Control of Cardiac Ca\(^{2+}\) Levels

Inhibitory Actions of Sphingosine on Ca\(^{2+}\) Transients and L-type Ca\(^{2+}\) Channel Conductance

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Abstract The naturally occurring second messenger sphingosine (SPH) was examined for its ability to influence cardiac myocyte Ca\(^{2+}\) regulation. SPH inhibited intracellular Ca\(^{2+}\) transients in adult and neonatal rat ventricular myocytes. The inhibition was steeply dose dependent, with complete blockage of the Ca\(^{2+}\) transients occurring in the 20- to 25-\(\mu\)mol/L range. Whole-cell patch clamping revealed substantial inhibition of the L-type Ca\(^{2+}\) channel current (I\(_{\text{Ca}}\)) by SPH. The ability of SPH to block both the Ca\(^{2+}\) transients and I\(_{\text{Ca}}\) was not dependent on protein kinases, since the general protein kinase inhibitor H\(_7\) failed to prevent the actions of SPH. The specificity of the effect of SPH was determined in experiments showing that SPH analogues did not produce comparable effects. Neither the naturally occurring ceramide, N-stearoyl SPH, nor the cell-permeant ceramide, N-acetyl SPH, had SPH-like actions on the Ca\(^{2+}\) transients or L-type channel conductances. Caffeine-induced Ca\(^{2+}\) transients were also inhibited by the actions of SPH on cardiac sarcoplasmic reticulum Ca\(^{2+}\) release, and the threshold for caffeine-induced Ca\(^{2+}\) release was raised. We conclude that SPH inhibits excitation-contraction coupling in cardiac myocytes by reducing the amount of entering “trigger Ca\(^{2+}\)” for Ca\(^{2+}\)-induced Ca\(^{2+}\) release and by simultaneously raising the threshold of the ryanodine receptor for Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Consequently, we propose that sphingolipids produced by the sphingomyelin signal transduction pathway could be physiologically relevant regulators of cardiac [Ca\(^{2+}\)], and therefore cardiac contractility. (Circ Res. 1994;75:981-989.)

Key Words • sphingosine • ceramide • ryanodine receptors • cardiac myocytes • L-type channel

Sphingosine (SPH) is a putative second messenger that may participate in signal transduction through its ability to modulate various protein kinases\(^{1-7}\) and phospholipases.\(^8-9\) Recent studies using nonmuscle cells have demonstrated that SPH is also involved in protein kinase–independent signal transduction wherein SPH or its derivatives act on intracellular Ca\(^{2+}\) stores. For example, sphingolipids have been implicated in Ca\(^{2+}\)-dependent stimulus-secretion coupling,\(^10-12\) neutrophil activation,\(^13,14\) and cell proliferation.\(^15-19\) A sphingolipid-gated Ca\(^{2+}\) channel has recently been described for microsomes isolated from rat basophilic leukemia cells.\(^20\) Further, SPH blocks depolarization-induced Ca\(^{2+}\) fluxes across brain synaptosomes, thus lowering cytoplasmic Ca\(^{2+}\).\(^21\) Importantly, SPH and possibly its metabolite, SPH-1-phosphate (S1P), modulate Ca\(^{2+}\) release in permeabilized smooth muscle cells,\(^22\) implicating SPH as a regulator of Ca\(^{2+}\)-signaling pathways of both muscle and nonmuscle cells.

In striated muscle, excitation-contraction (EC) coupling involves the release of Ca\(^{2+}\) through a channel in the sarcoplasmic reticulum (SR) membrane, which is referred to as the ryanodine receptor (RyR) because of its affinity for the plant alkaloid ryanodine. It is likely that the physiological trigger for EC coupling in heart muscle cells is Ca\(^{2+}\) itself, entering via the voltage-regulated L-type Ca\(^{2+}\) channel (dihydropyridine receptor [DHPR]) of the T tubules and triggering a Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from the SR,\(^22-24\) (see Reference 25 for review).

Because of the role sphingolipids play in cellular Ca\(^{2+}\) signaling, we examined the potential involvement of sphingolipids in regulating EC coupling of striated muscle cells. SPH was previously shown to be capable of inhibiting Ca\(^{2+}\) release from skinned skeletal muscle fibers and from isolated skeletal muscle SR membranes.\(^26\) SPH also reduces \(^{3}H\)ryanodine binding to both skeletal and cardiac membranes containing the release channel, a result that is consistent with the blocking of the RyR channel by SPH.\(^26,27\) We also observed that the threshold for CICR was shifted by SPH such that higher levels of “trigger” Ca\(^{2+}\) were required to release Ca\(^{2+}\) from isolated cardiac microsomes containing the RyR.\(^27\) In chemically “skinned” adult rabbit cardiomyocytes lacking a functioning sarcolemma, SPH reduced the characteristic sarcomere activation waves without affecting contractile protein function.\(^28\) Since CICR is responsible for the sarcomere activation waves in these cells, the SPH effect was interpreted as a putative action of SPH on the CICR mechanism of the SR.

