Sphingosine 1-phosphate evokes calcium signals in C₂C₁₂ myoblasts via Edg3 and Edg5 receptors

Elisabetta MEACCI*, Francesca CENCETTI*, Lucia FORMIGLI†, Roberta SQUECCO‡, Chiara DONATI*, Bruno TIRIBILLI§, Franco QUERCIOLO§, Sandra ZECCHI ORLANDINI†, Fabio FRANCINI‡ and Paola BRUNI*¹

*Dipartimento di Scienze Biochimiche, Università degli Studi di Firenze, Viale G. B. Morgagni 50, 50134 Florence, Italy, †Dipartimento di Anatomia, Istologia e Medicina Legale, Università degli Studi di Firenze, Viale G. B. Morgagni 85, 50134 Florence, Italy, ‡Dipartimento di Scienze Fisiologiche, Università degli Studi di Firenze, Viale G. B. Morgagni 63, 50134 Florence, Italy, and §Istituto Nazionale di Ottica Applicata, Laboratorio di Biofotonica, Viale G. B. Morgagni 85, 50134 Florence, Italy

Sphingosine 1-phosphate (SPP) is a bioactive lipid that exerts multiple biological effects in a large variety of cell types, acting as either an intracellular messenger or an extracellular ligand coupled to Edg-family receptors (where Edg stands for endothelial differentiation gene). Here we report that in C₂C₁₂ myoblasts SPP elicited significant Ca²⁺ mobilization. Analysis of the process using a confocal laser-scanning microscope showed that the Ca²⁺ response occurred in a high percentage of cells, despite variations in amplitude and kinetics. Quantitative analysis of SPP-induced Ca²⁺ transients performed with a spectrofluorimeter showed that the rise in Ca²⁺ was strictly dependent on availability of extracellular Ca²⁺. Cell treatment with pertussis toxin partially prevented the Ca²⁺ response induced by SPP, indicating that G_i-coupled-receptors were involved. Indeed, SPP action was shown to be mediated by agonist-specific Edg receptors. In particular, suramin, an antagonist of the SPP-

specific receptor Edg3, as well as down-regulation of Edg3 by cell transfection with antisense oligodeoxyribonucleotides (ODN), significantly reduced agonist-mediated Ca²⁺ mobilization. Moreover, an antisense ODN designed to inhibit Edg5 expression also decreased the SPP-induced rise in Ca²⁺, although to a lesser extent than that observed by inhibiting Edg3. On the contrary, the SPP response was unaffected in myoblasts loaded with antisense ODN specific for Edg1. Remarkably, the concomitant inhibition of Edg3 and Edg5 receptors abolished the SPP-induced Ca²⁺ increase, supporting the notion that Ca²⁺ mobilization in C₂C₁₂ cells induced by SPP is a receptor-mediated process that involves Edg3 and Edg5, but not Edg1.

Key words: calcium influx, calcium increase, Edg receptor, skeletal muscle.

INTRODUCTION

Studies performed over the last decade have identified sphingosine 1-phosphate (SPP) as a member of a new class of lipid mediators, able to elicit a number of different biological effects, including cell proliferation and differentiation, suppression of apoptosis and cytoskeleton-dependent responses such as contraction, adhesion and chemotaxis [1–3]. Although there is substantial agreement with regard to the variety of biological effects of SPP, the mode of action of this bioactive lipid is still debated. In particular, it is presently unclear whether various effects of SPP are due to its role as an extracellular mediator that signals via cell-surface receptors or whether it acts intracellularly as a second messenger. Even though these possibilities are not necessarily mutually exclusive, it is, however, important to define specific biological responses regulated by SPP as an extracellular mediator and those regulated by its intracellular action. In earlier studies, SPP was reported to act directly on the internal Ca²⁺ pool, inducing Ca²⁺ mobilization in permeabilized cells or in the purified endoplasmic reticulum [4,5]. Since then, a role for the bioactive lipid as a second messenger responsible for Ca²⁺ mobilization induced by epidermal growth factor [6], antigen FcεRI receptor [7] and some G-protein-coupled receptors (GPCRs) [8] has been proposed. However, the recent identification of surface receptors specific for SPP and initial studies of

their signal transduction have provided evidence that many intracellular signalling pathways can be triggered by the bioactive lipid as an extracellular ligand. The first high-affinity receptor for SPP to be identified was Edg1 (where Edg stands for endothelial differentiation gene), an integral membrane protein belonging to the family of GPCRs [9]. In different studies performed using Edg1-transfected cell lines, the receptor was found to be coupled to a multitude of downstream signalling cascades via the pertussis toxin (PTx)-sensitive heterotrimeric G-proteins G_i and G_o [10,11]. However, as regards the pattern of signalling pathways coupled to Edg1, conflicting results have emerged. Indeed, whereas Edg1 expression in HEL or Chinese hamster ovary cells was correlated with SPP-induced Ca²⁺ mobilization [10], when the protein was expressed heterologously in HEK-293 cells it was not found to be coupled to intracellular Ca²⁺ increase [12]. The subsequent identification of additional members of the Edg family as specific SPP receptors and the initial characterization of their signal transduction have provided evidence for the occurrence of their coupling to similar signalling pathways in a redundant manner. In particular, Edg3- and Edg5-transfected cells also exhibited SPP-induced Ca²⁺ mobilization [13,14], but, probably due to specific cellular settings, their effect was found to be sensitive [13,14] or largely insensitive [15] to PTx. When Edg3 or Edg5 mRNA was microinjected into *Xenopus* oocytes, SPP evoked an increase in Ca²⁺ via a G_q-dependent mechanism, although both

