Sphingosylphosphocholine modulates the ryanodine receptor/calcium-release channel of cardiac sarcoplasmic reticulum membranes

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Sphingosylphosphocholine (SPC) modulates Ca^{2+} release from isolated cardiac sarcoplasmic reticulum membranes; 50 μM SPC induces the release of 70–80 % of the accumulated calcium. SPC releases calcium from cardiac sarcoplasmic reticulum through the ryanodine receptor, since the release is inhibited by the ryanodine receptor channel antagonists ryanodine, Ruthenium Red and sphingosine. In intact cardiac myocytes, even in the absence of extracellular calcium, SPC causes a rise in diastolic Ca^{2+} , which is greatly reduced when the sarcoplasmic reticulum is depleted of Ca^{2+} by prior thapsigargin treatment. SPC action on the ryanodine receptor is Ca^{2+} -dependent. SPC shifts to the left the Ca^{2+} -dependence of [^3H]ryanodine binding, but only at

high pCa values, suggesting that SPC might increase the sensitivity to calcium of the Ca^{2+} -induced Ca^{2+} -release mechanism. At high calcium concentrations (pCa 4.0 or lower), where [^3H]ryanodine binding is maximally stimulated, no effect of SPC is observed. We conclude that SPC releases calcium from cardiac sarcoplasmic reticulum membranes by activating the ryanodine receptor and possibly another intracellular Ca^{2+} -release channel, the sphingolipid Ca^{2+} -release-mediating protein of endoplasmic reticulum (SCaMPER) [Mao, Kim, Almenoff, Rudner, Kearney and Kindman (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1993–1996], which we have identified for the first time in cardiac tissue.

INTRODUCTION

Excitation–contraction coupling in the heart involves Ca^{2+} release from the sarcoplasmic reticulum through the Ca^{2+} -activated channel known as the ryanodine receptor. The cardiac ryanodine receptor is regulated by Ca^{2+} entry through the L-type Ca^{2+} channel in the sarcolemma, by a process termed calcium-induced calcium release. Besides Ca^{2+} , various endogenous effector molecules modulate ryanodine receptor activity, including lipid-derived second messengers [1].

The naturally occurring second messenger sphingosine has been shown to modulate Ca^{2+} release from skeletal and cardiac muscle cells, in part through direct actions on the sarcoplasmic reticulum ryanodine receptor [2–4]. Sphingolipids mediate a variety of cellular responses, including Ca^{2+} -dependent stimulus–secretion coupling [5], neutrophil activation [6], cell proliferation [7,8] and apoptosis [9]. Ceramide, sphingosine and sphingosine 1-phosphate are three biologically active sphingolipid-derived second messengers [9,10]. Sphingosylphosphocholine (SPC) is able to induce Ca^{2+} release from sarcoplasmic reticulum membranes isolated from skeletal muscle [4]. SPC-induced Ca^{2+} release has also been reported in cultured smooth muscle cells, known to contain mainly inositol 1,4,5-trisphosphate (IP_3) receptors [11,12], and in Swiss 3T3 fibroblasts [7]. However, the SPC-induced release was insensitive to heparin, a blocker of IP_3 -induced Ca^{2+} release [12]. Recently we provided evidence that SPC elicits Ca^{2+} release from brain microsomes by activating the ryanodine receptor rather than the IP_3 receptor [13]. SPC-

induced Ca^{2+} release from intracellular stores has also been described in rat basophilic leukaemia cells [14] and in human endothelial cells [15] that involves a novel intracellular Ca^{2+} channel [sphingolipid Ca^{2+} -release-mediating protein of endoplasmic reticulum (SCaMPER)], which has recently been cloned and when expressed in oocytes caused SPC-induced Ca^{2+} release [16].

In the present study, we provide evidence that SPC is able to induce Ca^{2+} release through direct action on the cardiac ryanodine receptor Ca^{2+} -release channel, and we consider the possible involvement of SCaMPER [16].

EXPERIMENTAL

Materials

[9,21- ^3H]Ryanodine (30–60 Ci/mmol) was purchased from New England Nuclear, and unlabelled ryanodine was from RBI (Natick, MA, U.S.A.). SPC and sphingosine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [α - ^{32}P]dUTP was from Dupont NEN (Boston, MA, U.S.A.). All other chemicals were of analytical grade. For all the biochemical assays, sphingolipids were delivered as DMSO solutions, keeping the maximum solvent concentration below 1 %. In control experiments this concentration of DMSO did not have any effects on Ca^{2+} release measurements.

