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# Sphingosine 1-phosphate induces $Ca^{2+}$ transients and cytoskeletal rearrangement in $C_2C_{12}$ myoblastic cells

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*Departments of [1]Anatomy, Histology, and Forensic Medicine, [2]Physiological Sciences, and [3]Biochemical Sciences, University of Florence, 50134 Florence; and [4]Biophotonics Lab, National Institute of Applied Optics, 50125 Florence, Italy*

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**Formigli, Lucia, Fabio Francini, Elisabetta Meacci, Massimo Vassalli, Daniele Nosi, Franco Quercioli, Bruno Tiribilli, Chiara Bencini, Claudia Piperio, Paola Bruni, and Sandra Zecchi Orlandini.** Sphingosine 1-phosphate induces $Ca^{2+}$ transients and cytoskeletal rearrangement in $C_2C_{12}$ myoblastic cells. *Am J Physiol Cell Physiol* 282: C1361–C1373, 2002. First published January 30, 2002; 10.1152/ajpcell.00378.2001.—In many cell systems, sphingosine 1-phosphate (SPP) increases cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) by acting as intracellular mediator and extracellular ligand. We recently demonstrated (Meacci E, Cencetti F, Formigli L, Squecco R, Donati C, Tiribilli B, Quercioli F, Zecchi-Orlandini S, Francini F, and Bruni P. *Biochem J* 362: 349–357, 2002) involvement of endothelial differentiation gene (Edg) receptors (Rs) specific for SPP in agonist-mediated $Ca^{2+}$ response of a mouse skeletal myoblastic ($C_2C_{12}$) cell line. Here, we investigated the $Ca^{2+}$ sources of SPP-mediated $Ca^{2+}$ transients in $C_2C_{12}$ cells and the possible correlation of ion response to cytoskeletal rearrangement. Confocal fluorescence imaging of $C_2C_{12}$ cells preloaded with $Ca^{2+}$ dye fluo 3 revealed that SPP elicited a transient $Ca^{2+}$ increase propagating as a wave throughout the cell. This response required extracellular and intracellular $Ca^{2+}$ pool mobilization. Indeed, it was significantly reduced by removal of external $Ca^{2+}$, pretreatment with nifedipine (blocker of L-type plasma membrane $Ca^{2+}$ channels), and inositol 1,4,5-trisphosphate [Ins(1,4,5)$P_3$]-mediated $Ca^{2+}$ pathway inhibitors. Involvement of EdgRs was tested with suramin (specific inhibitor of Edg-3). Fluorescence associated with Ins(1,4,5)$P_3$Rs and L-type $Ca^{2+}$ channels was evident in $C_2C_{12}$ cells. SPP also induced $C_2C_{12}$ cell contraction. This event, however, was unrelated to $[Ca^{2+}]_i$ increase, because the two phenomena were temporally shifted. We propose that SPP may promote $C_2C_{12}$ cell contraction through $Ca^{2+}$-independent mechanisms.

calcium ion transients; cytoskeleton; cell contraction; confocal microscopy

SPHINGOSINE 1-PHOSPHATE (SPP) is a bioactive lysophospholipid mediator that is recognized as a highly versatile molecule capable of affecting many cellular processes, including cell proliferation and differentiation, apoptotic cell death, cell motility, and cytoskeletal organization (19). Some of these biological effects of SPP have long been attributed to its action as second messenger. Indeed, the mitogenic responses to several growth factors, such as platelet-derived growth factor, epidermal growth factor, nerve growth factor, and insulin, as well as the inhibition of apoptosis induced by antiblastic drugs have been related to the activation of sphingosine kinase and to the subsequent enhanced production of intracellular SPP (35, 36, 38). More recently, the identification of a subset of receptors belonging to the endothelial differentiation gene (Edg) receptor (R) family has suggested that SPP may also act as an extracellular lipid mediator. In agreement with this suggestion, SPP is released on platelet activation and is an important constituent of serum (18). Moreover, extracellular SPP stimulation is required for inhibition of cell motility in vascular smooth muscle and melanoma cells, neurite retraction, and stimulation of DNA synthesis in 3T3 fibroblasts (8, 39, 50, 52). Only very recently has a role for exogenous SPP in the pathogenesis of inflammatory diseases such as asthma also been proposed (2).

It is becoming apparent that both modes of action of SPP may involve $Ca^{2+}$ mobilization from intracellular stores and/or from the external pool. However, at present, the mechanisms by which SPP affects cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) are far from being fully delineated. Several studies have suggested that SPP, when acting as a second messenger, can directly promote $Ca^{2+}$ release from the endoplasmic reticulum (ER) through mechanisms independent of the activation of the ryanodine (Ry)R or inositol 1,4,5-trisphosphate [Ins(1,4,5)$P_3$]R pathways (5, 54). Consistent with this, a novel sphingolipid-gated $Ca^{2+}$-permeable channel has been discovered on isolated ER vesicles of *Xenopus* oocytes (26). In contrast, dissection of the $Ca^{2+}$ signaling pathways triggered by the interaction of SPP with its G protein-coupled receptors is of great difficulty and complexity considering that multiple ef-

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fector systems, including phospholipase C (PLC) and protein kinase C (PKC), may be involved (3, 37, 40). In particular, although activation of phospholipases has been reported in several cell types after SPP stimulation, only limited data have provided conclusive evidence for a role of Ins(1,4,5)$P_3$ in the SPP-induced $Ca^{2+}$ signaling pathway. Moreover, we recently found (28) in a myoblastic $C_2C_{12}$ cell line that SPP elicits a $Ca^{2+}$ response in the form of $Ca^{2+}$ waves that are dependent on extracellular $Ca^{2+}$, thus suggesting a role for the lipid metabolite in mediating the influx of the ion through plasma membrane $Ca^{2+}$ channels. Furthermore, in the same study we also showed that inhibition of Edg-3R and Edg-5R by specific antisense oligodeoxyribonucleotides totally abolished SPP-induced $Ca^{2+}$ response (28). Voltage-dependent dihydropyridine receptors (L-type $Ca^{2+}$ channels), located on the plasma membrane, represent the major $Ca^{2+}$ entry pathway in excitable cells. In particular, $Ca^{2+}$ influx through these channels is critical for the activation of $Ca^{2+}$-induced $Ca^{2+}$ release via RyR channels of the sarcoplasmic reticulum (SR) and for contractility stimulation in cardiac muscle cells (5). In contrast, L-type $Ca^{2+}$ channels were shown to couple conformationally with RyRs on depolarization to release $Ca^{2+}$ during contraction in mature skeletal muscle cells (33, 41, 49). Nevertheless, even though numerous studies exist on the physiological significance of L-type $Ca^{2+}$ channels in striated muscle cells, their possible role in SPP-induced $Ca^{2+}$ response remains to be studied.

Because a better understanding of the molecular basis of SPP action may be of crucial importance in understanding the physiological significance and possible pathological implications of this metabolite, it seemed worthwhile, in the present study, to further characterize the $Ca^{2+}$ response elicited by exogenous SPP in skeletal muscle cells, particularly in view of the crucial role exerted by $Ca^{2+}$ effector molecules in skeletal muscle development and differentiation. Confocal laser scanning microscopy equipped with Time Course software was then used to determine the spatiotemporal distribution of SPP-mediated $Ca^{2+}$ transients in a myoblastic $C_2C_{12}$ cell line and the extracellular and intracellular sources of $Ca^{2+}$ mobilization and to characterize the pattern of expression of voltage- and ligand-gated plasma membrane and intracellular $Ca^{2+}$ channels in these cells. The effects of SPP-induced $Ca^{2+}$ transients on the cytoskeletal reorganization were also considered in view of the well-known role that this ion plays in the regulation of cell contractility.

## MATERIALS AND METHODS

### *Cell Cultures*

Mouse skeletal $C_2C_{12}$ myoblasts (51) were obtained from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified minimum essential medium (DMEM) with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Sigma, Milan, Italy) at 37°C and exposed to a humidified atmosphere of 5% $CO_2$.

### *Confocal Analysis of Calcium Transients*

To reveal variations in intracellular concentrations of calcium in $C_2C_{12}$ cells incubated with SPP (Calbiochem, San Diego, CA), ~$2 \times 10^4$ cells were plated on glass coverslips and incubated at room temperature for 10 min in serum-free DMEM with 0.1% bovine serum albumin (BSA) containing fluo 3-acetoxymethyl ester as fluorescent calcium indicator at a final concentration of 10 μM and 0.1% anhydrous dimethyl sulfoxide and Pluronic F-127 (0.01% wt/vol) as dispersing agent (Molecular Probes, Eugene, OR). The cells were then washed and maintained in fresh medium for 10 min to allow complete deesterification of fluo 3. After that, the cells were placed in open slide flow-loading chambers that were mounted on the stage of a confocal Bio-Rad MRC 1024 ES scanning microscope (Bio-Rad, Hercules, CA) equipped with a krypton/argon laser source (15 mW) for fluorescence measurements. The microscope was also equipped with differential interference contrast (DIC) optics. The fluorescence of fluo 3-loaded cells was monitored by using a 488-nm wavelength and collecting the emitted fluorescence with a Nikon Plan Apo ×60 oil-immersion objective through a 510-nm long-wave pass filter. The time course analysis of $Ca^{2+}$ waves after SPP stimulation was performed with Time Course Kinetic software (Bio-Rad).