Abbreviations used: Edg, endothelial differentiation gene; SPP, sphingosine 1-phosphate; GPCR, G-protein-coupled receptor; PTx, pertussis toxin; PKC, protein kinase C; fluo-3/AM, fluo-3 acetoxymethyl ester; fura-2/AM, fura-2 acetoxymethyl ester; ODN, oligodeoxyribonucleotides; PLC, phospholipase C; DMEM, Dulbecco's modified minimal essential medium.

receptors were also shown to couple to G_i [16]. When over-expressed in Chinese hamster ovary cells, Edg6, a lymphoid-tissue-specific receptor, was also found to couple SPP to Ca^{2+} mobilization [17].

Given the extraordinarily complex mechanism of SPP signalling and the tissue-specific expression pattern of its receptors [1–3], understanding of the biological action of SPP and the molecular mechanisms involved can draw on the study of biological responses elicited by SPP in native cellular systems.

Previous studies performed in skeletal-muscle C_2C_{12} cells demonstrated that exogenous SPP increased phospholipase D activity via cell-surface GPCRs [18]. The bioactive lipid was also able to activate conventional and novel isoforms of protein kinase C (PKC) [18] as well as the monomeric GTPase RhoA [19]. However, no information was available on the possible effects of SPP on Ca^{2+} mobilization in these cells. Confocal laser-scanning microscopic analysis of mouse C_2C_{12} skeletal-muscle cells revealed that a high percentage of myoblasts were responsive to SPP, although Ca^{2+} transients varied in amplitude and kinetics. Moreover, the agonist-induced Ca^{2+} increase was found to be a receptor-mediated event and a role for Edg3 and Edg5 was identified.

EXPERIMENTAL

Materials

Biochemicals, cell-culture reagents, suramin, bradykinin, Dulbecco's modified minimal essential medium (DMEM) and fetal bovine serum were purchased from Sigma (Milan, Italy). C_2C_{12} cells were obtained from the A. T. C. C. (Manassas, VA, U.S.A.). Fluo-3 acetoxymethyl ester (fluo-3/AM), fura-2 acetoxymethyl ester (fura-2/AM) and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR, U.S.A.). SPP, Ro 31-8220 and GF109203X were obtained from Calbiochem (San Diego, CA, U.S.A.). U73122 and U73343 were purchased from Alexis Corporation (San Diego, CA, U.S.A.). PTx was from List Biological Laboratories (Campbell, CA, U.S.A.). Lipofectin was from Life Technologies (Paisley, Scotland, U.K.). Phosphothioate oligodeoxyribonucleotides (ODN), corresponding to the translation-initiation regions of Edg1 (5'-AGTGGACACCATAGCTGCTAA-3'), Edg3 (5'-ATGCTGGGTTGCCATGGCTTC-3') and Edg5 (5'-GTATAAGCCGCCCATGGTGGG-3'), were synthesized by MWG Biotech AG (Ebersberg, Germany). Serum from rabbit immunized with the N-terminal region of mouse Edg3 (MATTHAQGHQPVLGNDTLR) was obtained by Neosystem (Strasbourg, France). Anti-rabbit IgG1 conjugated to horseradish peroxidase was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Enhanced chemiluminescence (ECL) reagents and Protein A-Sepharose were obtained from Amersham Bioscience (Uppsala, Sweden). I-block was obtained from Tropix (Bedford, MA, U.S.A.).

Muscle-cell culture

C_2C_{12} cells were maintained in DMEM with 10% fetal calf serum. Myoblasts (2×10^4 cells) were seeded into 100-mm diameter dishes and 90% confluent cells were moved to serum-free DMEM containing 0.1% BSA for 16–24 h before being utilized in experiments.

Imaging of Ca^{2+} transients in mouse myoblasts

A Bio-Rad MRC 1024ES confocal scanning head (Bio-Rad, Hercules, CA, U.S.A.) was coupled to a Nikon TE300 inverted microscope. In all experiments a Nikon Plan Apo 60 $\times$ oil-

immersion objective lens with a numerical aperture of 1.4 was used. The detector pinhole was chosen to obtain an axial (z) resolution of about 1 μ m and an x-y resolution of 0.3 μ m. Illumination was provided by a 15 mW krypton/argon ion laser. The excitation wavelength (488 nm) was selected using interference filters and the emitted fluorescence was collected at wavelengths greater than 515 nm. The fluorescence beam was detected using a photomultiplier tube.

