Permissive role of protein kinase Cα but not protein kinase Cδ in sphingosine 1-phosphate-induced RhoA activation in C2C12 myoblasts

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

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

Received 1 August 2000; accepted 1 September 2000

Edited by Julio Celis

Abstract Rho GTPases participate in various important signaling pathways and have been implicated in myogenic differentiation. Here the first evidence is provided that in C2C12 myoblasts sphingosine 1-phosphate (SPP) rapidly and transiently induced membrane association of RhoA in a pertussis toxin-insensitive manner. The bioactive lipid preferentially relocalized the GTPase to Golgi-enriched membrane. Translocation of RhoA was abolished by inhibition or down-regulation of protein kinase C (PKC). Notably, treatment with Gö6976, an inhibitor of conventional PKCs, which selectively blocked PKCα in these cells, prevented SPP-induced RhoA translocation. Conversely rottlerin, a selective inhibitor of PKCδ, was without effect, demonstrating that SPP signaling to RhoA involves PKCα but not PKCδ activation. This novel functional relationship between the two proteins may have a role in SPP-mediated regulation of downstream effectors.

Key words: Sphingosine 1-phosphate; RhoA; C2C12 myoblast; Protein kinase Cα

1. Introduction

The Rho subfamily of small GTP binding proteins, which includes Rho, Rac and Cdc42, has been implicated in the regulation of a number of biological processes including cell motility, cell adhesion, cytokinesis, cell morphology and cell growth [1,2]. Rho GTPases act as molecular switches cycling between an inactive GDP-bound state and an active GTP-bound state. The two alternative protein states also differ in intracellular localization: upon activation the cytosolic GDP-bound form is recruited to the membrane. The major function of Rho GTPases is to regulate the assembly and the organization of the actin cytoskeleton [3]. Recent studies have shown the existence of a large number of upstream activators and regulators of Rho proteins as well as the occurrence of a variety of effectors or target molecules able to interact with active Rho proteins to initiate a downstream response [3,4], indicating a high level of complexity in the regulation of Rho protein function.

Several lines of experimental evidence support a role for Rho GTPases in the differentiation of skeletal muscle. Dominant negative forms of Rho family proteins and RhoGDI, a GDP dissociation inhibitor, suppressed transcription of muscle-specific basic helix-loop-helix transcription factors such as myogenin in C2C12 muscle cells [5]. Moreover, in mouse myoblasts RhoA, but not Rac or Cdc42, appears to be required for the expression of MyoD, another key operator in skeletal muscle differentiation [6]. Finally, Rac1 appears to play a critical role in primary avian myoblast differentiation [7].

Sphingosine 1-phosphate (SPP), a serum-borne bioactive lysophospholipid, evokes multiple cellular responses, at least in part, through the interaction with different specific G protein-coupled receptors belonging to the endothelial differentiation gene (Edg) family [8]. In a number of circumstances biological actions elicited by the bioactive lipid rely on cytoskeletal rearrangement and Rho protein activation [9]. Consistent with this, a role for RhoA has been established in SPP-induced invasion of T-cell lymphoma [10], neurite retraction in neuroblastoma cells [11] and fibronectin matrix assembly [12]. Moreover, Rho and Rac have been shown to be coupled to the vascular endothelial cell adherens junction assembly induced by SPP [13]. However, the signaling pathway responsible for SPP-induced Rho GTPase activation has not been clarified so far. In a previous study Edg1, Edg3 and Edg5 receptors were detected in C2C12 myoblasts and SPP was demonstrated to be capable of inducing protein kinase C (PKC) and phospholipase D signaling pathways [14], but no information is presently available about the possible regulation of Rho GTPases by the bioactive lipid in skeletal muscle.

Here we report that in C2C12 myoblasts SPP was capable of activating rapidly RhoA and that inhibition of PKCα but not PKCδ impaired RhoA activation, implying a permissive role for PKCα in the signaling of SPP to RhoA.

2. Materials and methods

2.1. Materials

Mouse skeletal muscle C2C12 cells were from the American Type Culture Collection (ATCC, Manassas, VA, USA). Biochemicals, cell culture reagents and monoclonal antibodies against Golgi p58 were from Sigma (St. Louis, MO, USA). SPP and Gö6976 were from Calbiochem (San Diego, CA, USA); pertussis toxin (PTx) was from List Biological Laboratories (Campbell, CA, USA). Rottlerin was from Alexis Corporation (San Diego, CA, USA). I-block was from Tropix (Bedford, MA, USA). Mouse monoclonal antibodies against RhoA, Rac1 and caveolin-1, polyclonal antibodies against Cdc42 and secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin G1) were from Santa Cruz (Santa Cruz, CA, USA). Mouse monoclonal anti-PKCα and anti-PKCδ antibodies were from Transduction Laboratories (Lexington, KY, USA). Rabbit polyclonal anti-Rab5 and anti-calnexin antibodies were purchased from Stressgene Biotech (Victoria, BC, Canada).