Some experiments were performed in $Ca^{2+}$-free, 2 mM $Mg^{2+}$-containing medium and/or after pretreatment of $C_2C_{12}$ cells with various modulators of known voltage- and ligand-gated calcium channels. In particular, caffeine (100 mM; Sigma), 2-aminoethyldiphenylborate (2-APB, 100 μM; Alexis, San Diego, CA), heparin (50 mM), and 1-[6-([(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-1*H*-pyrrole-2,5-dione (U-73122, 10 μM; Alexis) were used to inhibit any potential Ins(1,4,5)$P_3$R-mediated release, Ry (100 μM; Sigma) to inhibit RyR-$Ca^{2+}$ release channels, and nifedipine (100 nM; Sigma) to inhibit $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels. To test the involvement of EdgRs in the $Ca^{2+}$ response, the cells were treated with suramin (100 μM) before stimulation. SPP was dissolved in the medium by fast perfusion; the small size of the chamber used (0.2 ml) and the perfusion flux of ~0.2 ml/s allowed a complete replacement of the bathing medium in ~1 s.

Usually, cells did not reach confluence on coverslips; a single coverslip with adherent cells was used for only one experiment. For each cell preparation a variable number of cells ranging from 10 to 22 were analyzed. Multiple regions of interest (ROIs) of 25 $\mu m^2$ were selected in single cells to monitor the spatiotemporal distribution of $Ca^{2+}$ transients. Fluorescence signals are expressed as fractional changes above the resting baseline, ΔF/F, where F is the averaged baseline fluorescence before the application of SPP and ΔF represents the fluorescence changes from the baseline. The latency ($T_0$) of the $Ca^{2+}$ wave was measured as the lag between the addition of the agonist and the beginning of the fluorescence increase over the basal noise. The time to peak ($T_p$) was measured as the time interval between $T_0$ and the peak level. The time to half-decay ($T_{0.5}$) of fluorescence was measured as the time for the fluorescence to decay from the peak to half its peak value. The temporal delay to peak amplitude between adjacent ROIs was used to calculate the extent of synchronization vs. propagation of $Ca^{2+}$ transients. Usually, two ROIs were placed within the nucleus, whereas a variable number ranging from 3 to 10 ROIs were placed inside the cytoplasm. Confocal fluorescence images were also used to evaluate intracellular $Ca^{2+}$ spatial distribution with a purpose-developed software running under Interactive Data Language (Research Systems, Boulder, CO).

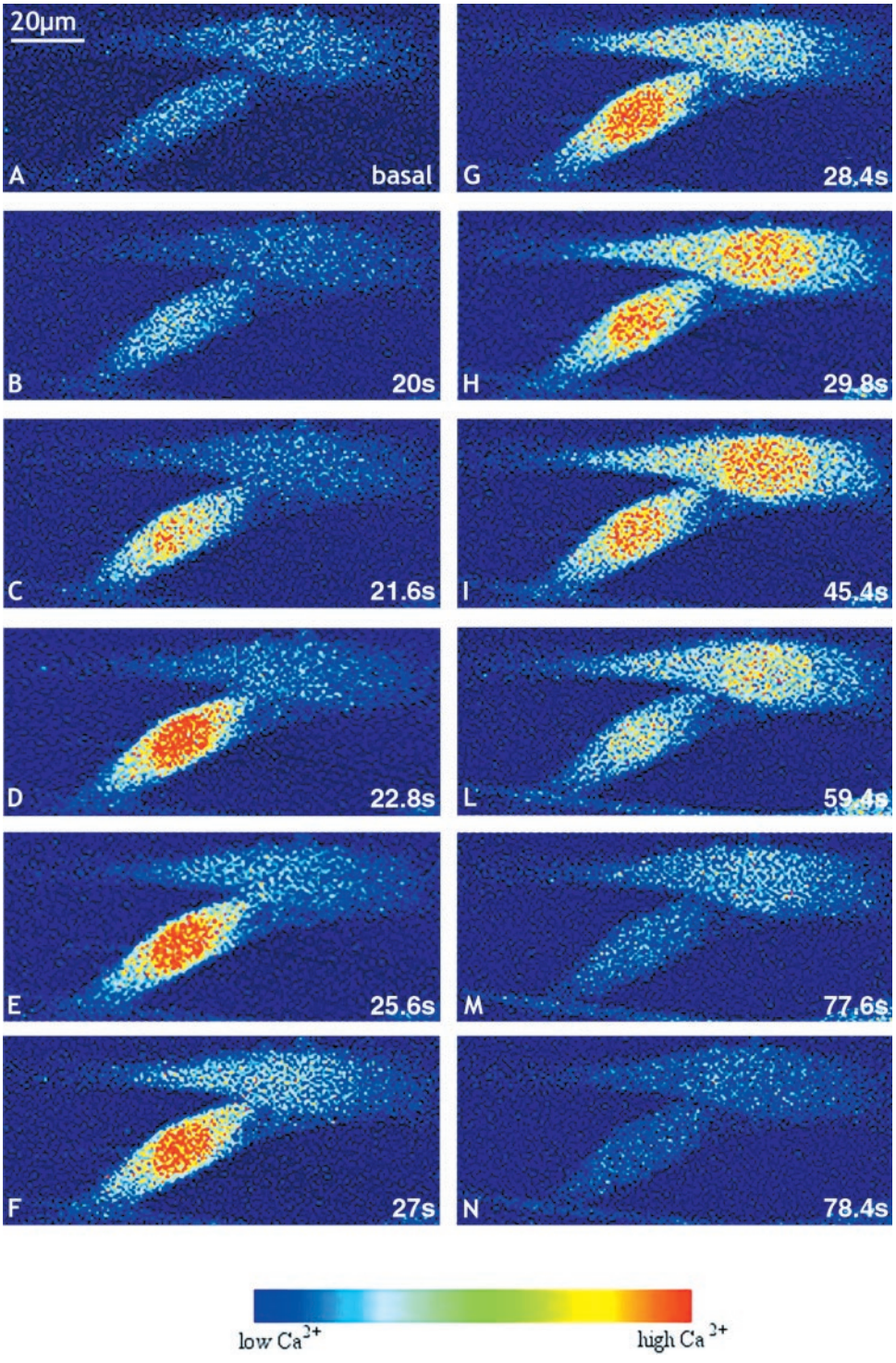


Fig. 1. Calcium wave images of fluo 3-loaded $C_2C_{12}$ cells by confocal microscopy. The pseudocoloring represents the absolute $Ca^{2+}$ increase as indicated by the color bar. Basal fluorescence is shown in *A*. Next images were taken ~20 s from sphingosine 1-phosphate (SPP; 1 μM) stimulation (*B*) and as indicated in *C–I*, *L–N*. In the *top* cell in each panel, a burst of fluorescence is initially visible on the *left* of the myoblast and then propagates onward, invading the nucleus. In the *bottom* cell, the fluorescence signal invades, at the same time, the cytoplasmic and nuclear regions.

*Determination of Inositol Phosphate Production*

Serum-starved $C_2C_{12}$ myoblasts were incubated for 24 h in inositol-free DME in the presence of 5 μCi/ml *myo*-[2-$^3$H(N)]inositol (25 Ci/mmol; NEN, Dreiech, Germany). Two hours before the beginning of the experiment the medium was changed, and 30 min before the addition of the agonist (1 μM SPP) the cells were incubated with 20 mM LiCl. Incubation was stopped by aspirating the medium and washing the monolayer twice with PBS. Inositol phosphate (InsP) accumulated in the cells was extracted with 5% ice-cold perchloric acid for 30 min. Cell extracts were neutralized with $K_2CO_3$, and InsP was separated onto Dowex (Bio-Rad) formiate form (1 ml) and quantified essentially as described previously (45).

*Determination of Diacylglycerol Production*

$C_2C_{12}$ cells were labeled with 5 μCi/ml [2-$^3$H]glycerol (14.2 Ci/mmol; NEN) for 24 h and then incubated for 30 s with 1 μM SPP. Lipid extraction and [$^3$H]diacylglycerol (DAG) sep-

aration by thin-layer chromatography was performed as described previously (31).

*Confocal Immunofluorescence*

$C_2C_{12}$ cells grown on coverslips were fixed in 4% buffered paraformaldehyde for 10 min at room temperature. The cells were then washed, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and blocked with a solution containing 0.5% BSA and 3% glycerol in PBS.

*Calcium channel immunodetection.* Cells were incubated with the following primary antibodies diluted in BSA-PBS: rabbit anti-$\alpha_{1c}$ L-type channels (recognizing $\alpha_{1c}$-subunit of voltage-gated $Ca^{2+}$ channel) and rabbit anti-$\alpha_{1D}$ L-type channels (reacting with all forms of $\alpha_{1D}$-subunit of voltage-gated $Ca^{2+}$ channel; 1:100; Chemicon International), mouse anti-Ins(1,4,5)$P_3$R [recognizing COOH-terminal cytoplasmic domain of Ins(1,4,5)$P_3$R types 1, 2, and 3; 1:200; Chemicon], and mouse anti-RyR (reacting with COOH-terminal domain of RyR; 500 kDa, 1:50; Chemicon) for 1 h at room temperature. After incubation with the primary antibodies, the cells were washed to remove unbound antibodies (Abs) and incubated with Alexa 488-conjugated anti-mouse or anti-rabbit secondary Abs (1:200 dilution; Chemicon). Counterstaining for F-actin was performed with rhodamine-phalloidin (Sigma).