On the day before the experiment, approx. 2×10^4 cells were plated on to a coverslip. Cells were loaded by incubation in serum-free DMEM, containing 0.1% BSA, the membrane-permeant fluorescent Ca^{2+} indicator fluo-3/AM (10 μ M), 0.1% anhydrous DMSO and 0.01% (w/v) Pluronic F-127 as a dispersing agent, for 10 min at room temperature. Then the cells were washed extensively with standard medium and maintained in the same medium to allow complete de-esterification of fluo-3/AM. The cells were placed successively in open slide flow-loading chambers, which were mounted on the stage of the confocal laser-scanning microscope. PBS containing 1 mM $CaCl_2$ was used as a flow perfusate in the presence or absence of SPP. The experiments were performed at room temperature. For time-course experiments, fluorescent digital images were obtained using Time Course Kinetic software (Bio-Rad). Excitation was performed at 488 nm wavelength and emission was monitored using a 515 nm long-pass filter and a high-sensitivity photomultiplier tube. Analysis of images was obtained using the free software Confocal Assistant (Bio-Rad).

Ca^{2+} assays

Ca^{2+} transients were measured in myoblasts loaded with the fluorescent dye fura-2/AM as described in [20]. Briefly, cells were trypsinized, collected by centrifugation (1000 g) and resuspended (7×10^5 cells/ml) in DMEM containing 0.1% BSA, 10 μ M fura-2/AM, 0.01% Pluronic F-127 and 0.1% anhydrous DMSO. The cells were incubated in the dark for 40 min at room temperature and washed twice with PBS containing 1 mM $CaCl_2$.

Excitation was alternated between 340 and 380 nm, with emission being read at 510 nm in a Shimadzu 9000 spectrofluorimeter equipped with a fast-filter device. The signal was calibrated by addition of 1 mM $CaCl_2$ and Triton X-100 to obtain maximal fluorescence. Extracellular Ca^{2+} was chelated with 25 mM EGTA in Tris base to elevate the pH above 8.3 to obtain minimal fluorescence. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated as described by Grynkiewicz et al. [21], using a K_d value of 224 nM for fura-2.

Measurement of [3H]diacylglycerol levels

C_2C_{12} cells were labelled with 5 μ Ci/ml [3H]glycerol for 24 h and then stimulated for 30 s with 1 μ M SPP. Lipid extraction and [3H]diacylglycerol separation by TLC were performed as described in Meacci et al. [22].

ODN transfection

For inhibition of Edg-receptor expression, ODN with non-conserved non-repetitive sequences corresponding to the region of translation initiation of Edg1, Edg3 and Edg5 were utilized. Receptor-specific scrambled ODN were used as a control. C_2C_{12} cells were treated with antisense ODN in the presence of Lipofectin reagent (1 mg/ml). Lipofectin was mixed with ODN (both prepared in DMEM), and complexes were allowed to form at room temperature for 40 min. Then the Lipofectin-ODN complexes were added with gentle agitation to C_2C_{12} cells plated

Figure 1 Spatio-temporal organization of Ca^{2+} increase induced by SPP in C_2C_{12} myoblasts

(A) The set of successive images represents the time course of Ca^{2+} increase induced by $1 \mu\text{M}$ SPP. The images were obtained at the indicated times. (B) A differential interference contrast image indicates the cells utilized for the analysis reported in (C). (C) Time course of fluorescence emission following SPP stimulation in the analysed cells indicated in (B). In (A and B) the side of each square = $225 \mu\text{m}$.

in 100 mm dishes. The final concentration of ODN in the incubation mixture was 20 μM . The treatment was allowed to proceed for 16 h. Following incubation, 6 ml of the complete growth medium (with serum) was added and the cells were incubated for 32 h.

Western-blot analysis

Aliquots (30 μg) of total membranes prepared as described previously [18] were separated by SDS/PAGE. Proteins were transferred to nitrocellulose membranes and incubated overnight in Tris-buffered saline containing 0.1% Tween (TTBS) and 0.2% I-block. Membranes were incubated for 1 h with anti-Edg3 antiserum purified previously by Protein A-Sepharose affinity chromatography, washed with TTBS and exposed to horseradish peroxidase-conjugated goat anti-rabbit IgG. The membranes were covered with ECL detection reagents. Autoradiographs were obtained by exposing Kodak X-Omat film to membranes. Quantitative analysis of the band corresponding to Edg3 was performed using Imaging and Analysis Software (Bio-Rad).

Data presentation

All experiments were performed in duplicate. The results of multiple observations are presented as means $\pm$ S.E.M. from at least three separate experiments unless otherwise stated. The data were analysed by Student's *t* test and $P < 0.05$ was considered significant.

RESULTS

SPP increases intracellular Ca^{2+} levels in C_2C_{12} myoblasts

The ability of SPP to elicit Ca^{2+} signals in C_2C_{12} myoblasts was investigated using a confocal laser-scanning microscope. The addition of 1 μM SPP triggered a transient increase of intracellular Ca^{2+} in 60% of cells ($n = 40$). In 40% of responsive

Figure 2 Dose-dependence of Ca^{2+} response to SPP in C_2C_{12} myoblasts

Trypsinized cells ($0.8 \times 10^6/\text{ml}$), loaded with fura-2/AM, were incubated with the indicated concentrations of SPP. Intracellular $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$) was calculated as described in the Experimental section. Data are means $\pm$ S.E.M. from three sets of experiments performed in duplicate. * $P < 0.05$, statistically significant.

cells ($n = 10$), the Ca^{2+} wave started in a localized region on the edge of the cell and it also propagated into the nuclei. Then the Ca^{2+} level decreased gradually and homogeneously throughout the cell. A representative set of images illustrating SPP-evoked Ca^{2+} transients is shown in Figure 1(A). Interestingly, the time courses of six cells (Figure 1B) showed differences in the duration of the Ca^{2+} signal, ranging from 30 to 70 s after SPP treatment was detected (Figure 1C). Moreover, from the same image it emerged that the examined cells were also heterogeneous in their magnitude of Ca^{2+} increase.