In our work, SPC action on the Ca^{2+} -release channel was tested in the presence of different amounts of membrane protein depending on the type of assay. Because of its amphipathic

Abbreviations used: SPC, sphingosylphosphocholine; SCaMPER, sphingolipid Ca^{2+} -release-mediating protein of endoplasmic reticulum; IP_3 , inositol 3,4,5-trisphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ORF, open reading frame.

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nature, SPC is known to be preferentially partitioned into membranes because of so-called membrane surface dilution [17]. Therefore the free sphingolipid concentration is critically dependent on the amount of membrane protein. For the purpose of taking into account the membrane surface dilution effect, in some figures SPC concentration values are also plotted as mol %, i.e. the molar percentage of the SPC added relative to the amount of membrane phospholipids present in the assay.

Sarcoplasmic reticulum membrane preparation

A microsomal membrane fraction rich in the ryanodine receptor Ca^{2+} -release channel was obtained from the left ventricle of adult canine hearts as previously described [2,18].

Calcium release measurements

Measurements of Ca^{2+} release were performed spectrophotometrically using the dye antipyrilazo III as a Ca^{2+} sensor according to modifications of procedures described previously [19,20]. Microsomes (200–300 mg/ml) were preloaded with Ca^{2+} at 37 °C in 1 ml of medium consisting of 100 mM KCl, either 7.5 mM sodium pyrophosphate or 12.5 mM potassium phosphate as precipitating anions, 0.25 mM antipyrilazo III, 1 mM MgATP, 5 mM phosphocreatine, 20 mg/ml creatine kinase and 20 mM Mops, pH 7.0. Calcium accumulation and release were followed by the difference in absorbance between 710 nm and 790 nm using a Hewlett–Packard 8451A diode array spectrophotometer. When all of the calcium had been taken up and the signal had returned to baseline, calcium release was initiated by the addition of SPC. The effects of various other compounds on release were tested by their addition just prior to the addition of SPC, except in the case of the ryanodine experiments, in which ryanodine was added prior to loading vesicles with Ca^{2+} .

[^3H]Ryanodine binding

[^3H]Ryanodine binding measurements were made on isolated cardiac microsome vesicles as described previously [2]. Samples of 50 μg of microsomal protein were incubated in duplicate at 37 °C for 3 h in 1.0 ml of 250 mM KCl, 15 mM NaCl, 20 mM Hepes, pH 7.1, and 5 nM [^3H]ryanodine. Various free Ca^{2+} concentrations were obtained by the addition of CaCl_2 alone or by buffering with 1 mM EGTA, as calculated on the basis of the stability constants published by Fabiato [21]. Bound [^3H]ryanodine was measured by Millipore filtration and liquid scintillation counting. Non-specific binding was determined in the presence of 10 μM unlabelled ryanodine.

Cell preparations and whole-cell patch-clamp

Adult rat ventricular myocytes were prepared using the method of Yazawa et al. [22], as previously described [3]. Measurements of Ca^{2+} transients were performed by monitoring Indo-1 fluorescence emission with a Photon Technologies Inc. (East Brunswick, NJ, U.S.A.) dual-emission photometry system interfaced to a Nikon Diaphot microscope [3]. Cells were electrically paced to contract at 0.3 Hz during the fluorescence measurements. Membrane potentials were monitored under a current clamp using a List EPC-7 patch-clamp. Calcium current measurements were performed under conditions whereby only the current carried by Ca^{2+} through the L-type channel was measured; the cells were held at -50 mV, and 70 ms depolarizing pulses were made to 0 mV every 10 s [3].

Isolation of total RNA

Total cellular RNA was isolated separately from rat heart and extensor digitorum longus muscles, brain, liver and kidney using the classical guanidinium isothiocyanate method (Ultraspec kit; Biotecx, Houston, TX, U.S.A.). The amount of RNA was estimated by UV absorption at 260 nm, and the ratio of absorbance at 260 nm to that at 280 nm was checked. Typically, 100 μg of total RNA was obtained from 100 mg of tissue. Degradation of RNA samples was monitored by the observation of appropriate 28 S/18 S rRNA ratios, as determined by ethidium bromide staining of the agarose gels.

cDNAs for RNA analysis

The cDNAs used as hybridization probes were as follows. (1) A canine 0.54 kb cDNA (SCaMPER-ORF) corresponding to the cDNA encoding SCaMPER. SCaMPER-ORF was ligated between the *Xba*I and *Eco*RI sites of a pBluescript SK vector, placing the 5' end of the open reading frame (ORF) downstream of the T7 promoter. Orientation was confirmed by restriction digestion and nucleotide sequencing. (2) A rat cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion, Austin, TX, U.S.A.) was used as internal marker for standardization of the amount of RNA. (3) A mouse 152 bp cDNA (clone C32) corresponding to the cytoplasmic loop linking repeats II and III of the cardiac isoform of the dihydropyridine receptor α 1-subunit. This cDNA was subcloned in the *Pst*I site of a pBluescript vector from a 928 bp cDNA kindly provided by Dr. N. Chaudhari (Colorado State University, CO, U.S.A.).