*Cytoskeletal protein immunodetection.* $C_2C_{12}$ cells were incubated with a monoclonal anti-myosin Ab (1:50 dilution; Sigma) and a monoclonal anti-vinculin Ab (1:100 dilution; Sigma) for 1 h at room temperature. The cells were subsequently incubated with Alexa 488-labeled anti-mouse IgG (Molecular Probes). Actin filaments were stained with tetramethylrhodamine-isothiocyanate-labeled phalloidin. After a series of washes the coverslips containing the immunostained cells were mounted with an antifade mounting medium (Biomeda; Electron Microscopic Sciences). Negative controls were performed by substituting blocking solution for the primary Abs. The fluorescence signals were revealed by a confocal laser scanning microscope (Bio-Rad). To this purpose, a series of optical sections (512 × 512 pixels) was taken through the depth of the cells with a thickness of 1 μm at intervals of 0.8 μm. Twenty optical sections for each examined sample were then projected as a single composite image by superimposition.

*Imaging of Dynamic Changes of Actin Cytoskeleton*

Alexa 488-labeled G-actin monomers were used as probes for live cell cytoskeleton. The monomers were introduced into $C_2C_{12}$ cells by the scrape-loading technique. Briefly, $C_2C_{12}$ cells grown to confluence on 100-mm petri dishes were washed twice with PBS and 2 ml of scraping buffer (in mM: 114 KCl, 1 NaCl, 5.5 $MgCO_3$, and 10 Tris·HCl). Fluorescent G-actin monomers (250 μg/ml; Molecular Probes) were added in 0.5 ml of scraping buffer, and the cells were gently scraped, suspended in DMEM-10% FCS, and split among six-well dishes on coverslips. After 1 h of incubation, we estimated that ~70% of the treated cells were viable. Labeled cells were then observed by confocal microscope and used to test the effect of SPP on dynamic changes of actin cytoskeleton.

*Statistics*

All data are expressed as means ± SD. Data were analyzed by one-way ANOVA with Bonferroni's correction for multiple comparisons. The α-value was at $P < 0.05$ for all tests.

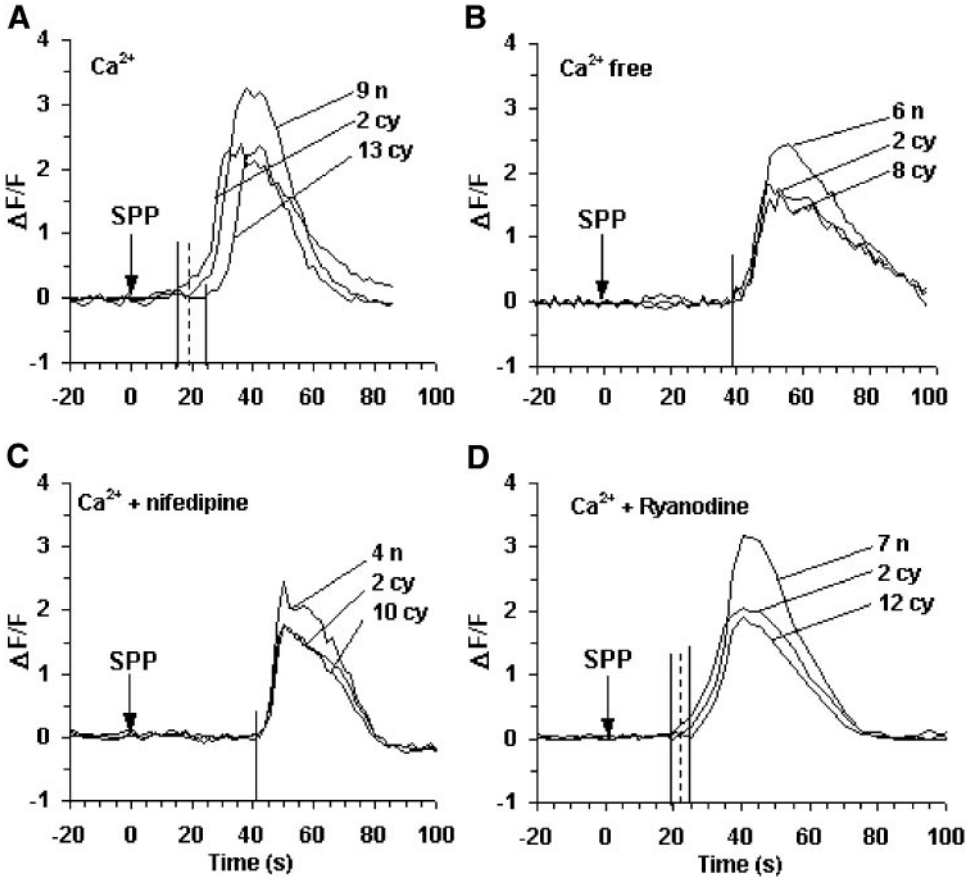


Fig. 2. SPP-mediated $Ca^{2+}$ waves in $C_2C_{12}$ myoblastic cells. Time course of $Ca^{2+}$ response from representative cells in $Ca^{2+}$-containing medium (*A*), $Ca^{2+}$-free medium (*B*), and $Ca^{2+}$-containing medium after pretreatment with nifedipine (100 nM; *C*) or ryanodine (100 μM; *D*). The data are from representative cells. *Inset*, number of regions of interest (ROIs; cy and n indicate ROIs taken in cytoplasm and nucleus, respectively). Arrows indicate the addition of SPP (*time 0*) . In *A* and *D*, the *left* vertical lines indicate latency ($T_0$) values in the cytoplasmic ROIs (2), the dashed vertical lines indicate $T_0$ in the nuclear ROIs (9 or 7), and the *right* vertical lines indicate $T_0$ in cytoplasmic ROIs (13 or 12) located at the opposite edge of the cells examined. In *B* and *C*, $T_0$ values are doubled and are the same in all ROIs. ΔF/F, fractional fluorescence changes above resting baseline.

## RESULTS

### *SPP-Induced $Ca^{2+}$ Transients in $C_2C_{12}$ Myoblasts*

$C_2C_{12}$ cells cultured on coverslips and observed under light microscopy showed variable morphology, being rounded or spindle-shaped with several cytoplasmic projections emanating from the cell surfaces and anchoring to the substrate. We first verified the responsiveness of the $C_2C_{12}$ myoblasts. To this aim, the cells were loaded with fluorescent $Ca^{2+}$ dye fluo 3 and ATP (1 mM) was added to $Ca^{2+}$-containing medium. In accordance with previous reports (24), ATP induced a significant intracellular $Ca^{2+}$ elevation in all cells (data not shown). The application of exogenous SPP (1 μM) to $C_2C_{12}$ cells also promoted a substantial increase in intracellular $Ca^{2+}$ that was evident in both the cytoplasmic and nuclear compartments. This increase was concentration dependent, with an $EC_{50}$ of ~50 nM. The SPP-induced $Ca^{2+}$ increase was transient and was followed by a return to near resting levels within 1 min (Fig. 1). In particular, a synchronous $Ca^{2+}$ increase was observed in some cells, whereas the $Ca^{2+}$ response propagated as a wave in others (Figs. 1 and 2*A*). As shown in Fig. 3*A*, almost 60% of the examined cells were responsive to SPP, exhibiting relative fluorescence changes significantly ($P < 0.001$) higher in the nuclear than in the cytoplasmic region (Fig. 3*B*). Moreover, differences in the time course of the fluorescence signal were found between the cytoplasmic and nuclear regions; in fact, $T_p$ and $T_{0.5}$ of the nucleus were significantly higher ($P < 0.001$) and lower ($P < 0.05$), respectively, compared with those of the cytoplasm (Fig. 3, *C–E*). The $Ca^{2+}$ response elicited by exogenous SPP could consist of at least two components: $Ca^{2+}$ influx across the plasma membrane and $Ca^{2+}$ release from the endogenous stores. To better investigate this issue, we stimulated $C_2C_{12}$ cells in $Ca^{2+}$-free, $Mg^{2+}$-containing medium (Fig. 2*B*). Under these particular conditions, the number of cells responsive to SPP was reduced to ~40% (Fig. 3*A*), and the cytosolic and nuclear ΔF/F increase in response to 1 μM SPP was significantly reduced by ~35% ($P < 0.001$) (Fig. 3*B*). Moreover, the $Ca^{2+}$ increase was evident as a synchronous rather than propagated $Ca^{2+}$ wave with a $T_0$ significantly increased with respect to controls (Figs. 2*B* and 3*C*). In myoblasts cultured in the absence of external $Ca^{2+}$, a slight increase in $T_p$ and a remarkable increase in $T_{0.5}$ ($P < 0.001$) in both the cytoplasmic and nuclear ROIs (Fig. 3, *D* and *E*) were found. All these data together suggested that SPP-induced $Ca^{2+}$ response was attributable to both extracellular and intracellular $Ca^{2+}$ pool mobilization.

A role for extracellular $Ca^{2+}$ influx was further confirmed in two-step experimental protocols consisting of addition of SPP to cells cultured in $Ca^{2+}$-free medium and subsequent readministration of $Ca^{2+}$ to the medium once the ion transients had occurred (Fig. 4, *A* and *B*). Indeed, $Ca^{2+}$ readministration caused a faster elevation of intracellular $Ca^{2+}$ in all cells in both the cytoplasmic and nuclear ROIs (Fig. 4, *A*, *B*, *F*, and *G*).

