FEBS Letters 473 (2000) 10–14 FEBS 23620

Receptor-activated phospholipase D is present in caveolin-3-enriched light membranes of C2C12 myotubes

Elisabetta Meacci*, Chiara Donati, Francesca Cencetti, Elena Romiti, Marta Farnararo, Paola Bruni

Dipartimento di Scienze Biochimiche, Università di Firenze, Viale G.B. Morgagni 50, 50134 Firenze, Italy

Received 2 March 2000; received in revised form 28 March 2000

Edited by Felix Wieland

Abstract Caveolin-3 (cav-3) is a key structural component of caveolar membrane in skeletal muscle. Cav-3-enriched light membrane (CELM) fractions obtained from C2C12 myotubes contain phospholipase D1 (PLD1) and its major regulators, RhoA and protein kinase Cα (PKCα). All these proteins were found bound to cav-3. An in vivo assay of PLD activity, which allows to localize the reaction product in CELMs, indicated that the enzyme associated to this membrane microdomain was active. Moreover, bradykinin (BK), thrombin and phorbol 12-myristate 13-acetate induced rapid stimulation of PLD activity in CELMs. The cav-3-PLD1 complex was not affected by BK treatment, whereas the agonist induced a marked increase of RhoA association with cav-3. Furthermore, BK-induced PLD activation in CELMs was dependent, at least in part, on PKCα.

Key words: Phospholipase D; Bradykinin; Caveolin-3; RhoA; C2C12 myotube

1. Introduction

Caveolae are plasma membrane invaginations which are thought to play a role in many cellular functions including transcytosis, receptor-mediated uptake, stabilization of lipid rafts and compartmentalization of a number of signaling events at the cell surface [1,2].

The principal component of caveolae membranes is caveolin (cav), an integral membrane protein which sequesters signaling molecules in the microdomain by direct interaction through a specific scaffolding domain. Three forms of cav have been identified and cloned [3]. While caveolin-1 (cav-1) and caveolin-2 (cav-2) are widely expressed in various tissues, caveolin-3 (cav-3) has been reported to be muscle-specific [4]. Caveolae are well represented in skeletal muscle where they have been identified in the plasma membrane as well as in the T-tubules [5]. Cav-3 has been shown to interact with the dystrophin–glycoprotein complex [4] and the finding that the density and size of caveolae are modified in the plasma membrane from Duchenne muscular dystrophy patients [6] strongly suggests that caveolae play a key role in the organization and stabilization of the dystrophin cytoskeletal network. A recent study showed that cav-3 expression is required for myoblast fusion and myotube formation, further supporting a role for caveolae in muscle cell physiology [7]. Similarly to cav-1, cav-3 functions as scaffold protein, interacting with various proteins containing specific cav-binding domains which recognize a specific domain located at the N-terminus of the protein [4].

Phospholipase D (PLD) catalyzes the hydrolysis of the major phospholipid, phosphatidylcholine (PtdCho), to generate phosphatidic acid (PtdOH) and this reaction is implicated in the regulation of different cellular processes, such as vesicular trafficking, cell secretion, proliferation and differentiation [8,9]. Two mammalian PLDs, PLD1 and PLD2, have been recently cloned, both widely expressed in many cell types and tissues [8,9]. Agonist-stimulated PLD activity requires phosphatidylinositol 4,5-bisphosphate as essential cofactor and appears to be highly regulated by a complex group of proteins, including protein kinase C (PKC), monomeric GTP-binding proteins, such as RhoA and ARF, as well as protein-tyrosine kinases [8,9].

Previously we demonstrated that in intact C2C12 myoblasts PLD activity is under the control of ligands acting through G-protein coupled receptors, such as bradykinin (BK), thrombin and sphingosine 1-phosphate (SPP) [10,11]. Recently, evidence has been provided for the localization of PLD activity in caveolar microdomains in keratinocytes and fibroblasts [12–14] suggesting that the enzyme participates in the signaling cascades initiated from these lipid platforms.

In the present study we report that PLD activity is associated with cav-3-enriched light membrane (CELM) fractions from C2C12 myotubes and it is regulated by BK, thrombin and phorbol 12-myristate 13-acetate (PMA). Moreover, BK treatment did not affect the immunocomplex PLD-1-cav-3 whereas it increased the level of RhoA, key activator of the enzyme, associated with cav-3.

