to membranes, which was similar to that induced by the rise of intracellular Ca^{2+} elicited by A23187 (Fig. 4A). However, the mechanism by which S1P activated Ral did not require Ca^{2+}, since the association of the GTPase to membranes was not significantly diminished in Ca^{2+}-depleted cells (Fig. 4B). In the same figure the effect of Ca^{2+} depletion on S1P-induced PKCα translocation is presented. Notably, in this experimental condition the agonist-dependent association of PKCα to membranes was

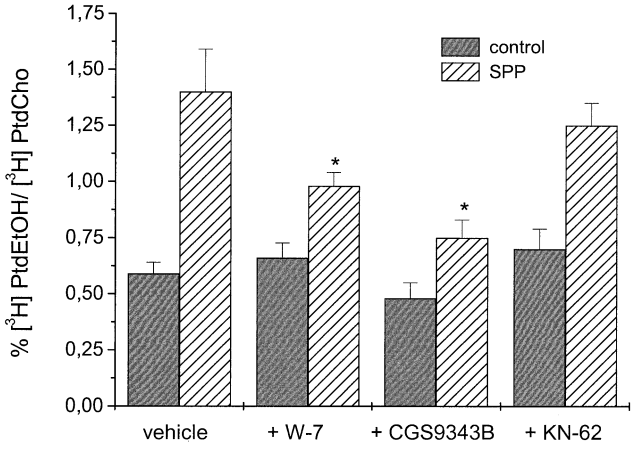

Fig. 3. Effect of W-7, CGS9343B and KN-62 on S1P-induced PLD activation in C2C12 cells. Serum-starved myoblasts were labeled with [^{3}H]glycerol and incubated with vehicle (DMSO, 0.1%), W-7 (1 μM), CGS9343B (40 μM) or KN-62 (1 μM) 30 min prior to the addition of S1P (1 μM) (hatched bar) for 10 min in the presence of 2% ethanol. Lipid extraction and [^{3}H]PtdEtOH measurement were performed as described in Section 2. Data are means ± S.E.M. of four independent experiments performed in duplicate. The addition of 0.1% DMSO (vehicle) did not change significantly basal PLD activity. The effect of the inhibition was statistically significant (Student's *t*-test, $^*P < 0.05$).

impaired, implicating a Ca^{2+}-dependent event in S1P-induced PKCα activation.

Finally, the potential involvement of calcineurin on S1P-induced PLD activity was examined. As illustrated in Fig. 5, cell treatment with 1 μM cyclosporin A, inhibitor of the CaM-dependent protein phosphatase [24], significantly reduced the extent of PLD activation by S1P, suggesting a role for calcineurin in PLD regulation.

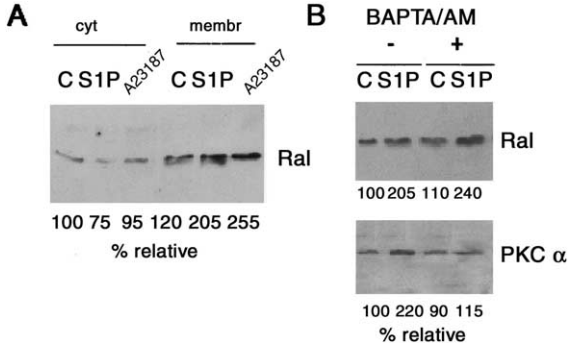

Fig. 4. Effect of S1P and Ca^{2+} on membrane translocation of Ral or PKCα in C2C12 cells. A: Serum-starved myoblasts were treated with S1P (1 μM) or A23187 (3 μM) for 30 s. Protein aliquots (15 μg) of cytosolic (cyt) and particulate fractions (membr) were separated by SDS–PAGE and transferred to nitrocellulose and immunodetected as described in Section 2. Experiment shown is representative of at least three. Band intensity is reported as percentage relative to control (no addition, 100%; mean ± S.E.M. was less than 15%). B: Serum-starved myoblasts were incubated with or without BAPTA/AM (15 μM) 1 h prior to the stimulation with S1P (1 μM). Cells were collected and samples (25 μg) of particulate fraction were subjected to Western analysis using monoclonal anti-Ral or polyclonal anti-PKCα antibodies. Experiment shown is representative of at least three. Band intensity is reported for each detected protein as percentage (mean ± S.E.M., $n = 4$) relative to each control set as 100%.