Although SPH has profound effects on isolated cardiac SR and permeabilized myocytes, the ability of SPH to influence Ca\(^{2+}\) levels in the intact cardiac cell has not previously been tested, nor has the effect of SPH on transsarcomemal Ca\(^{2+}\) fluxes been examined. Consequently, in the present study, we have examined the ability of SPH and various sphingolipid analogues to modulate the Ca\(^{2+}\) transients and L-type channel conductance of intact rat myocardial cells.

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Materials and Methods

Chemicals and Stock Solutions

SPH [(+)-erythro-2-amino-4-trans-octadecene-1,3-diol], sphingosylphosphorycholine (SPC [lysosphingomycin]), and C2 ceramide (N-acetyl-d-SPH) were purchased from Matreya, Inc; indo 1-AM, fluo 3-AM, and the pentaammonium salt of fluo 3 were obtained from Molecular Probes; and H7 was obtained from Seikagaku. All other chemicals, including stearoyl ceramide (N-stearoyl-d-SPH), DL-erythro-dihydrophosphoginosine (eDHSPH), and DL-threo-dihydrophosphoginosine (tDHSPH) were obtained from Sigma Chemical Co.

For the isolated myocyte experiments, stock solutions of SPH and other sphingolipids were prepared as complexes with fatty acid-free bovine serum albumin (BSA)13 so that the compounds could be delivered in the absence of micelles and without substantial solvent present. In control experiments, the delivery vehicle, BSA, did not by itself have any effects on the L-type Ca2+ current (ICa) or on the Ca2+ transients. Lambeth et al13 have shown that the dose-response curves are similar when SPH is added either as “free SPH” or as a complex with BSA. The ceramide, N-stearoyl-d-SPH, was prepared as a 400 chloroform:methanol (2:1)-stock solution, which was dried down in N2 before being diluted and dispersed into the final incubation media by bath sonication. Generally, sphingolipid solutions (0.1 mL) were added directly to the recording chamber (1.0-mL capacity) from a 1 × 10 stock solution containing BSA (direct-application method). In some experiments, sphingolipids were added by replacing the cell chamber contents by perfusion with 2.0 mL of the solution containing the final desired concentration of sphingolipid (slow perfusion method). The direct-application method yielded generally stronger effects than obtained by slow perfusion of the chamber but sometimes disturbed the gigaseals in patch-clamp experiments. The reason for the discrepancy is likely due to time-dependent differences in the amount of SPH initially seen by the cell under the two conditions. Unless otherwise indicated, results presented were obtained by the direct-application method.

Cell Preparation

Neonatal ventricular myocytes were dissociated and plated on fibronectin-coated glass coverslips in DMEM/F12 medium supplemented with 10% fetal calf serum (FCS) and antibiotics as previously described.29 After 24 hours in plating medium, the cells were rinsed and cultured for an additional 48 hours in medium containing 2% FCS.

Freshly dissociated adult ventricular myocytes were prepared from adult (200-350 g) Sprague-Dawley rat hearts by enzymatic dissociation as previously described.26 The Tyrode’s solution used for cell isolation contained (mmol/L) NaCl 140, KCl 5.4, MgCl2 0.5, CaCl2 1.0, HEPES 10, and NaH2PO4 0.25 (pH 7.3). Cells were stored in a KB solution31 containing (mmol/L) KCH2SO4 108, potassium phosphate 8.0, KCl 4.0, MgSO4 2.5, glucose 25, taunine 25, creatine 6.0, and pyruvic acid 6.0 (pH 7.3) at 4°C for a maximum of 24 hours before use, with little loss of viability. The adult rat myocytes were “Ca2+ tolerant” in that they did not supercontract in the presence of 1 mmol/L Ca2+ and were responsive to electrical field stimulation. To ensure prolonged viability, the adult cells were stored in KB solution and diluted into Tyrode’s solution before use.

Ca2+ Transient Methods

Fluorescence measurements were performed with a dual-emission photomultiplier system (Photon Technologies Inc) interfaced to a Nikon Diaphot microscope and a ×100 oil immersion objective. For the indo 1 measurements, excitation was at 355 nm. Fluorescence emission was split and monitored simultaneously at 405 and 485 nm (20-nm bandwidth) at a data collection rate of 20 Hz. The cells were loaded with the intracellular Ca2+ indicator indo 1 via 15-minute incubations with 3 μmol/L indo 1-AM. After loading, the cells were rinsed and placed in an air-compatible culture medium (bicarbonate-free DMEM buffered with 14 mmol/L HEPES and 15 mmol/L tricine-1.8 mmol/L Ca2+) or Tyrode’s solution (1 mmol/L Ca2+). Cells were electrically paced to contract at 0.3 Hz during the fluorescence measurements, unless otherwise noted. Fluorescence data is presented as the 405/485 emission ratio, which is a sensitive indicator of [Ca2+]i.32-34 Because of possible compartmentalization problems documented by others,32,35 all Ca2+ transient measurements were made at >1000 from nonnuclear regions of single myocytes. To further minimize compartmentalization, cells were loaded for brief (<15-minute) periods of time. Even with these short loading times, it is possible that some mitochondrial Ca2+ can contribute to the baseline indo 1 fluorescence; however, transmitochondrial Ca2+ fluxes contribute minimally (<2%) to whole-cell Ca2+ transients associated with cardiac contractions.26 The indo 1 fluorescence ratio was calibrated with solutions of varying Ca2+ (calibrations not shown) to ensure that the ratios reflected levels of intracellular Ca2+ in the micromolar range as reported by others.32-34