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 from Avanti Polar Lipids (Alabaster, AL, USA), solvents and silica gel 60 plates for thin-layer chromatography (TLC) from Merck (Darmstadt, Germany), BK, thrombin, other chemicals and Protein A- or Protein G-Sepharose beads were from Sigma (St. Louis, MO, USA). SPP and Gö6976 were from Calbiochem (San Diego, CA, USA). Mouse monoclonal antibodies against the N-terminal region (3–24) of cav-3 and PKCα were from Transduction Laboratories (Lexington, KY, USA). Rabbit polyclonal anti-Rab5 and rabbit polyclonal anti-calnexin were pur-

*Corresponding author. Fax: (39)-55-4222725.
E-mail: biocell@scibio.unifi.it

Abbreviations: PLD, phospholipase D; cav, caveolin; cav-3, caveolin-3; CELM, cav-3-enriched light membrane; BK, bradykinin; PMA, phorbol 12-myristate 13-acetate; PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid; PtdEtOH, phosphatidylethanol; PtdPro phosphatidylpropanol; SPP, sphingosine 1-phosphate; MBS, MES buffered saline; PKC, protein kinase C

PII: S0014-5793(00)01486-1

chased from Stressgen Biotech (Victoria, B.C., Canada). PLD1 antipeptide was a generous gift of Dr. S. Bourgoin (Ste-Foy, Que., Canada) and was generated as described [15]. Mouse monoclonal antibodies against RhoA and secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin G1) were from Santa Cruz (Santa Cruz, CA, USA).

2.2. Muscle cell culture

Mouse skeletal C2C12 myoblasts, were obtained from ATCC (Manassas, VA, USA), maintained as previously described [10] and, when at 70% confluent, were induced to differentiate in low mitogen medium (Dulbecco's modified Eagle medium containing 2% horse serum). Overt differentiation was indicated by the assembly of multinucleated syncytia. The myotubes were used 5–6 days after the cells were switched to low mitogen media.

2.3. Purification of cav-3-enriched membranes

CELMs were prepared from myotubes using a discontinuous sucrose gradient. Confluent differentiated cells were scraped into MES-buffered saline (MBS, 25 mM MES, pH 6.5, 0.15 M NaCl) containing a mixture of protease inhibitors and homogenized with 100 strokes of Dounce homogenizer. The homogenate was adjusted to 40% sucrose by 1:1 addition of 80% sucrose prepared in MBS, placed at the bottom of an ultracentrifuge tube and overlaid with two layers of 30 and 5% sucrose in MBS. The gradient was then centrifuged at $200\,000 \times g$ for 18 h using a Beckman SW50 rotor. When control and stimulated myotubes were compared, equal amounts of protein were fractionated in the discontinuous gradient. For analysis of the resulting gradient, 0.5 ml fractions were collected from the top of the gradient. The protein content was quantified according to the Coomassie Blue procedure [16].

2.4. Measurement of PLD activity

PLD activity was determined by measuring in sucrose gradient fractions [^{3}H]phosphatidylpropanol (PtdPro) or [^{3}H]phosphatidylethanol (PtdEtOH) produced via a PLD-catalyzed transphosphatidylation reaction performed in vivo in the presence of a primary alcohol such as 1-propanol or ethanol. Serum-starved myotubes were labeled for 24 h with 5 μCi/ml [^{3}H]glycerol. Basal PLD activity was measured incubating myotubes for 10 min with 2% 1-propanol. In parallel 2% 2-propanol was used to quantify unspecific radioactivity which was subtracted. Agonist-stimulated PLD activity was measured incubating the cells for 3 min with 2% ethanol prior agonist addition. Cells were washed twice in ice-cold PBS and scraped in methanol. Lipids were extracted and [^{3}H]PtdPro or [^{3}H]PtdEtOH was measured after TLC separation essentially as described [17]. [^{3}H]Phosphatidylalcohol was normalized to [^{3}H]PtdCho. Analysis of phospholipid species in [^{3}H]glycerol-labeled cells was determined after lipid extraction of the indicated sucrose density gradient fractions by TLC separation in a solvent mixture of chloroform:methanol:acetic acid (90:10:6) and quantified as described [10].

2.5. Immunoprecipitation procedures

Single or pooled (2–4) fractions from the sucrose density gradient, prepared from unstimulated or BK-stimulated myotubes, were diluted with MBS. The pellet obtained by centrifugation at $200\,000 \times g$, was resuspended in MBS containing 1% Triton X-100 and incubated overnight at 4°C with anti-PLD1 or anti-RhoA or anti-PKCα antibodies. The immunocomplexes were collected following 2 h incubation with Protein-A/G-Sepharose beads and washed extensively (five times) with MBS. The beads were resuspended in 10 μl of four-fold concentrated Laemmli electrophoresis buffer.