Subsequent experiments were performed by measuring the Ca^{2+} response with a spectrophotofluorimeter in approx. 1×10^6 cells loaded with 10 μM fura-2/AM and maintained in suspension. Under these experimental conditions the average duration of a Ca^{2+} transient was 50 s, which is comparable with the time course of individual cells reported in Figure 1(C).

As shown in Figure 2, SPP increased the intracellular Ca^{2+} concentration in a dose-dependent manner, with a maximal effect at 100 nM and a half-maximally effective concentration (EC_{50}) of approx. 35 nM.

Effect of extracellular Ca^{2+} chelation, PTx treatment and inhibition of phospholipase C (PLC) and PKC on the SPP-induced Ca^{2+} response

Next we studied the signalling mechanisms involved in the enhancement of intracellular Ca^{2+} induced by 1 μM SPP. Notably, in the absence of extracellular Ca^{2+} (0 mM Ca^{2+} plus 3 mM EGTA) the bioactive lipid was unable to affect Ca^{2+} levels in C_2C_{12} myoblasts, indicating that Ca^{2+} increase induced by SPP was strictly dependent on the ion influx through plasma-membrane Ca^{2+} channels (Figure 3A). Since the SPP-mediated Ca^{2+} increase is reportedly inhibited by PTx [1,2], which ADP-ribosylates and inactivates G_i , the effect of the bacterial toxin in myoblasts was evaluated. Treatment with PTx (100 ng/ml) for 16 h reduced the SPP-induced increase of Ca^{2+} by $40 \pm 5\%$ ($n = 6$; $P < 0.05$), supporting the involvement of G_i -coupled receptors (Figure 3A).

Given that in several sets of circumstances PLC activation has been reported as a main upstream event responsible for SPP-induced Ca^{2+} increase [10,15,17], this possibility was examined. As shown in Figure 3(B), treatment with the PLC inhibitor U73122 (10 μM) [23], but not with the inactive analogue U73343 (10 μM), 15 min prior to incubation with 1 μM SPP, reduced the SPP-evoked Ca^{2+} response significantly ($40 \pm 7\%$, $n = 4$; $P < 0.05$). Under these experimental conditions, treatment of C_2C_{12} cells with 1 μM SPP for 30 s induced a significant increase in $[\text{^3H}]$ diacylglycerol levels that was reduced markedly by 10 μM U73122 (inhibition of $80 \pm 9.5\%$, $n = 3$; $P < 0.05$).

Interestingly, as shown in Figure 3(C), a moderate but significant reduction in the efficacy of SPP on Ca^{2+} levels was observed when cells were incubated with the PKC inhibitors Ro 31-8220 (10 μM) and GF109203X (10 μM) prior to agonist addition (inhibition of $32 \pm 5\%$, $n = 4$, and $28 \pm 2\%$, $n = 3$, respectively; $P < 0.05$ in both cases). This indicated the involvement of PKC in SPP signalling leading to an increase in Ca^{2+} .

Involvement of Edg receptors in the SPP-induced Ca^{2+} response in myoblasts

In a previous study we reported that Edg1, Edg3 and Edg5 receptors are expressed, at least at the mRNA level, in C_2C_{12} myoblasts [18]. To investigate which Edg-receptor subtype was coupled to Ca^{2+} increase, initial experiments were performed in the presence of suramin, a polycyclic anionic compound reported

Figure 3 Effect of EGTA, PTx, and PLC or PKC inhibitor treatment on SPP-induced Ca^{2+} response in C_2C_{12} myoblasts

Fura-2/AM-loaded C_2C_{12} cells (1×10^6) were incubated without or with 3 mM EGTA for 30 s or 100 ng/ml PTx for 16 h (A), with 10 μ M U73122 or 10 μ M U73343 for 15 min (B), or with 10 μ M Ro 31-8220 or 10 μ M GF109203X for 30 min (C), prior to incubation with 1 μ M SPP. Arrows indicate the addition of agonist. Plots representative of at least three similar experiments performed in duplicate are presented.

to antagonize Edg3 but not Edg1 or Edg5 [16]. As can be observed in Figure 4, a marked reduction in the SPP-mediated Ca^{2+} increase was observed in C_2C_{12} myoblasts incubated with 100 μ M suramin 2 min prior to incubation with 1 μ M SPP (inhibition of $51 \pm 8\%$, $n = 5$; $P < 0.01$). In contrast, as also shown in Figure 4, suramin did not significantly affect the Ca^{2+} increase

induced by addition of 1 μ M bradykinin, which strongly suggested a specific effect of suramin on the Edg3 receptor.