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

Abbreviations: SPP, sphingosine 1-phosphate; Edg, endothelial differentiation gene; PKC, protein kinase C; TTBS, Tris-buffered saline containing 0.1% Tween-20; CELM, caveolin-enriched light membrane; PTx, pertussis toxin

0014-5793/00/$20.00
PII: S0014-5793(00)02039-1

2.2. Muscle cell culture

C2C12 mouse myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, as previously described [15]. The cells were seeded into 100 mm diameter dishes and when 90% confluent they were shifted to DMEM without serum containing 1 mg/ml bovine serum albumin for 24 h and then utilized for the experiments.

2.3. Cellular fractionation

Medium of control and agonist-treated C2C12 cells was removed and the cells were washed twice with ice-cold phosphate-buffered saline, scraped, and collected by centrifugation ($1000 \times g$). The cells were dispersed in a buffer solution containing 10 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM EDTA, 250 mM sucrose, 5 mM NaN_3, and protease inhibitors (1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.3 μM aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin) and disrupted in a Dounce homogenizer (120 strokes). Lysates were centrifuged (7 min, $500 \times g$) and the resulting supernatant was centrifuged again at $200\,000 \times g$ for 1 h to separate cytosolic and total particulate fractions. Protein content was quantified according to the Coomassie blue procedure using a commercially available kit (Bio-Rad).

2.4. Sucrose density fractionation

Control and SPP-treated C2C12 cells, collected as described above, were dispersed in a buffer solution containing 10 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM NaN_3, and protease inhibitors (1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.3 μM aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin) and disrupted in a Dounce homogenizer (120 strokes). Lysates were spun (7 min, $500 \times g$) and the resulting supernatant was centrifuged for 30 min at $200\,000 \times g$ to prepare Golgi. The pellet was gently dispersed in the same buffer containing 1.4 M sucrose. A sample (800 μl) was transferred to the bottom of a centrifuge tube that contained layers of 0.25, 0.85, 1.15 and 1.4 M sucrose in the same buffer without sucrose and protease inhibitors. After centrifugation (19 h, $200\,000 \times g$, Beckman SW50 rotor) the subcellular fractions localized at the interfaces of the sucrose layers were collected and used for Western analysis.

2.5. Western blot analysis

Proteins (30 μg) from cellular fractions of unstimulated and agonist-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 0.5% I-block. Hybridization for 1 h at room temperature 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 enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

2.6. Presentation of data

A blot representative of at least three similar experiments is presented. To test the statistical significance band intensities were measured using NIH Image and the control corresponding to no addition and time zero was set at 100% in each individual experiment.

3. Results

3.1. Effect of SPP on Rho GTPase translocation

To evaluate the possible activation of monomeric G proteins by SPP, the subcellular redistribution of RhoA, Cdc42 and Rac1 induced by the agonist was examined. To this end translocation from cytosol to membranes, known to be coupled with protein activation by replacement of bound GDP with GTP, was monitored. Incubation of cells for 30 s