Ribonuclease protection assay

Antisense RNA probes were produced using T3 and T7 polymerases (Stratagene, La Jolla, CA, U.S.A.) on linearized SCaMPER-ORF and GAPDH templates respectively in the presence of [α - ^{32}P]dUTP. Ribonuclease protection assays were carried out using the RPA II kit (Ambion), hybridizing 20–30 μg of total RNA from each tissue source to $(0.5\text{--}1.0) \times 10^5$ c.p.m. of the RNA probe. The RNA was run on a 5 % polyacrylamide/8 M urea gel. Undigested RNA probes were also run on the same gel to be used as size markers. The sizes of the full-length SCaMPER-ORF and GAPDH probes were ~ 290 and ~ 404 bases respectively. The expected sizes of the protected fragments were ~ 170 and ~ 350 bases respectively. Gels were exposed at -80 °C on X-ray films (X-OMAT AR; Eastman Kodak, Rochester, NY, U.S.A.) with two intensifying screens for up to 4 days.

Quantification of mRNA expression

Autoradiograms were subjected to densitometric analysis using the Lynx Densitometer program (They Lynx 5000 Digital Imaging Analysis System). The intensity of hybridization with the SCaMPER-ORF probe was corrected for variations in gel loading (using A_{260}), in some cases by normalizing to the intensity of hybridization of the same RNAs to the RNA probe for GAPDH.

RESULTS

SPC induces calcium release from cardiac microsomes rich in the ryanodine receptor. Cardiac microsomes were loaded with sequential additions of 12.5 nmol of Ca^{2+} and then challenged with SPC. A concentration of 50 μM SPC was able to induce significant Ca^{2+} release (0.95 ± 0.27 $\mu\text{mol}/\text{min}$ per mg of protein; mean $\pm$ S.E.M., $n = 4$); this was nearly completely abolished by preincubating the membranes with 300 μM ryanodine, indicating

Figure 1 SPC-induced calcium release from cardiac microsomes: effect of ryanodine

Canine cardiac microsomes (0.4 mg of protein) were actively loaded with 4×12.5 nmol of CaCl_2 (downward arrow; only the last dose is shown) in the presence of 12.5 mM potassium phosphate as a precipitating anion, as described in the Experimental section. After uptake was complete, Ca^{2+} release was induced by the addition of 50 μM SPC (upward arrow) in the absence and in the presence of 300 μM ryanodine. The final upward deflection (●) at the end of each trace represents 12.5 nmol of CaCl_2 added for calibration purposes. The data are representative of at least three experiments at different SPC and ryanodine concentrations which gave similar results.

that the observed Ca^{2+} release occurred through the ryanodine receptor calcium-release channel (Figure 1). The involvement of the ryanodine receptor was also confirmed by the ability of Ruthenium Red (3 μM) and sphingosine (20 μM) to antagonize SPC-induced Ca^{2+} release (Figures 2A and 2B respectively), since both of these agents [1,2,4] have been shown to block the ryanodine receptor.

To test the remote possibility that the effect of SPC on calcium release was a result of a severe inhibitory effect on the Ca^{2+} pump, we added 20 μM cyclopiazonic acid, an inhibitor of the Ca^{2+} pump [23], just before challenging the vesicles with 52 μM SPC. The resulting rate of SPC-induced Ca^{2+} release was increased in the presence of the Ca^{2+} pump inhibitor, as expected (Figure 2C), indicating that SPC stimulates the Ca^{2+} -release channel and does not cause Ca^{2+} release by inhibiting the sarcoplasmic reticulum Ca^{2+} pump.

The rate of SPC-induced calcium release increased with increasing SPC concentration, showing a steep dose-dependence (Figure 3). However, at high SPC concentrations, SPC-induced Ca^{2+} release was less sensitive to the action of Ruthenium Red. In fact, as shown in Figure 3, lower inhibition by Ruthenium Red was observed with increasing SPC concentration. The amount of SPC-induced Ca^{2+} release, calculated as the percentage of calcium released as compared with that released by 2 $\mu\text{g}/\text{ml}$ A23187, was approx. 70–80 % of loaded calcium over all SPC concentrations tested.