To suppress the functionality of the Edg3 receptor, an alternative approach was taken by the selective down-regulation of protein expression resulting from specific antisense ODN administration. As shown in Figure 5(A), polyclonal antibodies raised

Figure 4 Effect of suramin treatment on the SPP- or bradykinin (BK)-induced Ca^{2+} response in myoblasts

Fura-2/AM-loaded C_2C_{12} myoblasts (1×10^6) were incubated without (—) or with (+) 100 μM suramin 2 min prior to addition of 1 μM SPP or 1 μM bradykinin (arrows). Plots representative of at least three similar experiments performed in duplicate are presented.

against the N-terminal region of Edg3 recognized a single band of approx. 55 kDa, which was not recognized when the primary antibodies were neutralized with the immunogen peptide. Western-blot analysis of total membranes prepared from cells loaded with Edg3-specific antisense ODN demonstrated a significant reduction of protein expression in comparison with total membranes from cells loaded with scrambled ODN (Figure 5B). Importantly, in myoblasts in which Edg3 expression was down-regulated by Edg3-specific antisense ODN, SPP-induced Ca^{2+} responsiveness diminished strongly (inhibition of $68 \pm 6\%$, $n = 4$; $P < 0.05$), confirming the role of Edg3 in this SPP-mediated event. It is interesting to note that suramin did not affect SPP action on Ca^{2+} levels in myoblasts loaded with Edg3-specific antisense ODN, further supporting a specific effect of suramin on Edg3 (Figure 5C).

Besides Edg3, Edg1 and Edg5 receptors were also reported to be coupled to Ca^{2+} mobilization [1,2]. Therefore subtype-specific antisense ODN were used to investigate their possible role in the Ca^{2+} rise induced by SPP in C_2C_{12} myoblasts. As shown in Figure 6, in cells loaded with Edg1-specific antisense ODN the SPP response did not vary significantly (inhibition of $17 \pm 4\%$, $n = 4$;

$P = 0.07$), whereas the effect of suramin was comparable with that shown in Figure 4. In contrast, in cells transfected with Edg5-specific antisense ODN, the SPP-induced Ca^{2+} response was reduced by approx. $35 \pm 5\%$ ($n = 4$; $P < 0.01$). Notably, the SPP-mediated Ca^{2+} rise was not significant in Edg5-specific antisense ODN-loaded cells treated with suramin ($15 \pm 4\%$ above the control, $n = 3$; $P = 0.065$), supporting the existence of two independent signalling pathways coupled to Edg3 and Edg5 in the regulation of Ca^{2+} levels by SPP in C_2C_{12} myoblasts.

DISCUSSION

Although the capacity of SPP to increase intracellular Ca^{2+} in many cell types is well documented, its possible role in the mobilization of Ca^{2+} in skeletal-muscle cells has not yet been investigated. Here we provide the first evidence that in C_2C_{12} myoblasts SPP elicited a rapid and transient increase in Ca^{2+} levels, which was mediated by Edg3 and Edg5 receptors. This finding supports the notion that Ca^{2+} mobilization is a critical component in the SPP signalling machinery, which could be essential for the overall biological response elicited by the bioactive lipid. Confocal laser-scanning microscopic analysis, which allowed us to monitor Ca^{2+} in single cells, revealed that a significant fraction of the cell population was responsive to SPP. Interestingly, myoblasts were found to be heterogeneous with regard to the time course of the Ca^{2+} increase, as well as to the amplitude of the response. A similar temporal difference was observed recently in depolarization-induced Ca^{2+} transients in C_2C_{12} myotubes [24]. These findings suggest that one or more molecular components involved in the coupling of cell stimuli to Ca^{2+} mobilization could be represented differentially in individual cells or, although present, could be switched off in some cells.

A partial dependence of the SPP-mediated Ca^{2+} rise on extracellular Ca^{2+} has been reported [14,25], even though, under several sets of circumstances, $\text{Ins}(1,4,5)\text{P}_3$ generation through PLC activation was reported as the main upstream event responsible for agonist-induced Ca^{2+} increase [10,15,17]. Notably, this study shows that in C_2C_{12} myoblasts extracellular Ca^{2+} was critical for SPP-induced Ca^{2+} increase, suggesting that an eventual release of Ca^{2+} from intracellular stores could play only a secondary role in the control of Ca^{2+} levels by SPP. This assumption is in agreement with previous studies in skeletal-muscle cells [26]. In view of the hypothesis that the role of $\text{Ins}(1,4,5)\text{P}_3$ in SPP-induced Ca^{2+} rise in myoblasts could be only marginal, the observation that inhibition of PLC impaired the SPP-mediated Ca^{2+} response as well as [^3H]diacylglycerol formation suggests that the latter has a role in SPP signalling to Ca^{2+} . Consistently, the extracellular Ca^{2+} -dependent increase of Ca^{2+} induced by SPP was found to be reduced by the blockade of the main effector molecule of diacylglycerol, PKC, which is known to be stimulated by SPP in these cells [18], supporting a role for PKC in this SPP-mediated event. The molecular target of PKC-mediated phosphorylation implicated in Ca^{2+} influx triggered by the bioactive lipid is presently unknown. However, it is tempting to speculate that it could be represented by L-type calcium channels which are expressed in C_2C_{12} myoblasts (L. Formigli, F. Francini, E. Meacci, M. Vassalli, D. Nosi, F. Quercioli, B. Tiribilli, C. Bencini, C. Piperio, P. Bruni and S. Zecchi Orlandini, unpublished work). This hypothesis is supported by the recent observations that PKC-dependent activation of L-type calcium channels occurs in cardiac and portal vein myocytes [27,28].