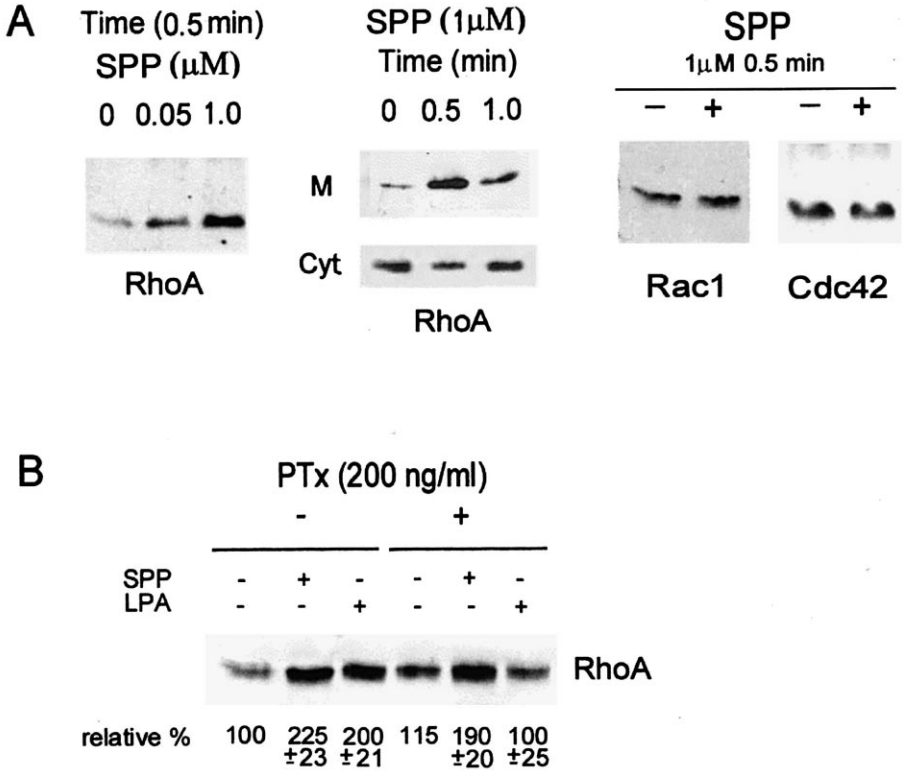

Fig. 1. Effect of SPP on Rho GTPase membrane translocation. A: Serum-starved confluent myoblasts were incubated without or with the indicated concentration of SPP for 30 s or with 1 μM SPP for the indicated time. Samples (30 μg protein) from cytosol (Cyt) or membrane fraction (M) of control or SPP-stimulated cells were separated by SDS–PAGE in 14% gel and transferred to nitrocellulose as described in Section 2. A blot representative of three similar experiments is presented. B: Confluent myoblasts were incubated in serum-free medium without or with 200 ng/ml PTx for 16 h before stimulation with 1 μM SPP or 5 μM lysophosphatidic acid for 30 s. Samples (30 μg protein) from membrane fraction were electrophoresed and transferred to nitrocellulose. A blot representative of three similar experiments is presented and band intensity is reported as percentage (mean ± S.D.) relative to control (no addition, 100%).

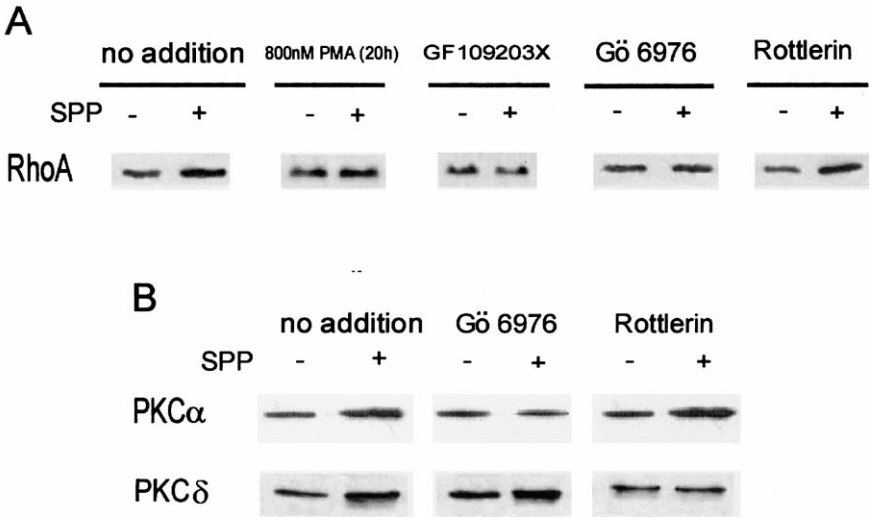

Fig. 2. Effect of inhibition of PKC on SPP-induced RhoA (A) and PKCα or PKCδ (B) membrane translocation. Upper panel: Serum-starved myoblasts were incubated with vehicle (0.05% dimethylsulfoxide), 10 μM GF109203X, 0.5 μM Gö6976 or 5 μM rottlerin for 30 min or incubated for 20 h with 800 nM phorbol 12-myristate 13-acetate to down-regulate PKC prior to stimulation with 1 μM SPP for 30 s. Proteins (25 μg) from membrane fraction were separated by SDS–PAGE and transferred to nitrocellulose. Blot is representative of those from at least three similar experiments. Lower panel: Densitometric analysis. Band intensity is reported as percentage (mean ± S.D.) relative to control (no addition, 100%). *Statistically significant ($P < 0.01$).