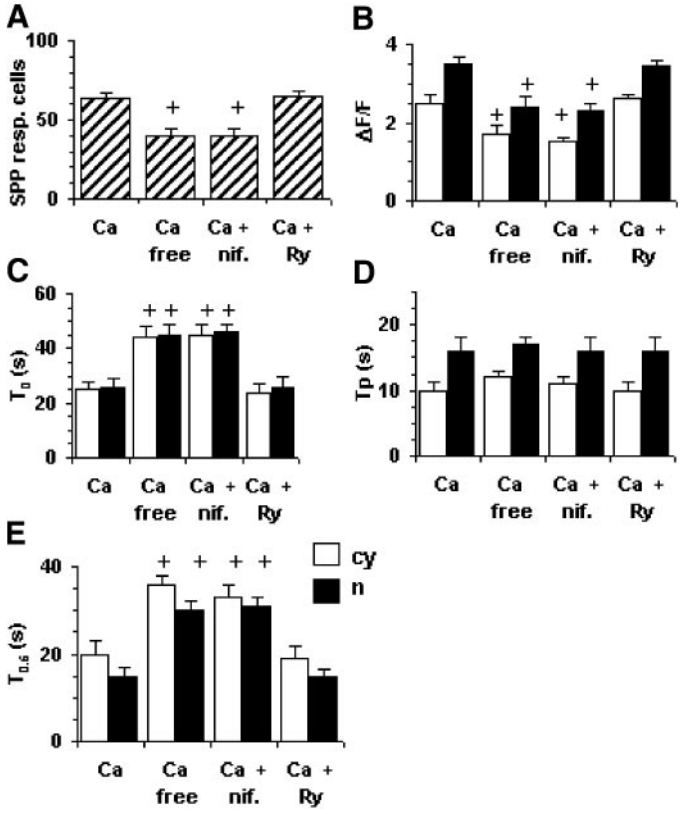


Fig. 3. SPP-mediated $Ca^{2+}$ waves in $C_2C_{12}$ myoblastic cells: statistical analysis. In *A–E*, bars indicated as Ca, Ca free, Ca + nif and Ca + Ry refer to experiments like those reported in Fig. 2, *A–D*. *A*: % of SPP-responsive cells. *B–E*: values of ΔF/F, $T_0$, time to peak ($T_p$), and time to half-decay ($T_{0.5}$) from the cytoplasmic (open bars) and nuclear (filled bars) ROIs examined are reported. Data are means ± SD from cells cultured on 10 (*A*), 9 (*B*), 9 (*C*), and 6 (*D*) different coverslips; the total number of the cells analyzed was 120 (*A*), 102 (*B*), 106 (C), and 80 (D). Significant differences were evaluated by 1-way ANOVA test. $^+$Data statistically different with respect to controls ($P < 0.001$). In all experimental condition ΔF/F values were significantly ($P < 0.01$) larger in nucleus than in corresponding cytoplasmic ROIs. No statistically significant differences were found between Ca free and Ca + nif data or between control (Ca) and Ca + Ry. nif., Nifedipine; Ry, ryanodine.

The $Ca^{2+}$ increase was transient but, in contrast to that elicited by SPP, it rapidly decayed (Fig. 4*H*) to steady-state intracellular $Ca^{2+}$ levels that remained elevated above the baseline. This latter response was absent when the two-step procedure was applied to cells not previously stimulated by SPP (data not shown), thus suggesting the existence of a SPP-dependent $Ca^{2+}$ influx pathway mediated by the activation of putative plasma membrane $Ca^{2+}$ channels.

To verify this latter hypothesis, $C_2C_{12}$ myoblasts were treated with nifedipine (100 nM), a prototypical blocker of plasma membrane L-type $Ca^{2+}$ channels, 20 min before stimulation with SPP (Fig. 2*C*). The presence of nifedipine in $Ca^{2+}$-containing medium significantly reduced the $Ca^{2+}$ transients, which became similar to those observed in $Ca^{2+}$-free medium (Figs. 2*B* and 3), supporting a role for nifedipine-sensitive receptors in SPP-mediated $Ca^{2+}$ influx.

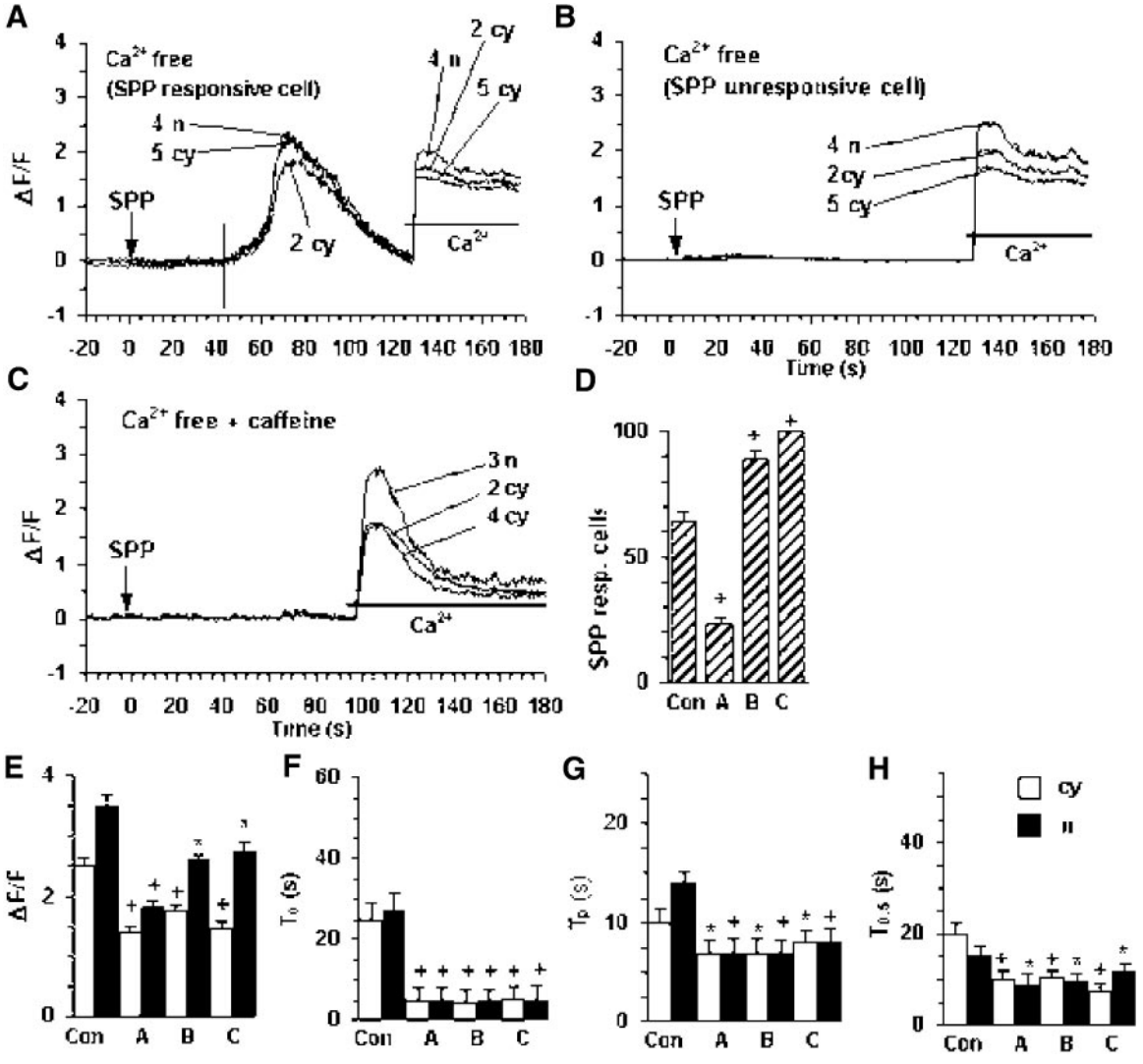


Fig. 4. Effects of $Ca^{2+}$ readministration on SPP-mediated $Ca^{2+}$ waves in $C_2C_{12}$ myoblasts from 2-step experimental protocol. *A* and *B*: cells were stimulated with SPP in $Ca^{2+}$-free medium, and $Ca^{2+}$ was readministered to the medium once intracellular $Ca^{2+}$ had returned to baseline. An SPP-responsive and an unresponsive cell are shown in *A* and *B*, respectively. Note that both cells respond to the readministration of $Ca^{2+}$ (indicated by horizontal line marked $Ca^{2+}$). *C*: experimental protocol as in *A* and *B* except that cells were pretreated with caffeine (100 mM). The cell is unresponsive to SPP but responds to the readministration of $Ca^{2+}$ to the medium. In *D–H*, bars labeled A, B, and C refer to the $Ca^{2+}$ response observed after readministration of $Ca^{2+}$ to the medium as reported in *A*, *B*, and *C*, respectively. The values obtained in cells in control conditions are similar to those in Fig. 3. Other symbols, labeling, and scales as in Fig. 3. Data are means ± SD from cells cultured on 8 (*A* and *B*) and 7 (*C*) different coverslips; the total number of cells analyzed was 90 (*A*), 80 (*B*), and 76 (*C*). Significant differences were evaluated by 1-way ANOVA test. Data statistically different with respect to controls: $^{*}P < 0.05$, $^{+}P < 0.001$. In all experimental conditions ΔF/F values were significantly ($P < 0.05$ for *A* and $P < 0.001$ for *B* and *C*) larger in nucleus than in corresponding cytoplasmic ROIs. In *D* and *E*, which indicate the number of responsive cells, ΔF/F values of *A* were significantly reduced ($P < 0.001$) with respect to *B* and *C*. In *F–H*, no statistical differences were found among *A*, *B*, and *C* data.