In several studies SPP was reported to act as an intracellular mediator, able to mobilize Ca^{2+} from internal Ca^{2+} pools

Figure 5 Expression of the Edg3 receptor in C₂C₁₂ myoblasts (A), and the effect of Edg3-specific antisense ODN administration on Edg3 expression (B) and SPP-induced Ca²⁺ response (C)

(A) Aliquots (30 μ g of protein) of total membranes obtained from C₂C₁₂ myoblasts were separated by SDS/PAGE, transferred on to nitrocellulose membrane and hybridized with anti-Edg3 antibodies (1:2000) neutralized (+) or not (–) with the peptide (100 ng) used as immunogen. (B) Aliquots (30 μ g of protein) of total membranes obtained from cells transfected with scrambled (–) or Edg3-specific (+) antisense ODN were separated by SDS/PAGE, transferred on to nitrocellulose membrane and hybridized with anti-Edg3 antibodies. For (A) and (B), blots representative of three similar experiments are presented. (C) Cells transfected with scrambled or Edg3-specific antisense ODN were loaded with fura-2/AM. Cells were incubated for 2 min prior to agonist addition with or without 100 μ M suramin, and 1 μ M SPP was also added (arrows). Plots representative of three similar experiments performed in duplicate are shown.

insensitive to Ins(1,4,5)P₃, independent of binding to specific GPCRs [4,5]. In contrast, the present results show that in C₂C₁₂ myoblasts, as also demonstrated in other cell types [1–3], SPP-induced Ca²⁺ increase was a receptor-coupled process. This notion is supported by our observation that the effect of SPP had EC₅₀ values in the physiological concentration range and was saturable at 100 nM. Moreover, inactivation of G_i by PTx treatment significantly reduced the rise of Ca²⁺ levels promoted by the bioactive lipid, further supporting the occurrence of a receptor-mediated event. Notably, although Edg1, Edg3 and Edg5 are expressed in C₂C₁₂ myoblasts [18] and all have been found to be coupled to SPP-induced Ca²⁺ increase in overexpressing cells [10–17], endogenous Edg3 and Edg5, but not Edg1, were here identified as mediators of SPP signalling to Ca²⁺. Indeed, suramin, antagonist of the SPP-specific receptor Edg3 [16], but also known for its non-specific pharmacological properties [29], was able to markedly reduce SPP- but not bradykinin-induced Ca²⁺ response, supporting the concept of a role for Edg3. The notion that Edg3 represents an important transducer of Ca²⁺ mobilization triggered by SPP in C₂C₁₂ cells is sustained further by the significant inhibition of SPP responsiveness in

myoblasts loaded with Edg3-specific antisense ODN. In agreement, a role for endogenous Edg3 in SPP signalling to Ca²⁺ was reported in HL-60 leukaemia cells [30]. Regarding the other two Edg-receptor subtypes (Edg1 and Edg5), the administration of subtype-specific antisense ODN suggests that only Edg5 is implicated in SPP signalling leading to a rise on Ca²⁺, although to a lesser extent than Edg3. Moreover, the Ca²⁺ response to SPP was abolished when both Edg3 and Edg5 receptors were inhibited, reinforcing the notion that the SPP-induced Ca²⁺ rise in C₂C₁₂ cells is a receptor-mediated event and further excluding a role for Edg1. This latter finding is in contrast with reports in rat cardiomyocytes [31] and endothelial cells [32], where Edg1 was involved, suggesting that receptor coupling to a Ca²⁺ response depends on specific cellular settings. In overexpressing cells, Edg3- and Edg5-mediated Ca²⁺ mobilization has been reported to be inhibited moderately by PTx [13,14]. In agreement, our study in a native cell system demonstrated a partial coupling to G_i of SPP signalling to Ca²⁺, consistent with the view that Edg3 and Edg5 act via multiple heterotrimeric G-proteins.

The SPP-induced Ca²⁺ response in skeletal-muscle cells reported here helps to clarify the signalling pathways activated by SPP

Figure 6 Effect of Edg1- and Edg5-specific antisense ODN transfection on SPP-induced Ca^{2+} response in C_2C_{12} myoblasts

C_2C_{12} myoblasts were transfected with scrambled, or Edg1- or Edg5-specific antisense ODN. Then, 2 min prior to incubation with $1 \mu\text{M}$ SPP (arrows), cells transfected with Edg1- and Edg5-specific antisense ODN, loaded with fura-2/AM, were treated (+) or not with $100 \mu\text{M}$ suramin. Plots representative of at least three experiments with similar results performed in duplicate are presented.

in this cell type. It also sheds some light on the functional coupling of endogenous SPP receptors.

In view of the crucial role exerted by Ca^{2+} effector molecules in skeletal-muscle development and differentiation [33–35], and the broad spectrum of biological activities exhibited by SPP, which also embrace cell differentiation, it will be worth investigating whether the agonist-induced Ca^{2+} response participates in specific biological events triggered by SPP.