with SPP resulted in a transient translocation of RhoA from cytosol to membrane fraction which was concentration-dependent (Fig. 1A). A significant increase in membrane-associated RhoA was observed with 50 nM SPP, the maximal effect being reached with 1 μM. RhoA translocation was maximal within 1 min. Conversely, no increase in membrane association of Rac1 or Cdc42 was observed at 30 s (Fig. 1A). Altogether, these findings indicated that RhoA, but not other monomeric G proteins, was involved in early SPP-induced signaling pathways. In addition, at 2.5 min a significant association of Rac1 with the membrane fraction could be detected (data not shown), suggesting a possible involvement of this protein in later SPP-mediated events. Given that the various SPP-specific Edg receptors are coupled to multiple heterotrimeric G proteins, the possible involvement of Gi/Go in the signaling pathway leading to RhoA activation was investigated. In pilot experiments designed to optimize the modification of Gi/Go proteins by PTx treatment (200 ng/ml, 16 h), the cells were incubated with [^{32}P]nicotinamide adenine dinucleotide and activated toxin. In membranes from cells treated with toxin, no further labeling of Gi/Go was observed (data not shown). Membrane translocation of RhoA induced by the bioactive lipid was not inhibited in myoblasts treated with PTx. In contrast, the toxin completely prevented RhoA membrane translocation induced by lysophosphatidic acid, a lipid molecule similar in structure to SPP (Fig. 1B).

3.2. Inhibition of PKCα impairs SPP-induced RhoA translocation

In a previous study we demonstrated that SPP rapidly activated PKCα, PKCδ and PKCλ [14]. To investigate whether the PKC signaling pathway may interfere with SPP-induced RhoA activation, PKC activity was inhibited by prolonged exposure to phorbol 12-myristate 13-acetate (800 nM, 20 h) or by treatment with GF109203X (10 μM), an inhibitor of all PKCs. As shown in Fig. 2A, SPP-induced RhoA translocation was abolished in PKC-down-regulated myoblasts as well as when all PKC isoforms were blocked. To investigate this point deeply, similar experiments were performed in the presence of Gö6976 (0.5 μM), a selective inhibitor of conventional PKCs, or rottlerin (5 μM), a specific inhibitor of the novel isoform PKCδ. As expected, the two inhibitors blocked membrane recruitment of PKCα or PKCδ, respectively, promoted by SPP in C2C12 cells (Fig. 2B). Remarkably, Gö6976 prevented the SPP-induced increase in membrane-associated RhoA whereas rottlerin was inefficacious (Fig. 2A).

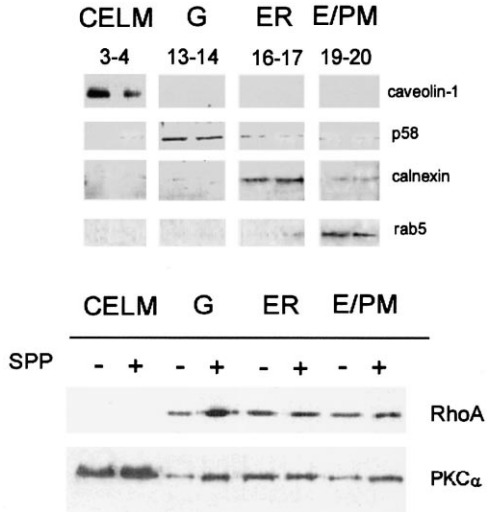

Fig. 3. Subcellular distribution of SPP-activated RhoA and PKCα. Cell lysates were fractionated as described in Section 2. Samples (25 μg) of membrane fractions (CELM; fractions 3–4), Golgi-enriched membranes (G; fractions 13–14), endoplasmic reticulum (ER; fractions 16–17), endosomes/plasma membranes (C/PM; fractions 19–20) were separated by SDS–PAGE, transferred to nitrocellulose and analyzed for the presence of marker proteins. Membrane fractions prepared from myoblasts incubated without or with 1 μM SPP for 30 s and positive for the specific compartment marker were collected and analyzed for the expression of RhoA and PKCα. A blot representative of three similar experiments is presented.

3.3. Subcellular distribution of SPP-activated RhoA and PKCα

Next the subcellular membrane distribution of RhoA and PKCα in control and SPP-stimulated C2C12 cells was examined. For this purpose cell lysates were fractionated on a discontinuous sucrose density gradient and fractions analyzed for the presence of specific subcellular markers: fractions 3–4 were identified as caveolin-enriched light membrane (CELM) fractions, by the detection of caveolin-1; fractions 13–14 corresponded to Golgi-enriched membranes based on the presence of p58; fractions 16–17, which were enriched in calnexin, preferentially contained membranes of endoplasmic reticulum; and fractions 19–20 corresponded to endosomes/plasma membranes based on the enrichment in Rab5. As shown in Fig. 3, RhoA was detectable to a similar extent in all examined subcellular compartments, except for the caveolar microdomains. Following SPP stimulation activated RhoA preferentially associated with Golgi while a slight increase could also be detected in endosomes/plasma membranes. Instead, PKCα was ubiquitous in the examined fractions and was highly enriched in CELMs; in stimulated cells the kinase translocated in all compartments except endoplasmic reticulum.