We further investigated the action of SPC on the ryanodine receptor by studying the activation of [^3H]ryanodine binding to cardiac microsomes induced by SPC, since ryanodine binding to the receptor is dependent on the functional state of the channel, i.e. it is maximal when the channel is fully open and minimal when it is closed [24,25]. We tested the effects of SPC at pCa 6.0 (Figure 4), a condition where the ryanodine receptor calcium channel is only partially open. We found that, at pCa 6.0, 2 μM SPC increased [^3H]ryanodine binding from 0.225 ± 0.026 to 0.393 ± 0.017 pmol/mg of protein (means $\pm$ S.E.M., $n = 12$, $P < 0.001$). The effect of SPC was specific, as sphingomyelin did not

Figure 2 SPC-induced calcium release from cardiac microsomes: effects of Ruthenium Red and sphingosine

Canine cardiac microsomes (0.4 mg of protein) were loaded with 8×25 nmol of CaCl_2 (downward arrowheads; only the last three doses are shown) in the presence of 7.5 mM sodium pyrophosphate as a precipitating anion, as described in the Experimental section. After uptake was complete, 52 μM SPC (upward arrows) was added to elicit Ca^{2+} release from the vesicles. Ruthenium Red (RR, 3 mM; **A**), 20 μM sphingosine (SPH; **B**) or 20 μM cyclopiazonic acid (CA; **C**) was added just prior to SPC addition. The final upward deflection at the end of each trace (●) represent 12.5 nmol of CaCl_2 added for calibration purposes in the presence of the different compounds used. The data in (**B**) and (**C**) are representative of at least two experiments which gave similar results. Results with Ruthenium Red were quite variable; of six experiments performed, that shown here (**A**) gave the second largest inhibitory effect.

augment [^3H]ryanodine binding under identical conditions (results not shown), whereas sphingosine inhibited binding with an IC_{50} of approx. 2 μM (Figure 4). At pCa 4.0, a Ca^{2+} concentration where the ryanodine receptor is maximally activated, SPC was not able to stimulate [^3H]ryanodine binding further (Figure 5). On the other hand, sphingosine showed the same inhibitory effects on ryanodine binding at pCa 4.0 and at pCa 6.0, as previously shown [2].

That the activation of ryanodine binding by SPC is limited to low Ca^{2+} concentrations was confirmed by investigating the effect of SPC on the calcium-dependence of ryanodine binding. Figure 6 shows the typical bimodal, bell-shaped, dependence of [^3H]ryanodine binding on pCa. The data indicate that 2 μM SPC was able to shift the bell-shaped curve, but only at pCa values between 6 and 5, suggesting that SPC might increase the sensitivity to calcium of the calcium-induced calcium-release mechanism.

We have demonstrated that sphingosine is able to block myoplasmic calcium transients and L-type channel conductance in primary neonatal and adult rat myocytes [3], and we could distinguish the actions on the ryanodine receptor from those on the L-type Ca^{2+} channel. In the present work we performed similar experiments, testing SPC for its effects on intracellular Ca^{2+} transients in adult rat ventricular myocytes. Ca^{2+} transients were first recorded from cultured myocytes paced electrically. After the electrical pacing was turned off, normal Tyrode's solution was replaced twice by a Ca^{2+} -free Tyrode's solution. The subsequent addition of 25 μM SPC induced a significant and

Figure 3 Dose-dependent SPC-induced calcium release from cardiac microsomes

Canine cardiac microsomes (0.4 mg of protein) were actively loaded as described in the legend to Figure 2, and the rate of calcium release after SPC addition was measured (○). Experiments performed in the presence of 3 μM Ruthenium Red (●) are also shown. The broken line represents the curve fitting of the data ($r = 0.84$) showing the reduced effect of Ruthenium Red at increasing SPC concentrations. SPC concentration is expressed both as μM and as mol% (see the Experimental section). Data are means $\pm$ S.E.M. of the numbers of experiments indicated in parentheses.