This work was supported by funds from the Italian Telethon Foundation (project no. 1086 to P. B., and project no. 945 to F. F.) and Cofinanziamento Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) Progetti di Interesse Nazionale 1999 (to P. B., F. F. and S. Z. O.).

REFERENCES

- Pyne, S. and Pyne, N. J. (2000) Sphingosine 1-phosphate signalling in mammalian cells. *Biochem. J.* **349**, 385–402.
- Spiegel, S. and Milstein, S. (2000) Functions of a new family of sphingosine 1-phosphate receptors. *Biochim. Biophys. Acta* **1484**, 107–116.
- Pyne, S. and Pyne, N. (2000) Sphingosine 1-phosphate signalling via the endothelial differentiation gene family of G-protein-coupled receptors. *Pharmacol. Therap.* **88**, 115–131.
- Ghosh, T. K., Bian, J. and Gill, D. L. (1994) Sphingosine 1-phosphate generated in the endoplasmic reticulum membrane activates release of stored Ca^{2+} . *J. Biol. Chem.* **269**, 22628–22635.
- Mattie, M., Brooker, G. and Spiegel, S. (1994) Sphingosine-1-phosphate, a putative second messenger, mobilizes calcium from internal stores via an inositol trisphosphate independent pathway. *J. Biol. Chem.* **269**, 3181–3188.
- Meyer zu Heringdorf, D., Lass, H., Kuchar, I., Alemany, R., Schmidt, M. and Jacobs, K. H. (1999) Role of sphingosine kinase in Ca^{2+} signalling by epidermal growth factor receptor. *FEBS Lett.* **461**, 217–222.
- Choi, O. H., Kim, J. H. and Kinet, J. P. (1996) Calcium mobilization via sphingosine kinase in signalling by the Fc epsilon RI antigen receptor. *Nature (London)* **380**, 634–638.
- Meyer zu Heringdorf, D., Lass, H., Alemany, R., Laser, K. T., Neumann, E., Zhang, C., Schmidt, M., Rauen, U., Jakobs, K. H. and van Koppen, C. J. (1998) Sphingosine kinase-mediated Ca^{2+} signalling by G-protein-coupled receptors. *EMBO J.* **17**, 2830–2837.
- Lee, M.-J., Van Brocklyn, J. R., Thangada, S., Liu, C. H., Hand, A. R., Menzeleev, R., Spiegel, S. and Hla, T. (1996) Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science* **279**, 1552–1555.
- Okamoto, H., Takuwa, N., Gonda, K., Okazaki, H., Chang, K., Yatomi, Y., Shigematsu, H. and Takuwa, Y. (1998) EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to multiple signaling pathways, including phospholipase C activation, Ca^{2+} mobilization, Ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition. *J. Biol. Chem.* **273**, 27104–27110.
- Zondag, G. C., Postma, F. R., Van Elten, A., Verlaan, I. and Moolenaar, W. H. (1998) Sphingosine 1-phosphate signalling through the G-protein-coupled receptor Edg-1. *Biochem. J.* **330**, 605–609.
- Van Brocklyn, J. R., Lee, M.-J., Menzeleev, R., Olivera, A., Edsall, L., Cuvillier, O., Thomas, D. M., Coopman, P. J. P., Thangada, S., Liu, C. H. et al. (1998) Dual actions of sphingosine-1-phosphate: extracellular through the Gi -coupled receptor Edg-1 and intracellular to regulate proliferation and survival. *J. Cell Biol.* **142**, 229–240.
- Kon, J., Sato, K., Watanabe, T., Tomura, H., Kuwabara, A., Kimura, T., Tamama, K.-I., Ishizuka, T., Murata, N., Kanda, T. et al. (1999) Comparison of intrinsic activities of the putative sphingosine 1-phosphate receptor subtypes to regulate several signaling pathways in their cDNA-transfected Chinese hamster ovary cells. *J. Biol. Chem.* **274**, 23940–23947.
- Gonda, K., Okamoto, H., Takuwa, N., Yatomi, Y., Okazaki, H., Sakura, T., Kimura, S., Sillard, R., Harii, K. and Takuwa, Y. (1999) The novel sphingosine 1-phosphate receptor AGR16 is coupled via pertussis toxin-sensitive and -insensitive G-proteins to multiple signalling pathways. *Biochem. J.* **337**, 67–75.
- Okamoto, H., Takuwa, N., Yatomi, Y., Gonda, K., Shigematsu, H. and Takuwa, Y. (1999) EDG3 is a functional receptor specific for sphingosine 1-phosphate and sphingosylphosphorylcholine with signaling characteristics distinct from EDG1 and AGR16. *Biochem. Biophys. Res. Commun.* **260**, 203–208.