We next tested $C_2C_{12}$ myoblasts for the presence of voltage-dependent ionic channels in $C_2C_{12}$ cells by adding KCl (100 mM) to the medium. High extracellular $K^+$ was not able to elicit any $Ca^{2+}$ response in undifferentiated $C_2C_{12}$ cells. This finding indicated the absence of voltage-dependent $Ca^{2+}$ channels and demonstrated that the observed nifedipine-sensitive $Ca^{2+}$ influx in response to SPP was independent from the existence of functional voltage-dependent L-type $Ca^{2+}$ channels.

Subsequently, we explored the involvement of intracellular receptors in the $Ca^{2+}$ mobilization elicited by SPP by adding caffeine (100 mM), a known agonist of RyRs, to the medium. Caffeine was without effect on induction of $Ca^{2+}$ mobilization in all $C_2C_{12}$ cells examined. Moreover, pretreatment with Ry (100 μM) did not affect SPP-mediated intracellular $Ca^{2+}$ transients (Figs. 2*D* and 3), suggesting that RyR-mediated $Ca^{2+}$ mobilization was absent in $C_2C_{12}$ myoblasts.

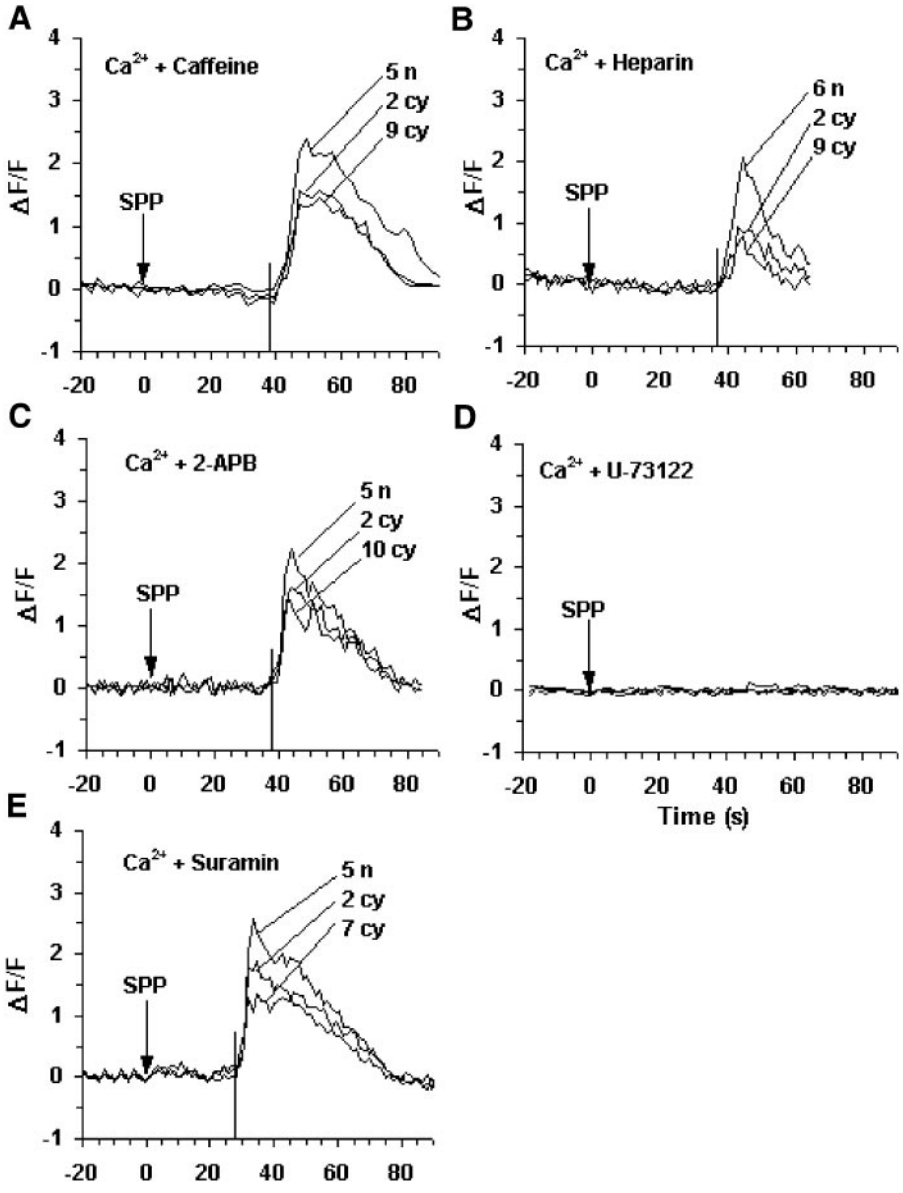


Fig. 5. Effects of pretreatment with inhibitors of inositol-1,4,5-trisphosphate [Ins(1,4,5)$P_3$]-mediated $Ca^{2+}$ pathway and of inhibitor of endothelial differentiation gene (Edg) receptors (Rs) on SPP-mediated intracellular $Ca^{2+}$ elevation in $C_2C_{12}$ myoblasts. Time course of the $Ca^{2+}$ response from cells in $Ca^{2+}$-containing medium is shown. Data are referred to representative cells. Cells were pretreated with caffeine (100 mM; *A*), heparin (50 mM; *B*), 2-aminoethyldiphenylborate (2-APB, 100 μM; *C*), U-73122 (10 μM; *D*), and suramin (100 μM; *E*).

Interestingly, pretreatment with caffeine 1 min before SPP stimulation (Fig. 5*A*) significantly reduced the number of responsive cells ($P < 0.05$) and the peak of $Ca^{2+}$ transients in both the cytoplasmic and nuclear ROIs by ~35% ($P < 0.001$; Fig. 6, *A* and *B*). Moreover, $T_0$ and $T_{0.5}$ of the $Ca^{2+}$ response were significantly increased with respect to controls in both the cytoplasm and nuclear ROIs ($P < 0.001$; Fig. 6). Because it has been reported that caffeine, besides activating RyRs, inhibits Ins(1,4,5)$P_3$Rs (53), these latter data are suggestive for an involvement of the Ins(1,4,5)$P_3$ signaling pathway in the cytosolic $Ca^{2+}$ response. As it occurred in the cells cultured in $Ca^{2+}$-free medium, the pretreatment with caffeine before stimulation with SPP elicited a $Ca^{2+}$ response in the form of a synchronous wave (Fig. 5*A*). Quite similar results were obtained by pretreating $C_2C_{12}$ cells with other inhibitors of the Ins(1,4,5)$P_3$ signaling $Ca^{2+}$ pathway such as heparin and 2-APB, both known blockers of Ins(1,4,5)$P_3$Rs (Figs. 5, *B* and *C*, and 6). In contrast, pretreatment with U-73122, a specific inhibitor of PLC activation, completely blocked the occurrence of $Ca^{2+}$ transients elicited by SPP (Figs. 5*D* and 6). These data are in favor of a role of PLC activation not only in the Ins(1,4,5)$P_3$ signaling pathway but also in mediating plasma membrane $Ca^{2+}$ influx.

The involvement of EdgRs in the $Ca^{2+}$ signaling pathway induced by SPP was tested by pretreating $C_2C_{12}$ cells with suramin, an inhibitor of Edg-3R (Ref. 4; Fig. 5*E*). Pretreatment with suramin, although significantly ($P < 0.001$) reducing the number of responsive cells (by ~40%), only slightly affected the time course of the intracellular $Ca^{2+}$ transients evoked by SPP in $C_2C_{12}$ myoblasts (Figs. 5*E* and 6).

The dual origin, extracellular and intracellular, of $Ca^{2+}$ transients elicited by SPP was further supported by the finding of a complete inhibition of SPP-mediated $Ca^{2+}$ response after pretreatment with caffeine in