2.6. Western blot analysis

Proteins (5 μl) from sucrose density gradient fractions or immunocomplexes from immunoprecipitation experiments were separated by SDS–PAGE and immunoblotting 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. In immunoblot experiments a blot representative of at least three similar experiments is presented and to test the statistical significance, band intensities were measured using NIH Image.

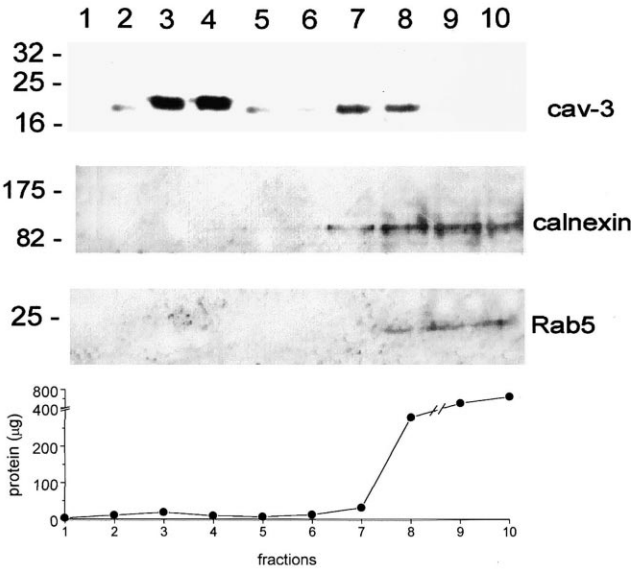

Fig. 1. Sucrose gradient fractionation of C2C12 myotubes. Cellular lysates from myotubes were fractionated by flotation in a discontinuous sucrose density gradient and the fractions were analyzed for cav-3, calnexin and Rab5 immunoreactivity and protein concentration as described in Section 2. Aliquots (5 μl) of the sucrose gradient fraction were subjected to SDS–PAGE and immunoblotting with the specific antibodies.

3. Results

To isolate CELMs, myotube lysates were fractionated on a discontinuous sucrose density gradient and the obtained fractions (0.5 ml) were analyzed for cav-3 immunoreactivity. As it can be observed from Fig. 1, cav-3 was enriched in fractions 3 and 4 (CELMs) which corresponded to the turbid interphasic band between 30 and 5% sucrose equilibrium density. Cav-3 was also present in fractions 7 and 8 which sedimented at higher sucrose density. CELMs excluded intracellular membrane proteins such as calnexin and endosomal and plasma membrane markers such as Rab5 which were instead enriched in fractions 8–10 (Fig. 1). Analysis of the protein profile demonstrated that the bulk of protein was present in the high-density sucrose fractions (Fig. 1). Typically fractions 2–4 contained $2 \pm 0.6\%$ of the total protein content. We then examined whether PLD1 and two of its most important regulators, RhoA and PKCα, were present in CELMs and were associ-

Table 1
Effect of mitogens on PLD activity in CELMs

Treatment	PLD activity [^{3}H]PtdEtOH/[^{3}H]PtdCho
None	2.2 ± 0.4
BK, 1 μM	3.8 ± 0.2*
PMA, 100 nM	4.8 ± 0.6*
Thrombin, 0.2 U/ml	3.9 ± 0.3*
SPP, 1 μM	2.7 ± 0.3

Confluent [^{3}H]glycerol labeled myotubes were stimulated with the indicated concentration of BK, PMA, thrombin or SPP for 1 min in presence of 2% ethanol. Cells were scraped in MSB and low density membranes prepared on sucrose gradient as described in Section 2. Fractions 2–4 were pooled and the formation of [^{3}H]PtdEtOH was measured as described in Section 2. Data are mean ± S.E.M. of at least three separated experiments performed in duplicate (*$P < 0.05$).