Figure 4 Dose-dependent effects of SPC and sphingosine on ryanodine binding at pCa 6.0

Ryanodine binding was measured using 50 μg of cardiac microsomes incubated for 3 h at 37 °C in 1.0 ml of 20 mM Hepes (pH 7.1), 0.25 M KCl, 15 mM NaCl, 1 mM EGTA, 0.816 mM CaCl₂ and 5 nM [³H]ryanodine in the presence of 2 μM SPC or 10 μM sphingosine (SPH). The free calcium concentration (pCa 6.0) was calculated on the basis of the stability constants published by Fabiato [21]. Bound [³H]ryanodine was determined by Millipore filtration and liquid scintillation counting. Non-specific binding was measured in the presence of 10 μM unlabelled ryanodine. The sphingolipid concentration is expressed both as μM and as mol% (see the Experimental section). Data are means $\pm$ S.E.M. of four experiments performed in triplicate.

progressive rise in the diastolic calcium concentration that soon reached values able to evoke the appearance of spontaneous calcium transients, the frequency of which consistently increased

Figure 5 Dose-dependent effects of SPC and sphingosine on ryanodine binding at pCa 4.0

Ryanodine binding was measured as in Figure 4 using 50 μg of cardiac microsomes incubated with 20 mM Hepes (pH 7.1), 0.25 M KCl, 15 mM NaCl, 5 nM [³H]ryanodine and 100 μM CaCl₂ for 3 h at 37 °C, in the presence of either 2 μM SPC or 10 μM sphingosine (SPH). Bound [³H]ryanodine was determined by Millipore filtration and liquid scintillation counting. Non-specific binding was measured in the presence of 10 μM unlabelled ryanodine. The sphingolipid concentration is expressed both as μM and as mol%. Data are means $\pm$ S.E.M. of four experiments performed in triplicate.

Figure 6 Effect of SPC on the activation of ryanodine binding by free calcium

Ryanodine binding was measured using 50 μg of cardiac microsomes incubated either in the absence or in the presence of 2 μM SPC in 20 mM Hepes (pH 7.1), 0.25 M KCl, 15 mM NaCl and 5 nM [³H]ryanodine for 3 h at 37 °C. Free calcium concentrations (from pCa 7 to 5) were adjusted with 1 mM EGTA using the stability constants published by Fabiato [21]. Data are means $\pm$ S.E.M. of three experiments performed in triplicate.

with the increase in diastolic calcium concentration (Figure 7A). To investigate whether SPC mobilizes Ca²⁺ from the sarcoplasmic reticulum or from a different intracellular store, diastolic Ca²⁺ changes were also monitored after the sarcoplasmic reticulum had been depleted of calcium by thapsigargin treatment (Figure 7B). Thapsigargin added to paced myocytes caused a progressive decrease in the amplitude of the Ca²⁺ transients, culminating in the complete suppression of the response. The subsequent addition of caffeine failed to release calcium from the sarcoplasmic reticulum, confirming that thapsigargin had effectively unloaded all the sarcoplasmic reticulum calcium. Under these

Figure 7 Effects of SPC in cultured adult ventricular myocytes

(A) Adult rat ventricular myocytes were paced for 40 s, and then the pacing was turned off. At 60 and 74 s the BSA/Tyrodé's buffer was removed and replaced with a Ca-free Tyrodé's. At 136 s, 50 μ M SPC was added. (B) To adult paced rat myocytes was added 0.75 μ M thapsigargin after 40 s. The amplitude of the calcium transients was almost completely inhibited. At 120 and 140 s, BSA/Tyrodé's was removed and replaced with a Ca-free Tyrodé's. To verify that the sarcoplasmic reticulum was empty of Ca^{2+} , 5 mM caffeine was added. SPC (50 μ M) was added at the indicated time. The intracellular free Ca^{2+} concentration is expressed as the ratio of indo-1 fluorescence emission at 405 nm and 485 nm. Traces are typical of several experiments with similar results.

conditions, the rise in diastolic calcium concentration produced by subsequent SPC addition was greatly reduced and no spontaneous calcium transients were observed (Figure 7B). These data suggest that the major calcium compartment acted upon by SPC is the sarcoplasmic reticulum. Furthermore, the rise in intracellular calcium evoked by SPC was not caused by depolarization of the membrane to the threshold value, since we found that 50 μ M SPC did not alter the resting membrane potential, causing less than 5 mV depolarization (results not shown). We excluded the possibility of sarcolemmal leakiness, since myocytes retained the indo-1 stain.

We also tested the possibility that SPC might cause Ca^{2+} transients by stimulating L-type calcium channel activity. The effect of SPC on calcium currents was investigated using the whole-cell configuration of the patch-clamp technique. Whole-cell calcium currents were recorded under conditions where only L-type currents were measured [3]. Under these conditions, 25 μ M SPC did not cause any increase in L-type calcium current (Figure 8A), that could be attributed to the BSA carrier (Figure 8C and [26]). As expected, sphingosine (25 μ M) inhibited L-type calcium currents (Figure 8B), as demonstrated previously [3].