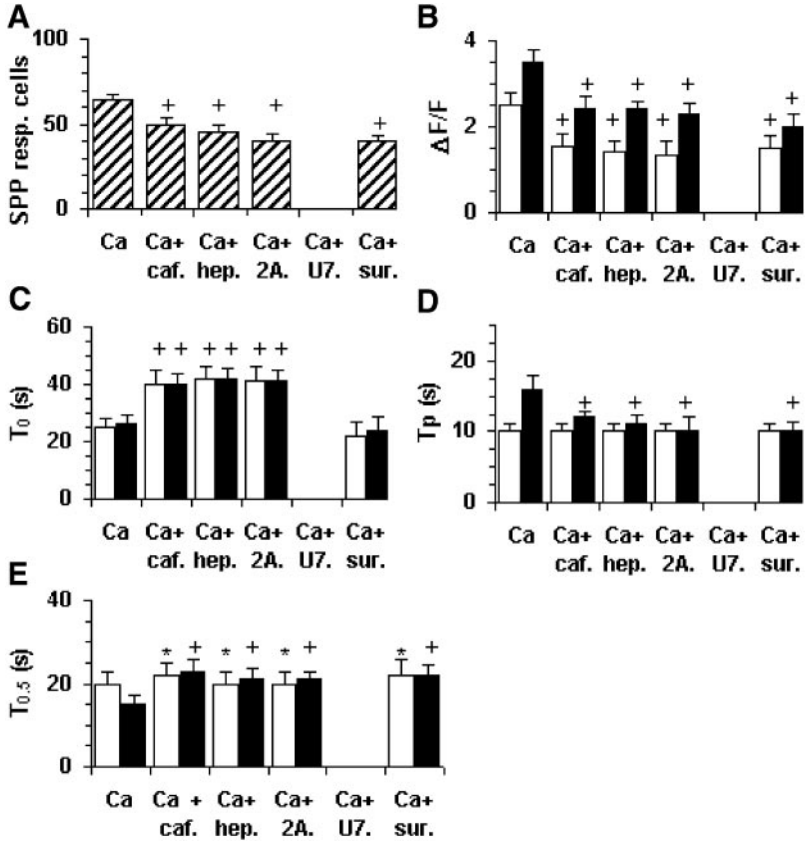


Fig. 6. Effects of pretreatment with inhibitors of Ins(1,4,5)$P_3$R-mediated $Ca^{2+}$ pathway on SPP-mediated intracellular $Ca^{2+}$ elevation in $C_2C_{12}$ myoblasts: statistical analysis. The values obtained in cells in control conditions, indicated as Ca, are as in Fig. 3. In *A–E*, Ca + caf, Ca + hep, Ca + 2A, Ca + U7, and Ca + sur refer to experiments like those reported in Fig. 5, *A–E*, respectively. Other symbols, labeling, and scales as in Fig. 3. Data are means ± SD from cells cultured on 10 (*A*), 9 (*B*), 9 (*C*), 9 (*D*), and 10 (*E*) different coverslips; the total number of cells analyzed was 99 (*A*), 80 (*B*), 86 (*C*), 102 (*D*), and 108 (*E*). Significant differences were evaluated by 1-way ANOVA test. Data statistically different with respect to controls (Ca): $^{*}P < 0.05$, $^{+}P < 0.001$. In all experimental conditions, ΔF/F values were significantly ($P < 0.001$) larger in nucleus than in corresponding cytoplasmic ROIs. No statistical differences for ΔF/F, $T_0$, and $T_p$ were found among Ca + caf, Ca + hep, and Ca + 2-APB. caf, Caffeine; hep, heparin; 2A, 2-APB; U7, U-73122; sur, suramin.

$Ca^{2+}$-free medium (Fig. 4*C*). The subsequent readministration of $Ca^{2+}$ caused fast elevation of intracellular $Ca^{2+}$ in all cells examined (Fig. 4*D*). The $Ca^{2+}$ increase was transient and decreased to steady-state intracellular $Ca^{2+}$ levels that remained elevated over baseline. A complete inhibition of the SPP-mediated $Ca^{2+}$ response in $C_2C_{12}$ cells was also observed after pretreatment with caffeine and nifedipine in $Ca^{2+}$-containing medium (data not shown), strongly suggesting that the plasma membrane $Ca^{2+}$ channels implicated in the $Ca^{2+}$ influx were sensitive to nifedipine.

### *InsP and DAG Production*

To further study the characteristics and mechanisms of the $Ca^{2+}$ response elicited by SPP, we measured the production of radiolabeled total InsP as well as [$^3$H]DAG after SPP stimulation. Treatment of $C_2C_{12}$ cells with 1 μM SPP increased the cellular levels of both InsP and DAG after 30 s by ~25% ± 3 [10,141 ± 697 (control) vs. 13,747 ± 1,107 disintegrations/min (dpm) InsP/$10^6$ dpm labeled phospholipid (SPP); $n = 4$, $P = 0.05$] and 41% ± 7 [18,756 ± 2,014 (control) vs. 27,530 ± 2,950 dpm DAG/$10^6$ dpm labeled phospholipid (SPP); $n = 3$, $P < 0.01$], respectively.

### *Confocal Immunocytochemistry*

To further probe the $Ca^{2+}$ sources implicated in SPP-induced $Ca^{2+}$ mobilization, we evaluated, by confocal immunofluorescence, the expression of several known plasma membrane as well as intracellular $Ca^{2+}$ channels in $C_2C_{12}$ myoblastic cells. We found that some cells abundantly expressed L-type $Ca^{2+}$ channels in the form of small, granular fluorescence bodies, whereas others displayed virtually no reactivity (Fig. 7, *A* and *B*). When present, both the $\alpha_{1c}$- and $\alpha_{1d}$-subunits were distributed in a diffuse pattern within the cytoplasm and also in close association with the plasma membrane. Ins(1,4,5)$P_3$Rs were also markedly expressed and were distributed throughout the cytoplasm with a denser staining in the perinuclear regions, whereas the cell surfaces remained virtually unstained (Fig. 7*C*). However, not all the cells were stained with the same intensity, and cells negative for Ins(1,4,5)$P_3$Rs were also present. In agreement with the lack of effects of ryanodine on the $Ca^{2+}$ transients stimulated by SPP, RyRs showed a low and often barely detectable intracellular labeling (data not shown). The latter data strongly suggest that these receptors, which are specific markers for SR $Ca^{2+}$ release channels of striated muscle fibers, may develop in later phases of myogenic differentiation.

### *Cytoskeletal Modifications Induced by SPP*

We finally examined whether the SPP-dependent effects on intracellular $Ca^{2+}$ mobilization were associated with variations in cytoskeletal organization. We first analyzed the organization of the cytoskeletal apparatus in fixed $C_2C_{12}$ cells and found that actin myofilaments represented the main cytoskeletal components in myoblastic cells at this stage of differentiation;

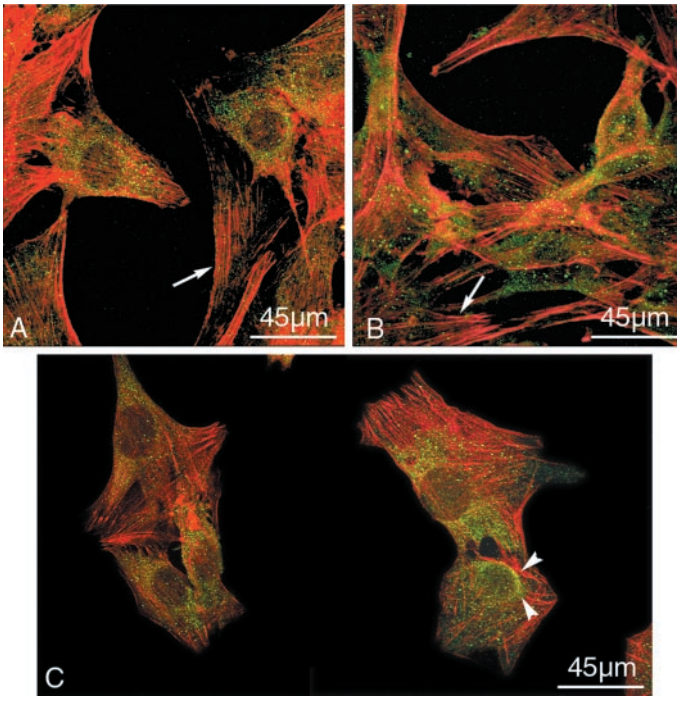


Fig. 7. Localization of L-type channels and Ins(1,4,5)$P_3$Rs in $C_2C_{12}$ myoblasts. Confocal immunofluorescence microscopy of $C_2C_{12}$ myoblasts that double-stained either with polyclonal antibodies to the $\alpha_{1c}$- and $\alpha_{1D}$-subunits of L-type channels (*A* and *B*) or with monoclonal antibodies to Ins(1,4,5)$P_3$Rs (*C*) and with tetramethylrhodamine isothiocyanate-labeled phalloidin is shown. *A* and *B*: both subunits of L-type channels appear as bright granular bodies scattered in the cytoplasm and in close association with the plasma membrane (brighter dots). Note that some cells (arrows) display lower immunolabeling and even absence of immunolabeling. *C*: Ins(1,4,5)$P_3$R staining also shows a spotlike pattern that is diffuse throughout the cytoplasm, tending to concentrate in the perinuclear regions (arrowheads).

actin filaments were arranged in a weblike structure that invaded all the cytoplasm, anchoring to the plasma membrane, and terminated in typical focal adhesion sites containing vinculin immunostaining (Fig. 8). A less defined reaction was, however, observed for myosin filaments, which appeared as scattered fluorescent small cytoplasmic aggregates (data not shown). These results indicated that the cytoskeletal organization of these cells was quite different from the orderly arrays of myofilaments forming the sarcomeric units of mature skeletal muscle cells. Interestingly, in $Ca^{2+}$-containing medium, SPP stimulated contraction and shortening of living $C_2C_{12}$ cells, but, unexpectedly, this phenomenon did not correlate with the onset of $Ca^{2+}$ rise in these cells. In fact, by comparing time-lapse video imaging obtained by DIC with the fluorescence $Ca^{2+}$ images, we found that 88.4% of the cells underwent cytoskeletal contraction whereas only 64% displayed $Ca^{2+}$ transients in response to SPP stimulation. Moreover, whenever the two phenomena coexisted in the same cell, a clear temporal shift existed between SPP-stimulated cell contractility and SPP-stimulated intracellular $Ca^{2+}$ increase (Fig. 9). In fact, cell contraction was a very rapid event, occurring within 3–5 s, whereas the rise in intracellular $Ca^{2+}$ became evident within ~14–35 s (mean value 24 ± 1.5 s; Figs. 1, 2*A*, and 3*C*) from SPP stimulation. These data were further confirmed by statistical analyses of the time behaviors of the intracellular spatial $Ca^{2+}$ distribution (Fig. 10). By plotting together the time course of the area occupied by fluo 3 fluorescence with the temporal behavior of the total fluo 3 fluorescence, it was found that significant modifications in the fluorescence area (i.e., an initial decrease followed by a return to basal levels, indicating cell contraction and cell relaxation, respectively) occurred before the beginning of $Ca^{2+}$ transients . The small increase in the fluorescence signal ($\Delta F/F = 0.05–0.1$) corresponding to the time of cell contraction was probably due to a reduction in the cell volume caused by contraction rather than to a small intracellular calcium elevation.