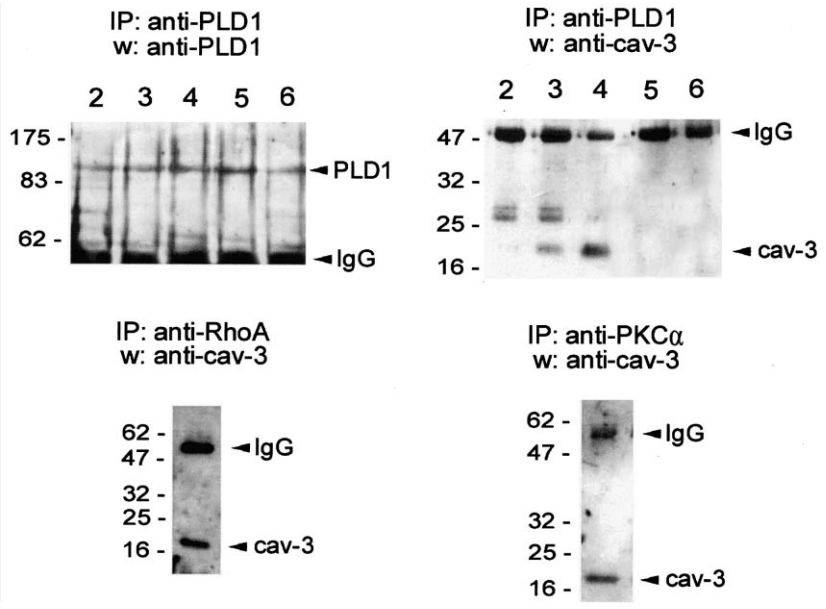

Fig. 2. Coimmunoprecipitation of cav-3 with PLD1 or RhoA or PKCα in CELMs from C2C12 myotubes. Single or pooled (2–4) fractions from the sucrose density gradient were used for immunoprecipitation with polyclonal anti-PLD1 antibodies or mouse monoclonal anti-RhoA or anti-PKCα antibodies. The immunoprecipitated (IP) complexes were processed as described in Section 2 and analyzed by SDS–PAGE using 8 or 14% polyacrylamide gels. PLD1 and cav-3 were immunodetected (w) using specific antibodies.

ated with cav-3. Detection of PLD1 by Western analysis was unsuccessful and required protein immunoprecipitation: PLD1 was widely distributed throughout the sucrose density fractions, being however detectable also in CELMs fractions (2–4). In Fig. 2 the selective immunoprecipitation of cav-3 by the addition of anti-PLD1 or anti-RhoA or anti-PKCα to CELMs is shown. Two bands at approximately 26 and 28 kDa, slightly higher than the molecular mass of cav-3 (18 kDa), were also specifically recognized in the immunocomplexes with anti-PLD1 by anti-cav-3 antibodies (fractions 2 and 3), suggesting that PLD1, besides interaction with native cav-3, was associated also with a higher molecular weight form of the scaffold protein, possibly resulting from a covalent modification. Any attempt to detect the PLD2-cav-3 complex in CELMs failed.

The distribution of PLD activity in the sucrose gradient fractions was then examined utilizing an in vivo assay system, described in Section 2, based on the PLD-catalyzed transphosphatidylation reaction. As shown in Fig. 3, all fractions exhibited PLD activity and two peaks were found corresponding to fractions 2–4 and fractions 8 and 9. Notably, no significant difference in the levels of [^{3}H]glycerol-labeled major phospholipid species between fraction 3 and fraction 8 was observed (Fig. 3, inset). Remarkably, the enzymatic activity present in CELMs (fractions 2–4) comprised 37% ± 1.1 ($n = 8$) of total cellular PLD activity and it was characterized by the highest specific activity. The similar observed pattern of distribution of PLD1-cav-3 immunocomplex and the PLD product [^{3}H]PtdPro in CELMs suggested that PLD activity was, at least in part, due to PLD1.

As shown in previous studies [10,11], total PLD activity in C2C12 myoblasts is rapidly stimulated by mitogens such as BK, thrombin and SPP. These observations, together with the results described above, led us to investigate whether also CELMs-associated PLD activity in myotubes was regulated by these agonists. Treatment with 1 μM BK, 100 nM PMA or 0.2 U/ml thrombin for 1 min induced a significant increase in enzymatic activity, while SPP was ineffective (Table 1).