The presence of significant Ruthenium Red-insensitive SPC-induced Ca^{2+} release suggested the possible involvement of another SPC-sensitive efflux mechanism in cardiac sarcoplasmic reticulum. Since SPC has been shown to induce Ca^{2+} release from intracellular stores in other cell types containing ScaMPER [14–16], we utilized an RNase protection assay to determine the

Figure 8 Effects of SPC on L-type Ca^{2+} currents in rat ventricular myocytes

Representative traces of calcium currents obtained by depolarizing adult dissociated ventricular myocytes from -50 to 0 mV as previously described [3]. Ca^{2+} currents were recorded from adult rat ventricular myocytes before and after the application of 25 μ M SPC (A), 25 μ M sphingosine (SPH) (B) or the BSA carrier alone (C).

Figure 9 Ribonuclease protection assay showing the presence of ScaMPER mRNA in various rat tissues

(A) The levels of GAPDH and ScaMPER mRNAs were revealed using the following amounts of total RNA: 35 μ g of heart RNA (lanes 1 and 2), 16 μ g of extensor digitorum longus (EDL) skeletal muscle RNA (lane 3) and 30 μ g of brain RNA (lane 4). (B) The levels of cardiac dihydropyridine receptor (Card DHPR) and ScaMPER mRNAs are compared using 35 μ g of total heart RNA in each lane.

Table 1 Quantification of ribonuclease protection assay results for ScaMPER

Data are expressed in all cases relative to the value for basophilic leukaemia cells, and are means $\pm$ S.E.M. of the numbers of measurements indicated in parentheses.

	ScaMPER mRNA, normalized with respect to:	
	Total RNA applied	GAPDH mRNA
Heart	1.40 $\pm$ 0.14 (4)	2.07 $\pm$ 0.37 (4)
Skeletal muscle	1.05 $\pm$ 0.17 (4)	2.79 $\pm$ 1.56 (3)
Brain	1.13 $\pm$ 0.23 (3)	1.35 $\pm$ 0.17 (3)
Basophilic leukaemia cells	1.0 (2)	1.0 (2)

expression of ScaMPER in certain rat tissues, including heart. Whether results were normalized with respect to the total amount of RNA applied to the gels or to the levels of GAPDH mRNA,

significant amounts of mRNA encoding SCA_{MPER} were found, not only in cardiac muscle, but also in skeletal muscle and brain. As seen in Figure 9(A) and Table 1, levels in these tissues were comparable with, if not higher than, those found in the rat basophilic leukaemia cells originally used to characterize the SCA_{MPER}-mediated Ca²⁺ release induced by SPC [14]. In addition, as much SCA_{MPER} mRNA as mRNA for the cardiac dihydropyridine receptor was found in rat heart (Figure 9B).

DISCUSSION

Numerous studies have demonstrated that the sarcoplasmic reticulum calcium-release channel is regulated by various endogenous modulators, including Ca²⁺, Mg²⁺, ATP, calmodulin and lipid-derived second messengers such as phosphoinositides, fatty acid acyl esters and sphingosine (see [1] for a review).

SPC is a putative lipid second messenger, derived from sphingomyelin by *N*-deacylation, that is known to alter a variety of cellular functions [10]. Foremost among the effects of SPC is its ability to induce the release of Ca²⁺ from intracellular pools in a variety of cell systems [5,11,12,14,15,26]. It has been proposed that control of cellular Ca²⁺ by SPC may be the basis of its potent mitogenic activity [7,8]. We have previously shown that SPC induces Ca²⁺ release from skeletal muscle terminal cisternae by a direct action on the ryanodine receptor [4] and, more recently, we presented evidence that SPC is able to induce Ca²⁺ release from brain microsomes by mobilizing ryanodine-sensitive Ca²⁺ pools [13]. Furthermore, a direct effect of SPC on Ca²⁺ release has also been shown in cultured smooth muscle cells [12], in rat pancreatic acinar cells [5], in rat basophilic leukaemia cells [14] and in human endothelial cells [15]. In all cases, the release was not mediated through the IP₃ receptor, since heparin, a potent blocker of IP₃-induced Ca²⁺ release [27], was ineffective in preventing SPC-induced Ca²⁺ release. A rapid rise in intracellular calcium without an increase in the levels of inositol phosphates has also been observed in Swiss 3T3 fibroblasts [7,8]. On the other hand, it has been suggested that, in thyroid FRTL-5 cells [28] and HL60 leukaemia cells [29], SPC induces Ca²⁺ release from IP₃-sensitive Ca²⁺ pools. More recently, van Koppen et al. [30] demonstrated that the exogenous addition of sphingosine 1-phosphate and SPC mobilizes Ca²⁺ in many different cell types by interaction with a G-protein-coupled receptor in the plasma membrane.