To confirm that SPP was able to affect the cytoskeletal reorganization, $C_2C_{12}$ cells were preloaded with fluorescent probes for actin. With a time interval quite similar to that observed with DIC video imaging (~5 s), a remarkable reorganization of the actin cytoskeleton could be visualized (Fig. 11). Indeed, small fluorescent dots, representing G-actin short polymers, tended to move coordinately and concentrate toward the nucleus in response to SPP.

## DISCUSSION

In the present study we have shown that SPP is capable of producing intracellular $Ca^{2+}$ transients in myoblastic $C_2C_{12}$ cells. In light of our previous findings (28), this response was mediated by the interaction of

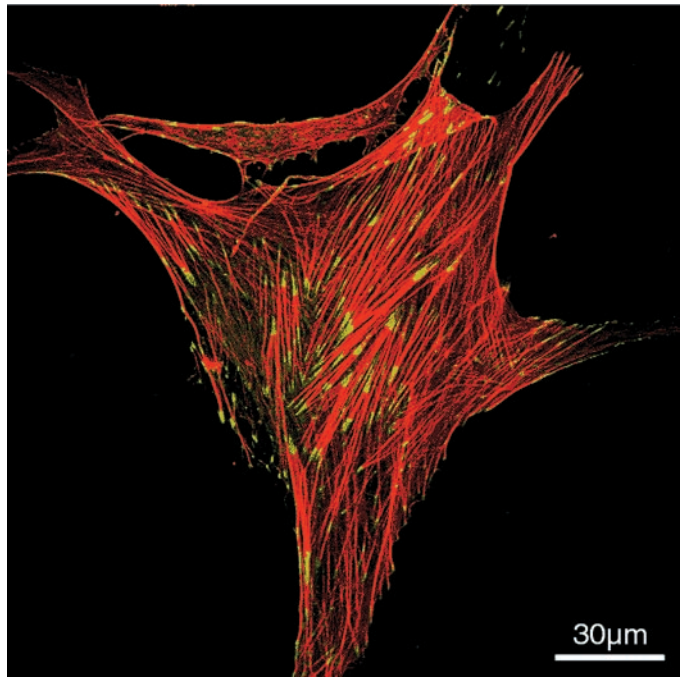


Fig. 8. Localization of vinculin and actin in $C_2C_{12}$ myoblasts. Confocal fluorescence microscopy of $C_2C_{12}$ myoblasts double-stained with polyclonal antibodies to vinculin and with tetramethylrhodamine isothiocyanate-labeled phalloidin to reveal actin filaments is shown. A myoblast exhibits a well-developed actin cytoskeleton with stress fibers converging toward focal adhesion complexes that contain vinculin (green staining).

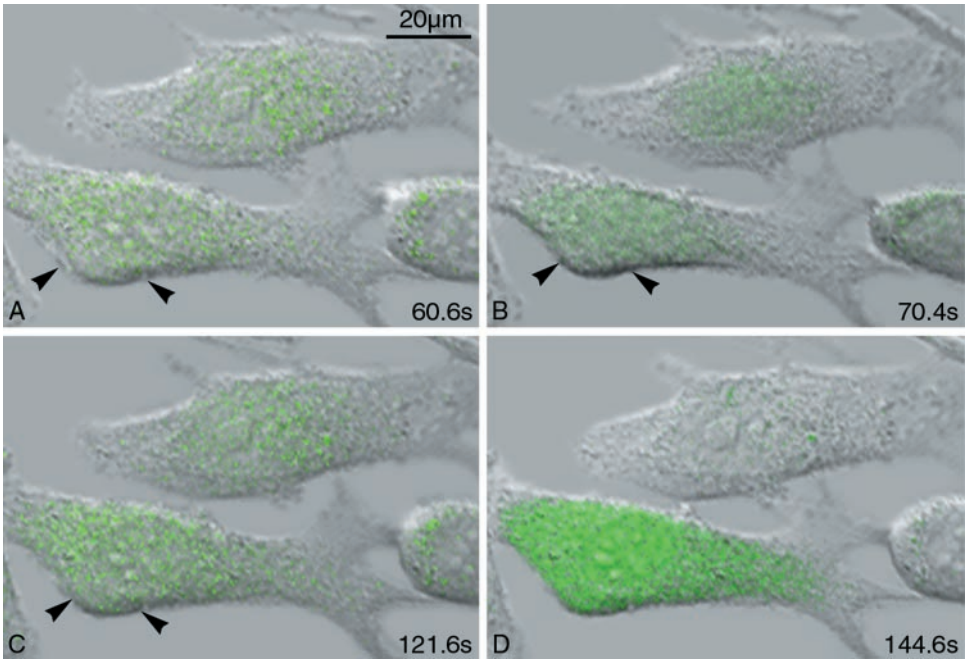


Fig. 9. Differential interference contrast and $Ca^{2+}$ fluorescence time-lapse images of $C_2C_{12}$ myoblasts. Images were acquired sequentially according to the indicated times. Two consecutive applications of PBS were made before SPP stimulation to avoid misinterpretation of the data. SPP was added at 65 s from the beginning of the experiment. The images were acquired before SPP addition (*A*) and 10 (*B*), 56 (*C*), and 85 (*D*) s from the agonist addition. Basal distribution of fluorescence is seen in *A*; note that the $Ca^{2+}$ dye is particularly concentrated in some regions corresponding to the nucleus and perinuclear areas and is absent in the cytoplasmic projections anchoring the cell to the substrate. *B*: after stimulation with SPP remarkable changes in the cell size (arrowheads), strongly indicative for cell contraction, become visible, whereas $Ca^{2+}$ signals are absent, apart from the apparent brighter fluorescence staining, probably due to the redistribution of the fluorescence signal during contraction. *C*: after contraction the cells return to the original size of *A*. *D*: $Ca^{2+}$ transient is now clearly visible both in the cytoplasmic and nuclear regions of 1 $C_2C_{12}$ cell. Arrowheads point to identical spots of the same cell in parallel images.

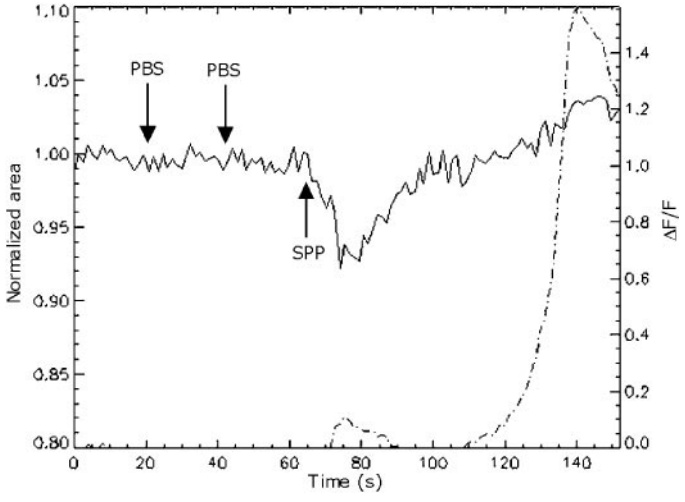


Fig. 10. Statistical evaluation of intracellular $Ca^{2+}$ distribution. The SPP-responsive cell shown in Fig. 9 is considered. The solid line represents the normalized area occupied by fluo 3 fluorescence, and the lower dot-dashed line indicates the total increase in intracellular $Ca^{2+}$. Addition of PBS within the first 50 s does not modify the characteristics of the fluorescence distribution. In contrast, after the addition of SPP, a substantial decrease in the fluorescence area (solid line), suggestive of cell contraction, is clearly evident. During the relaxation phase, the fluorescence area returns to the basal levels whereas the $Ca^{2+}$ transients become visible.

the bioactive lipid with specific EdgRs. Evidence is reported here that the $Ca^{2+}$ response elicited by SPP involved both the cytoplasmic and nuclear compartments as propagated or synchronous waves and required contributions from intracellular and extracellular $Ca^{2+}$ sources. In trying to dissect the $Ca^{2+}$ signaling pathway we also found that intracellular Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ release channels [Ins(1,4,5)$P_3$Rs] and nifedipine-sensitive $Ca^{2+}$ channels (undifferentiated/non-voltage-dependent L-type channels) of the plasma membrane were likely involved in the SPP action on myoblasts. Our experimental evidence to support this hypothesis includes the ≤40% reduction of the SPP-mediated $Ca^{2+}$ response in cells in the absence of external $Ca^{2+}$; the requirement of functional PLC cascade and Ins(1,4,5)$P_3$Rs for this response, as evidenced by its reduction up to 60% on pretreatment with inhibitors of Ins(1,4,5)P3Rs, such as heparin, caffeine and 2-APB, or with U-73122, an inhibitor of PLC; the ability of nifedipine, a prototypical blocker of L-type $Ca^{2+}$ channels, to affect significantly the $Ca^{2+}$ transients elicited by SPP; and, finally, the complete inhibition of the $Ca^{2+}$ response in cells pretreated with caffeine and nifedipine in $Ca^{2+}$-containing medium or with caffeine in $Ca^{2+}$-free medium.