The possible mechanisms involved in the stimulation of PLD by BK in CELMs were then investigated. Analysis of immunoprecipitates obtained from CELMs purified from un-

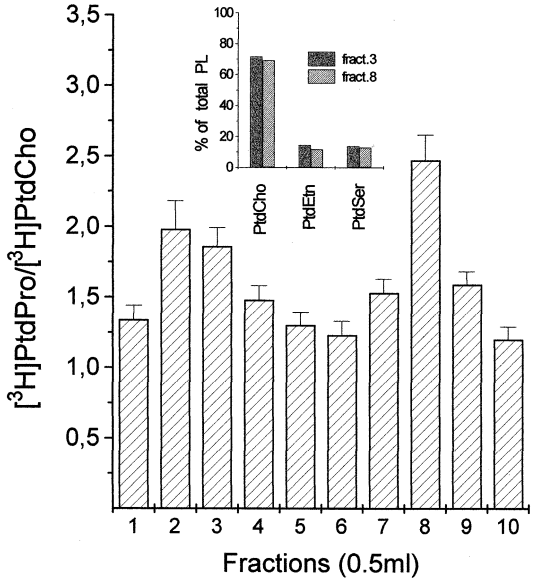

Fig. 3. Basal PLD activity distribution in sucrose gradient fractions from C2C12 myotubes. Fractions from the discontinuous sucrose density gradient, prepared from unstimulated myotubes were analyzed for basal PLD activity as described in Section 2. [^{3}H]PtdPro was normalized to [^{3}H]PtdCho in the TLC. Data are mean ± S.E.M. of four separated experiments. Inset: Comparison of labeled PtdCho, phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEtn) levels in sucrose gradient fractions 3 and 8. Phospholipid (PL) composition was determined as described in Section 2. A representative experiment of those from three similar experiments is reported.

stimulated and BK-stimulated myotubes indicates that the level of PLD1 associated to cav-3 did not change following agonist treatment for 1 min (Fig. 4) or 5 min (data not shown). The possible redistribution in CELMs of the monomeric GTP-binding protein RhoA, known to act as direct activator of PLD1 [8,9,19], was then examined. As shown in Fig. 5A, addition of anti-PLD1 antibodies to fractions 2–4 resulted in the selective immunoprecipitation of RhoA, indicating that the two proteins directly interact in vivo in CELMs. Interestingly, association of RhoA with cav-3 increased in BK-treated cells (Fig. 5B). All these findings support the possible involvement of RhoA in the BK-induced PLD regulation. Given that in caveolae obtained from rat fibroblasts PKCα has been reported to participate in PLD1 regulation [13], we verified whether it similarly acted in BK-stimulated PLD localized in muscle CELMs. Notably, treatment of myotubes with 250 nM Gö6976, specific inhibitor of PKCα in these cells [11,18], 30 min prior to treatment with BK, significantly reduced agonist-stimulated PLD activity (73% ± 8 versus 38% ± 5; $n = 3$) indicating that CELM-associated PLD activity is, at least in part, regulated by PKCα.

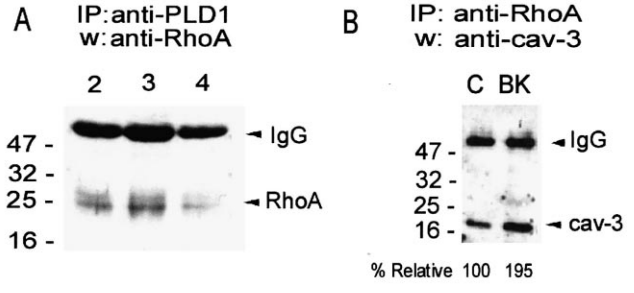

Fig. 4. Effect of BK on PLD1 association with cav-3 in CELMs from C2C12 myotubes. Cav-3-enriched membrane fractions (2–4) obtained from myotubes stimulated or not with 1 μM BK for 1 min were incubated with anti-PLD1 antibodies and immunoprecipitation was performed as described in Section 2. A blot representative of three similar experiments is presented. Band intensity is reported as percentage relative to control.

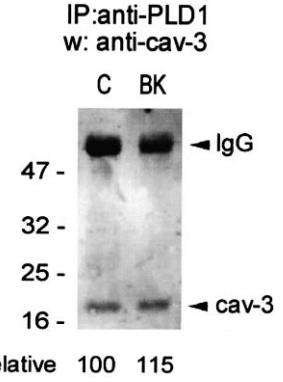

Fig. 5. Association of PLD1 with RhoA (A) and effect of BK on RhoA-cav-3 immunocomplex (B) in CELMs. A: Immunoprecipitation with anti-PLD1 antibodies was performed as described in Fig. 2 and anti-RhoA antibodies were used to specifically detect the protein. B: Cav-3-enriched membrane fractions obtained from myotubes stimulated or not with 1 μM BK for 1 min were incubated with anti-RhoA antibodies and immunoprecipitation experiment performed as described in Section 2. Cav-3 was detected using mouse monoclonal anti-cav-3 antibodies. A blot representative of three similar experiments is presented. Band intensity is reported as percentage relative to control.