In the present study we provide evidence that SPC stimulates Ca²⁺ release from the cardiac sarcoplasmic reticulum by activating the ryanodine receptor. SPC selectively releases Ca²⁺ from cardiac microsomal vesicles that are rich in the ryanodine receptor calcium-release channel. The action of SPC on the ryanodine receptor is demonstrated by the observation that ryanodine, Ruthenium Red and sphingosine, potent ryanodine receptor blockers [1], antagonized the SPC-induced Ca²⁺ release. In the absence of ATP, SPC also stimulated [³H]ryanodine binding to isolated cardiac sarcoplasmic reticulum vesicles, further suggesting a direct SPC-ryanodine-receptor interaction. We tested the effect of SPC at Ca²⁺ concentrations in the micromolar range, a condition where the ryanodine receptor channel is partially open and is susceptible to further opening [24,25]. At pCa 6.0, 2 µM SPC nearly doubled the binding of ryanodine. Higher SPC concentrations were less effective in stimulating binding.

Ryanodine binding experiments also showed that SPC opens the Ca²⁺-release channel in a Ca²⁺-dependent manner. The binding of [³H]ryanodine to isolated cardiac sarcoplasmic reticulum membranes is progressively activated by free Ca²⁺ concentrations from pCa 7 to pCa 4, and inactivated in the pCa

range 2–4 (Figure 6) [1,31]. This biphasic Ca²⁺ dependence is due to the existence in the Ca²⁺-release channel of both high-affinity and low-affinity Ca²⁺-binding sites. The occupancy by Ca²⁺ of these sites leads to activation or inactivation of the channel respectively [1]. Our data indicate that SPC modulates the channel by influencing the high-affinity Ca²⁺-binding sites only, while it is ineffective on the low-affinity sites, since SPC did not modify ryanodine binding at Ca²⁺ concentrations higher than pCa 4. We therefore suggest that the action of SPC on the Ca²⁺-release channel might be to increase the sensitivity to calcium of the calcium-induced calcium-release mechanism, i.e. for the same calcium concentration, the ryanodine receptor is open to a greater extent in the presence of SPC and, as a consequence, more calcium is released.

The above interpretation is also supported by the results obtained on exogenous addition of SPC to rat cardiac myocytes. SPC produced a significant increase in the diastolic calcium concentration in intact adult cardiac myocytes to levels that lead to the occurrence of spontaneous calcium transients. Other lipid second messengers also affect the L-type Ca²⁺ channel [32,33]. Our results indicate that SPC is effective on the ryanodine receptor rather than the dihydropyridine receptor. The ability of SPC to cause Ca²⁺ release occurred in the absence of extracellular calcium and was almost completely abolished when the sarcoplasmic reticulum was depleted of calcium by thapsigargin treatment.

SPC-induced Ca²⁺ release, especially at high SPC concentrations, was not completely blocked by the ryanodine receptor blockers. Although it has been reported that ryanodine-insensitive leakage may still involve ryanodine receptors [34], a component of SPC-induced Ca²⁺ release still remained in the combined presence of bastadin 5 and Ruthenium Red. Thus we considered it necessary to investigate the possible involvement of other Ca²⁺-release pathways. Cl[−]-dependent calcium release not involving the ryanodine receptor has been reported in cardiac sarcoplasmic reticulum [35]. However, 10 mM clofibrate acid, a blocker of Cl[−]-induced Ca²⁺ release [35], was unable to antagonize SPC-induced Ca²⁺ release even in the presence of Ruthenium Red. Similarly, 100 µM verapamil, an inhibitor of nicotinic acid adenine dinucleotide phosphate induced Ca²⁺ release [36] as well as of L-type channels, did not abolish SPC-induced Ca²⁺ release even in the presence of Ruthenium Red. Recently a new intracellular SPC-gated calcium channel, SCA_{MPER}, distinct from other previously identified channels, has been discovered in rat basophilic leukaemia cells [14] and in human endothelial cells [15]. SCA_{MPER} has been cloned, and when expressed in oocytes it causes SPC-induced Ca²⁺ release from internal stores [16]. Using ribonuclease protection assays we have determined that SCA_{MPER} mRNA is expressed in rat heart at levels comparable with those of dihydropyridine receptor mRNA (Figure 9). In addition, SCA_{MPER} has been detected at similar levels in other tissues (brain, skeletal muscle) that yield SPC-sensitive microsomes that exhibit both Ruthenium Red-sensitive and -insensitive components of Ca²⁺ release [13,37]. However, since no specific antagonist of this channel has been described so far, we have been unable to test further its possible involvement in SPC-induced Ca²⁺ release from cardiac muscle sarcoplasmic reticulum.