- 16 Ancellin, N. and Hla, T. (1999) Differential pharmacological properties and signal transduction of the sphingosine 1-phosphate receptors EDG-1, EDG-3, and EDG-5. *J. Biol. Chem.* **274**, 18997–19002
- 17 Yamazaki, Y., Kon, J., Sato, K., Tomura, H., Sato, M., Yoneya, T., Okazaki, H., Okajima, F. and Ohta, H. (2000) Edg-6 as a putative sphingosine 1-phosphate receptor coupling to Ca^{2+} signaling pathway. *Biochem. Biophys. Res. Commun.* **268**, 583–589
- 18 Meacci, E., Vasta, V., Donati, C., Farnararo, M. and Bruni, P. (1999) Receptor-mediated activation of phospholipase D by sphingosine 1-phosphate in skeletal muscle C2C12 cells. A role for protein kinase C. *FEBS Lett.* **457**, 184–188
- 19 Meacci, E., Donati, C., Cencetti, F., Romiti, E. and Bruni, P. (2000) Permissive role of protein kinase C alpha but not protein kinase C delta in sphingosine 1-phosphate-induced RhoA activation in C2C12 myoblasts. *FEBS Lett.* **482**, 97–101
- 20 Lipp, P. and Niggli, E. (1993) Ratiometric confocal Ca^{2+} -measurements with visible wavelength indicators in isolated cardiac myocytes. *Cell Calcium*. **14**, 359–372
- 21 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440–3450
- 22 Meacci, E., Vasta, V., Faraoni, P., Farnararo, M. and Bruni, P. (1995) Potentiated bradykinin-induced increase of 1,2-diacylglycerol generation and phospholipase D activity in human senescent fibroblasts. *Biochem. J.* **312**, 799–803
- 23 Bleasdale, J. E., Thakur, N. R., Gremban, R. S., Bundy, G. L., Fitzpatrick, F. A., Smith, R. J. and Bunting, S. (1990) Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. *J. Pharmacol. Exp. Ther.* **255**, 756–768
- 24 Estrada, M., Cárdenas, C., Liberona, J. L., Carrasco, M. E., Mignery, G. A., Allen, P. D. and Jaimovich, E. (2001) Calcium transients in 1B5 myotubes lacking ryanodine receptors are related to inositol phosphate receptors. *J. Biol. Chem.* **276**, 22868–22874
- 25 An, S., Bleu, T. and Zheng, Y. (1999) Transduction of intracellular calcium signals through G protein-mediated activation of phospholipase C by recombinant sphingosine 1-phosphate receptors. *Mol. Pharmacol.* **55**, 787–794
- 26 Berridge, M. J., Lipp, P. and Bootman, M. D. (2000) The versatility and universality of calcium signalling. *Nature Rev. Mol. Cell Biol.* **1**, 11–21
- 27 Aiello, E. A. and Cingolani, H. E. (2001) Angiotensin II stimulates cardiac L-type Ca^{2+} current by a Ca^{2+} - and protein kinase C-dependent mechanism. *Am. J. Physiol.* **280**, H1528–H1536
- 28 Zhong, J., Hume, J. R. and Keef, K. D. (2001) beta-Adrenergic receptor stimulation of L-type Ca^{2+} channels in rabbit portal vein myocytes involves both alphas and betagamma G protein subunits. *J. Physiol.* **531**, 105–115
- 29 Voogd, T. E., Vansterkenburg, E. L. M., Wilting, J. and Janssen, L. H. M. (1993) Recent research on the biological activity of suramin. *Pharmacol. Rev.* **45**, 177–203
- 30 Sato, K., Murata, N., Kon, J., Tomura, H., Nochi, H., Tamoto, K., Osada, H., Ohta, H., Tokumitsu, Y., Ui, M. and Okajima, F. (1998) Down-regulation of mRNA expression of EDG3, a putative sphingosine 1-phosphate receptor coupled to Ca^{2+} signaling, during differentiation of HL-60 leukaemia cells. *Biochem. Biophys. Res. Commun.* **253**, 253–256
- 31 Nakajima, N., Cavalli, A. L., Biral, D., Glembofski, C. C., McDonough, P. M., Ho, P. D., Betto, R., Sandomà, D., Palade, P. T., Dettbarn, C. A. et al. (2000) Expression and characterization of Edg-1 receptors in rat cardiomyocytes calcium deregulation in response to sphingosine 1-phosphate. *Eur. J. Biochem.* **267**, 5679–5686
- 32 Lee, M.-J., Thangada, S., Claffey, K. P., Ancellin, A., Liu, C. H., Kluk, M., Volpi, M., Sh'afi, R. I. and Hla, T. (1999) Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine 1-phosphate. *Cell* **99**, 301–312
- 33 Ferrari, M. B., Rohrbough, J. and Spitzer, N. C. (1996) Spontaneous calcium transients regulate myofibrillogenesis in embryonic *Xenopus* myocytes. *Dev. Biol.* **178**, 484–497
- 34 Friday, B. B., Horseley, V. and Pavlath, G. K. (2000) Calcineurin activity is required for the initiation of skeletal muscle differentiation. *J. Cell Biol.* **149**, 657–666
- 35 Delling, U., Tureckova, J., Lim, H. W., De Windt, L. J., Rotwein, P. and Molkekin, J. D. (2000) A calcineurin-NFATc3-dependent pathway regulates skeletal muscle differentiation and slow myosin heavy-chain expression. *Mol. Cell. Biol.* **20**, 6600–6611

Received 2 March 2001/8 November 2001; accepted 7 December 2001

¹ To whom correspondence should be addressed (e-mail biocell@scibio.unifi.it).