4. Discussion

Experimental evidence for the localization of PLD in caveolae is limited: in Kim et al. [13] it has been shown that cav-1 coimmunoprecipitates with PLD1 in fibroblasts and, based on in vitro experiments, a modulatory role of cav-1 on PLD activity has been suggested [13]. Similar functional interaction between PLD and cav-1 in human keratinocytes was reported also by Czamy et al., although exclusively PLD2 was found in caveolar microdomains [12,14].

Skeletal muscle caveolae have been proposed to exert a fundamental role in the organization of cytoskeletal network and are characterized by the specific expression of cav-3. So far only nitric oxide synthase (neuronal and inducible forms) and phosphofructokinase M enzymatic activities have been found to be bound and regulated by cav-3 in skeletal muscle [20–22].

Here we provide experimental data for the localization of PLD1 in myotube CELMs and its association to cav-3. A significant amount of basal PLD activity was detectable in CELMs, however PLD activity and PLD1 distribution in the low density gradient fractions did not exactly coincide. Although this finding could be attributable to an at least partial redistribution of lipids during the membrane purification procedure, alternative explanations may exist: PLD1 in the various gradient fractions could display a different activation status as consequence of differential distribution of PLD cofactor (PIP2) or PLD regulators (PKC isoenzymes, Rho proteins, ARFs) required to render active otherwise unfunctional PLD1; in addition at present it cannot be ruled out that PLD2 is also presenting low density membranes and participates to the bulk of PLD activity detected in CELMs. Interestingly, although the contribution of caveolae to clathrin-independent endocytosis is unclear [2], the observed PLD activity in CELMs suggests that the enzyme, known to have a role in vesicle trafficking, could participate in the physiological maintaining of caveolae, which could be exerted by PtdOH itself or by another PtdOH-derived lipid molecule. This hypothesis is supported by a recent study in which the PtdCho-specific PLD/PtdOH phosphohydrolase pathway has been shown to be active in cav-1-enriched domains in fibroblasts [23], and proposed to regulate the selective environment for the actions of enzymes involved in signaling.

Remarkably, CELMs associated-PLD activity in myotubes appears to be rapidly stimulated by PMA, a direct activator of PKC and physiological agonists, such as BK and thrombin. This finding suggests that PLD activity present in caveolar domains may also play an important role in cell signaling, modulating the lipid composition and physical factors operating in these compartments in response to extracellular stimuli.

Cell stimulation with BK did not increase the association of PLD1 to cav-3 in CELMs suggesting that, upon agonist challenge, PLD1 did not redistribute to these microdomains and the enzyme could be regulated in situ either by selective translocation of one or more of its regulators or by activation of resident population of regulators. Intriguingly, a direct interaction between PLD1 and RhoA does exist in CELMs and the observation that BK increased the association of RhoA to cav-3, supports a role for the recruitment of the cytosolic monomeric GTPase in the mechanism of PLD stimulation by BK in this compartment. The relocalization of endogenous

RhoA induced by platelet-derived growth factor and lysophosphatidic acid in caveolae was reported also in rat-1 fibroblasts [24].

Moreover, the observations that PMA significantly increased CELMs-associated PLD activity and that BK-stimulated PLD activity was significantly reduced by treatment of myotubes with Gö6976, indicate that in agreement with what observed in rat fibroblasts [13] PKCα acts as regulator for caveolar PLD in C2C12 myotubes.

In conclusion, native PLD was identified in muscle cav-3-enriched membranes with a possible role in the physiological maintaining of caveolar microdomains; the enzymatic activity appears to be regulated by agonists acting through seven transmembrane receptors with a mechanism independent from enzyme recruitment in CELMs. There is growing evidence that altered levels of cav-3 and changes in the number and size of caveolae take place in the plasma membrane from muscular dystrophy patients [6,25]; in this connection it will be worthy to investigate whether any impairment in PLD membrane microdomain location and signaling occurs in cultured cells from patients affected by this genetic disease.

Acknowledgements: We thank Dr. S. Bourgoin (Centre de Recherche du CHUL Rhumatologie et Immunologie, Ste-Foy, Que., Canada) for kindly providing PLD1 antiserum. This work was supported by the Italian Telethon Foundation (Grant number 1086 to P.B.) and Italian MURST (COFIN 99).

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