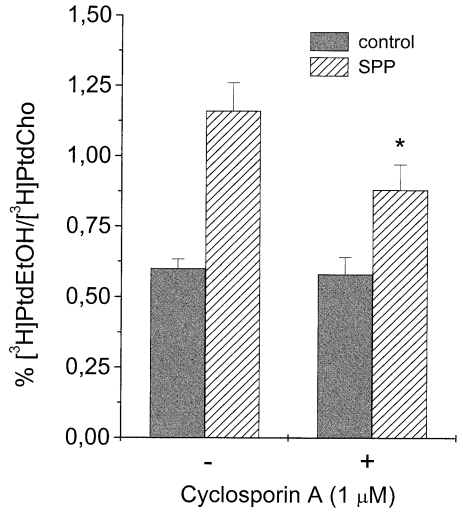

Fig. 5. Effect of cyclosporin A on S1P-induced PLD activation in C2C12 cells. Serum-starved myoblasts were labeled with [^{3}H]glycerol and incubated with vehicle (DMSO, 0.1%) (−) or cyclosporin A (1 μM) (+) for 10 min in the presence of 2% ehtanol. Lipid extraction and [^{3}H]PtdEtOH measurement were performed as described in Section 2. Data are means ± S.E.M. of four independent experiments performed in duplicate. The effect of the inhibition was statistically significant (Student's *t*-test, $*P < 0.05$).

4. Discussion

A multitude of different studies have presented evidence for a highly complex regulation of PLD by extracellular ligands. In the present report it is clearly shown that stimulation of PLD activity by S1P through specific G-protein-coupled receptors, besides PKC and Rho proteins activation [6,10], also requires Ca^{2+} increase. The here reported results confirm previous studies in which a role for Ca^{2+} in agonist-regulated PLD was proposed, on the basis of the stimulatory effect of the enzymatic activity exerted by Ca^{2+} ionophores [25] and the notion that both PLD isoforms, PLD1 and PLD2, are sensitive to Ca^{2+} ions when overexpressed in insect Sf9 cells [14]. Ca^{2+} increase evoked by S1P in mouse myoblasts was here found to be required for PLD activation, although it was not a prerequisite for agonist-dependent stimulation of PLD. This latter finding is consistent with the previously observed role of PKCδ in PLD regulation by S1P [6] and the dispensable role of Ca^{2+} in this signaling pathway. Notably, PLD activity in S1P-stimulated myoblasts resulted to be influenced by Ca^{2+} through different molecular mechanisms. First of all, Ca^{2+} could activate PLD via PKCα regulation, as suggested by two lines of evidence: the reduction of the effect of the Ca^{2+} ionophore A23187 on PLD activity by the conventional PKC inhibitor Gö6976 and the inhibition of PKCα translocation to membranes induced by S1P in Ca^{2+}-depleted cells. Secondly, the stimulatory effect of Ca^{2+} on PLD activity induced by S1P was found to be dependent on Ca^{2+}–CaM complex, as indicated by the sensitivity of the event to two structurally different inhibitors, W-7 and CGS9343B. In agreement with previous studies [26–28] Ca^{2+}–CaM was found implicated in agonist-regulated PLD in C2C12 myoblasts. However, Ca^{2+}–CaM-dependent protein kinase II was not apparently involved in PLD regulation, differently from what was observed in smooth muscle cells [28]. In search for a possible effector of CaM able to mediate Ca^{2+}-dependent signaling of S1P to PLD, regulation of Ral by the bioactive lipid was here investigated. Remarkably, the monomeric GTPase, known to participate to agonist-dependent PLD regulation [20,21], was relocalized to membranes by S1P, indicating that it may play a role in S1P signaling. However, despite translocation of Ral to membranes resulted to be promoted by Ca^{2+} rise, the S1P effect on Ral was unaffected by cytosolic Ca^{2+} depletion. This finding rules out that Ral could act as effector of Ca^{2+} in the signaling of S1P to PLD in myoblasts, although it does not exclude the involvement of Ral in PLD regulation through a Ca^{2+}-independent signaling cascade.

Another major finding of this study is that calcineurin very likely acts as effector of Ca^{2+}–CaM in the S1P-mediated PLD activation, as inferred by the significant reduction of S1P effect by cyclosporin A. However, the molecular mechanism by which the serine/threonine protein phosphatase exerts a positive effect on PLD activity is at present unknown. It can be speculated that PLD acts as substrate of calcineurin and is more active in the dephosphorylated form. This hypothesis is in keeping with the finding that PLD2 is inhibited by serine/threonine phosphorylation [29]. Alternatively, calcineurin could indirectly influence PLD activity by acting on one or more of its numerous regulators.

From this study it emerges that Ca^{2+} response evoked by S1P plays a role in the control of PLD and that CaM and calcineurin are implicated in this molecular event. In view of the key role exerted by these proteins in muscle cell biology [30], it will be of interest to examine whether calcineurin is critical for biological action of S1P in skeletal muscle and PLD activation represents a key element in its signaling machinery.

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