Both the cytosolic and nuclear $Ca^{2+}$ signals elicited by SPP action were dependent on intracellular $Ca^{2+}$

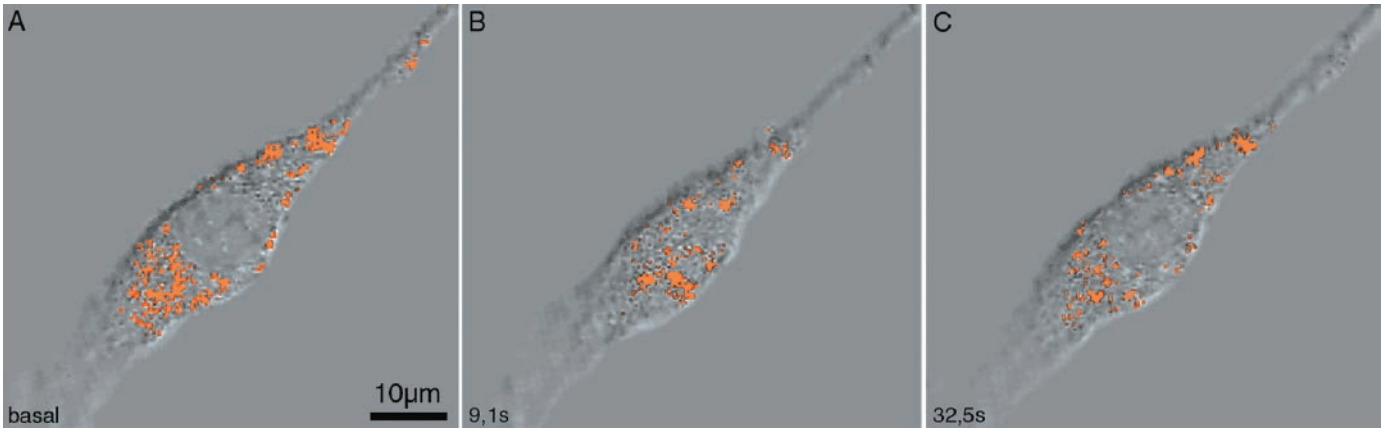


Fig. 11. Dynamic changes of actin cytoskeleton in SPP-stimulated $C_2C_{12}$ myoblasts. The cells were previously loaded with fluorescence-conjugated G-actin monomers. The images were acquired before SPP addition (*A*) and 9.1 (*B*) and 32.5 (*C*) s from agonist stimulation. A substantial redistribution of G-actin polymers, suggestive of activation of cell contractility, is clearly evident after the addition of SPP to the culture medium.

increase, regardless of whether it originated from external or endogenous stores. Indeed, removal of external $Ca^{2+}$ and pretreatment with nifedipine or caffeine significantly affected the latency time of the SPP-mediated $Ca^{2+}$ response. In these particular circumstances, in fact, $T_0$ was about twofold that of control myoblasts. A possible explanation of these results may be that $Ca^{2+}$ influx, namely through nifedipine-sensitive channels, and $Ca^{2+}$ release from the endogenous stores in the cytoplasm, perinuclear, and nuclear regions may be a $Ca^{2+}$-sensitive, autocatalytic processes. Moreover, under these particular experimental conditions the decrease in the number of cells responsive to SPP with respect to that of control conditions may be linked to the expression of different levels of $Ca^{2+}$ channels in these cells.

The involvement of Ins(1,4,5)$P_3$R and L-type $Ca^{2+}$ channels in the SPP-mediated $Ca^{2+}$ response in both the cytoplasmic and nuclear compartments was further confirmed by confocal immunofluorescence studies. Ins(1,4,5)$P_3$Rs were, in fact, found throughout the cytoplasm and in association with the nuclear envelope, whereas L-type channels were localized both at the plasma membrane and inside the cytoplasm. However, not all the cells revealed the same degree of fluorescence labeling. SPP did not affect $Ca^{2+}$ release through RyR channels in the myoblastic cells, in agreement with the absence of any detectable immunostaining associated with anti-RyR antibody in $C_2C_{12}$ cells and with previous findings on the absence of RyRs in proliferating undifferentiated skeletal muscle cells (1, 23, 25, 27).

The role for Ins(1,4,5)$P_3$R in the generation of $Ca^{2+}$ signals has been well established in several cell types, including immature and developing skeletal muscle cells (12, 20). In particular, $C_2C_{12}$ myoblasts have been shown to express several isoforms of G protein-coupled SPP receptors (Edg-1, Edg-3, and Edg-5 ; Ref. 30), and numerous studies on signal transduction have demonstrated that these receptors, activated by SPP, stimulate PLC and, in turn, promote Ins(1,4,5)$P_3$ generation (34, 37, 40). In contrast, $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels in myoblastic cells has not been investigated in depth. Voltage-dependent, dihydropyridine-sensitive L-type $Ca^{2+}$ channels are multisubunit transmembrane proteins that allow $Ca^{2+}$ influx necessary for the excitation-contraction coupling of cardiac fibers and for modulation of RyR $Ca^{2+}$-release channels of skeletal muscle cells (14). Their expression is developmentally regulated in embryos and in muscle cell lines, because their plasma membrane density sharply increases on muscle differentiation (23, 44). In such a view, the presence of the $\alpha_1$-subunit of nifedipine-sensitive L-type $Ca^{2+}$ channels in the cytoplasm of $C_2C_{12}$ cells probably denotes that they are on course to be transferred to the cell surface during this stage of myogenic differentiation. Moreover, the presence of these receptors at the cell surface, where no voltage-gated $Ca^{2+}$ currents were ever seen, further indicates the existence of "immature" L-type channels in undifferentiated $C_2C_{12}$ myoblasts. Nevertheless, a possible effect of SPP on these channels during muscle cell differentiation should be taken into account in view of the findings that L-type $Ca^{2+}$ channels of cardiac muscle cells (21) and developing skeletal muscle cells (10, 46) are modulated by PKC activation, which is a critical step in the signal transduction of exogenous SPP in $C_2C_{12}$ cells (30). Moreover, activation of L-type $Ca^{2+}$ channels, which involves phosphorylation of both α- and β-subunits, may occur whether or not the auxiliary subunits are coexpressed (42). Therefore, it is likely that SPP interacting with Edg cell surface receptors expressed on myoblastic cells triggers a signaling pathway that, through DAG production and PKC activation, targets regulation of immature plasma membrane L-type $Ca^{2+}$ channels, thus promoting extracellular $Ca^{2+}$ influx in $C_2C_{12}$ cells.

In searching for a possible morphological-functional correlation of the SPP-mediated $Ca^{2+}$ transients, we examined whether the $Ca^{2+}$ response was associated with corresponding changes in the cytoskeletal organization in the myoblastic cells, particularly in view of

the well-known effects that the sphingolipid has on cytoskeletal remodeling in several cell types (2, 6, 39, 48). Interestingly, we found that SPP promoted cell contractility in the myoblastic cells, but this event did not require intracellular $Ca^{2+}$ mobilization. Indeed, cell contraction was a rapid event that occurred early after stimulation, whereas the intracellular $Ca^{2+}$ changes become evident only after longer times, thus suggesting that binding of SPP to Edg receptors was able to activate multiple signaling pathways. Indeed, differential coupling to G proteins and effector systems have been shown for Edg-3 and Edg-5 receptors, which are involved in $Ca^{2+}$ mobilization and cytoskeletal remodeling, respectively (17, 32, 37, 40). In particular, Edg-5 receptors ligated by SPP stimulate Rho proteins and Rho-dependent kinases have been shown to play an important role in the regulation of smooth muscle and nonmuscle cell contractility by modulating the levels of phosphorylation of myosin light chain (16, 22, 43). Interestingly, in a previous study we demonstrated (29) that RhoA activation also occurs in $C_2C_{12}$ myoblasts after SPP stimulation. In view of that finding and in consideration of the findings reported here that $C_2C_{12}$ cytoskeleton has structural similarities to that of nonmuscle cells, being formed by an extensive network of actin filaments rather than a highly ordered array of myofilaments forming the contractile units of striated fibers, it may be speculated that $Ca^{2+}$-independent/Rho-dependent contraction of the actin cytoskeleton may also occur in undifferentiated skeletal muscle cells. Although not directly addressed here, it seems likely, on the basis of these data, that $Ca^{2+}$ transients elicited by exogenous SPP in $C_2C_{12}$ cells may play a role in the myogenic differentiation program rather than in the regulation of cell contractility. This hypothesis would be consistent with an involvement of calcineurin in skeletal muscle differentiation (15) and with recent data showing that $C_2C_{12}$ myoblasts subjected to genetic and metabolic mitochondrial stress release high $Ca^{2+}$ transients that are capable of enhancing the expression of RyR-1 and of modifying the activity of several $Ca^{2+}$-dependent transcription factor pathways (7). In keeping with all these findings, it is becoming apparent that $Ca^{2+}$ released from the muscle $Ins(1,4,5)P_3Rs$ may not be significantly involved in muscle cell contraction (20, 47).

In conclusion, the present study, taking advantage of updated techniques and instrumentation, substantially contributes to the understanding of the molecular and functional properties of the $Ca^{2+}$ signaling pathway triggered by exogenous SPP in myoblastic cells. It appears that SPP exerts profound biological effects on myoblasts that could have physiological and pathophysiological implications. Indeed, very recently, it was shown that cystic fibrosis transmembrane regulator (CFTR), also expressed in skeletal muscle (13), mediates cellular uptake of SPP, thus attenuating SPP signaling (9). In view of this finding, it is tempting to speculate that alterations of the SPP signaling and skeletal muscle cell response may occur in cystic fibrosis, accounting for the peripheral muscle weakness observed in this pathology (11).

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