On the other hand, SPC was without effect on L-type channel conductance and on the resting membrane potential. However, it is also possible that, besides its prevalent action on the sarcoplasmic reticulum, SPC may act on cardiac myocytes as it does in other cell types. It has been reported that SPC induces Ca²⁺ mobilization from internal stores by interaction with a G-protein-coupled receptor, leading to IP₃ production by phospholipase C activation [29,30], and IP₃-induced Ca²⁺ release has been reported

from cardiac sarcoplasmic reticulum [38]. However, 100 $\mu\text{g/ml}$ heparin (an inhibitor of IP_3 binding and IP_3 -induced Ca^{2+} release [27]) and 500 μM tetrapentylammonium and 100 μM quinidine (non-competitive inhibitors of IP_3 -induced Ca^{2+} release [39]) were unable to antagonize SPC-induced Ca^{2+} release.

We excluded a non-specific detergent-like effect of SPC because, in the Ca^{2+} release experiments, high concentrations of SPC did not increase membrane leakiness non-specifically, since the addition of the Ca^{2+} ionophore A23187, after SPC-induced Ca^{2+} release, caused additional Ca^{2+} release (results not shown). A non-specific detergent-like effect of SPC has been also excluded by other workers using the same SPC concentrations in permeabilized smooth muscle cells [11] and in thyroid FRTL-5 cells [28]. Furthermore, data from our cardiac myocyte experiments also demonstrated that SPC does not induce membrane leakiness, as judged by the ability of the myocytes to retain indo-1 and to remain polarized.

SPC and other lysosphingolipids, as well as sphingosine, have been proposed to act as endogenous modulators controlling a variety of cellular activities [10,17]. Most of their actions appear to be a direct consequence of the potent inhibition of protein kinases [17]. Our data indicate that SPC elicits Ca^{2+} release independently of its action on protein kinases, since SPC-stimulated ryanodine binding occurred in the absence of ATP, so effects of ryanodine receptor phosphorylation on ryanodine binding can be excluded. Furthermore, inhibition of kinases by the non-selective kinase inhibitor H-7 (25 μM) did not affect the ability of SPC to induce Ca^{2+} release (results not shown).

In conclusion, our data indicate that the putative second messenger SPC modulates intracellular Ca^{2+} levels in cardiac muscle by directly activating the ryanodine receptor Ca^{2+} -release channel of the sarcoplasmic reticulum. Because of technical problems in the quantitative extraction of SPC from muscle cells and other tissues, there are no reliable data on the SPC content of different cell systems. In preliminary unpublished studies using a modification of previously published procedures [2,40] used to measure endogenous sphingosine in cardiac tissue, we determined that rabbit cardiac muscle contains at least 12.7 ± 4.6 nmol of SPC/g wet wt. of muscle ($n = 3$), corresponding to approx. 15.6 μM . Similar amounts of SPC were found in rat cardiac muscle. Because of the above-mentioned technical problems, these values are likely to be underestimates. In any case, it seems that in cardiac muscle there is as much SPC as sphingosine [2,40], so that endogenous SPC levels seem to be similar to the range of concentrations for which we observed effects on cardiac muscle sarcoplasmic reticulum, suggesting that SPC may be a physiological regulator of cardiac calcium levels, particularly when sphingomyelinase is activated. The identification of ScaMPER as a novel calcium channel modulator in cardiac cells provides intriguing possibilities for future investigations, including whether or not ScaMPER is involved in excitation-contraction coupling or cardiac dysfunction.

This work was supported by institutional funds from the Consiglio Nazionale delle Ricerche and the Ministero della Pubblica Istruzione of Italy, and by grants from Telethon Italy (no. 469; R.B.), FATMA (G.S.), National Institutes of Health (no. HL42527; P.T.P.), Muscular Dystrophy Association (R.A.S.) and the American Heart Association (no. 941459; R.A.S.).

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