4. Discussion

Evidence is emerging that RhoA GTPase plays a critical role in the signaling of SPP, although so far its involvement in the transduction machinery activated by the bioactive lipid has been demonstrated almost exclusively on the basis of *Clostridium botulinum* C3 exoenzyme sensitivity of biological processes which require active RhoA [12,13,16,17]. In the present report a rapid and transient translocation of RhoA is shown to occur following stimulation of C2C12 myoblasts with SPP. Interestingly, a detailed analysis of subcellular distribution revealed that RhoA was relocalized by SPP mainly to Golgi-enriched membranes, suggesting that agonist-activated protein acts in highly defined subcellular compartments. Interestingly, the short-term translocation of RhoA was not accompanied by membrane recruitment of other Rho GTPases, although Rac1 was activated at a more prolonged time of agonist treatment. A hierarchical cascade linking Rho, Rac and Cdc42 was initially described in cultured fibroblasts [2] and a role for Rac in the activation of Rho has been proposed [18]. Here the occurrence of such a kind of regulation can be ruled out taking into account the more rapid time course of RhoA activation in comparison with that of Rac1.

In agreement with other studies [13,16] SPP-induced RhoA translocation was insensitive to PTx treatment, suggesting that the signaling pathway involves heterotrimeric $G_{12/13}$ and/or Gq protein. However, given that Edg3 and Edg5 [16,19] as well as Edg1 [20] have been implicated in Rho-dependent pathways and all these receptor subtypes are expressed in C2C12 myoblasts [14] it appears difficult to attribute the observed increased RhoA translocation to one or more Edgs. The here reported activation of RhoA by SPP, in view of the importance of RhoA functioning for myogenic differentiation [5–7], suggests that the bioactive lipid is responsible for key morphogenetic changes in these cells.

A major finding of the present study is represented by the evidence for a functional relationship between a specific PKC isoform and RhoA translocation in SPP-stimulated cells; indeed, catalytic activity of conventional PKCs but not PKCδ was required for the ligand-induced membrane association of the GTPase protein. Given that in C2C12 myoblasts [14] and in rat muscle [21] PKCβ was undetectable, the inhibition of conventional PKCs obtained in the presence of Gö6976 implied a selective blocking of PKCα. Therefore on this basis it is possible to ascribe specifically to PKCα a permissive role in SPP signaling leading to RhoA activation. The mechanism by which PKCα interferes with the agonist-mediated membrane recruitment of RhoA is at present unknown, however several hypotheses can be advanced. One theoretical possibility is represented by a direct interrelationship between these two molecules. Evidence for this stems from studies which showed that Pkc1, the yeast homologue of classical mammalian PKC, coimmunoprecipitated with Rho1, corresponding to RhoA in mammalian systems [22]. The broad subcellular distribution of PKCα and its relocalization after SPP challenge of C2C12 myoblasts do not exclude that a direct interaction between the two proteins may take place. Alternatively, PKCα could indirectly regulate RhoA activation status and its cellular distribution, for instance through the phosphorylation of Rho-specific nucleotide exchange factor. The identification of PKCα as an upstream regulator of RhoA translocation induced by SPP, however, does not necessarily imply that PKC activation can independently activate RhoA, because other Edg receptor-mediated signals, in addition to PKC activation, may be needed to relocalize RhoA.

This study represents the first evidence that SPP signaling to RhoA specifically involves PKCα activation. This finding may be physiologically relevant in view of the key role played by PKC and RhoA in the control of crucial biological processes. Consistently, RhoA has been reported to participate to stimulation of SPP-induced phospholipase D activity [23] and similarly PKC is known to act as a positive regulator in the SPP-mediated enzyme activation [14,24]. Another possible common target of the action of RhoA and PKC could be represented by immediate early genes which are induced through the activation of serum response elements [25]. Therefore, it will be worth investigating whether such a functional relationship between RhoA and PKC has a role in SPP-mediated regulation of downstream effectors.

Acknowledgements: This work was supported by funds from the Italian Telethon Foundation (Grant 1086 to P.B.) and Cofinanziamento Ministero dell'Universitá e della Ricerca Scientifica e Tecnologica (MURST) Progetti di Interesse Nazionale (PRIN) 1999 (to P.B.).

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