Whole-Cell Patch-Clamp Methods for Determination of ICa

Ca2+ currents were recorded as described by Zahradnik and Palade27 by use of the patch-clamp technique in the whole-cell configuration28 and a Dagan 3900 amplifier. The cells were bathed in external solution containing (mmol/L) NaCl 140, CsCl 3.0, MgCl2 0.5, CaCl2 1.0, HEPES 10, and NaH2PO4 0.25, along with 10 μmol/L tetrodotoxin (pH 7.3). Pipettes had resistances of 3 to 5 MΩ when filled with internal solution containing (mmol/L) cesium aspartate 120, CsCl 20, Na2ATP 3.0, disodium phosphocreatine 3.0, EGTA 10, CaCl2 0.65, MgCl2 3.5, and HEPES 5.0 (pH 7.3). All electrophysiological experiments were performed at room temperature (21°C to 23°C). Current signals were filtered at 2 kHz and sampled at 4 kHz. Data were analyzed with an MS-DOS personal computer and PCLAMP software (Axon Instruments).

Patch-clamp recordings were performed in the presence of tetrodotoxin to block inward Na+ currents and by replacing intracellular K+ with Cs+ in the pipette to block outwardly directed K+ currents. Thus, at a holding potential of ~50 mV, which inactivates T-type Ca2+ channel activity, the currents recorded under voltage-clamp conditions represent current carried exclusively by Ca2+ through the L-type channel. ICa was elicited and monitored by 70-millisecond depolarizing pulses to 0 mV every 10 seconds with a holding potential of ~50 mV. Peak ICa was elicited at +0 mV. During the monitoring of ICa for 5 minutes in the control condition, no significant rundown of ICa was observed (see Fig 6).

Simultaneous Measurements of ICa and Fluor 3 Ca2+ Transients

In a few experiments, adult myocytes were loaded with fluo 3 by 20-minute (room temperature) incubation in Tyrode’s solution containing 4 μmol/L fluo 3-AM. These cells were then patch-clamped as described above with 50 μmol/L fluo 3 pentaammonium salt added to the pipette solution instead of the EGTA/Ca2+ specified above. Ca2+ transients were recorded simultaneously with a Nikon Diaphot microscope interfaced to a Newmont digital video system and collected on a 505-nm dichroic mirror through a long-working ×20 fluor objective (Nikon). The emitted light was collected from the same objective and dichroic mirror and filtered with a 530-nm bandpass filter (Omega Optical). The optical tracing was filtered at 500 Hz and sampled at 4 kHz.

Results

SPH Blocks Cardiac Ca2+ Transients

Fig 1 shows the inhibitory effects of SPH on Ca2+ transients in a neonatal cardiac myocyte. SPH was
capable of rapidly (in 10 to 30 seconds) inhibiting the electrically evoked transients in both neonatal and adult cell preparations. The negative inotropic effect of SPH was independent of the type of myocyte used and was also present when several different bathing solutions and temperatures were used. For example, SPH inhibited intracellular Ca\(^{2+}\) transients when recordings were carried out either in air-compatible DMEM or Tyrode's solution at both room temperature and at 37°C without appreciable differences in response (data not shown). On the other hand, the myocytes were more sensitive to SPH when the agent was added by the direct-application method rather than by slow perfusion (see "Materials and Methods"). There were also some differences in the kinetics of SPH inhibition. For example, sometimes the transients diminished progressively before they were eliminated (as in Fig 1), and occasionally they abruptly ended (eg, see below [Fig 4]); these differences possibly reflect cell-to-cell variations in response to SPH.

Fig 2 shows that exogenously applied SPH reduced the magnitude of the Ca\(^{2+}\) transients of neonatal cultured myocytes in a dose-dependent manner. The curve was particularly steep in the 20- to 25-μmol/L range, with complete blockage of the transients occurring between 25 and 50 μmol/L SPH. In other experiments involving Ca\(^{2+}\) release from isolated SR vesicles (not shown), we have determined that the IC\(_{50}\) for SPH action depends on the ratio of SPH to total cellular lipid, and we have calculated that the IC\(_{50}\) is consistently ~5 mol%, which is similar to the amount of SPH (~0.7 μmol/L) required to inhibit protein kinase C (PKC).

The depressant effects of SPH on intracellular Ca\(^{2+}\) transients were largely reversible, as evidenced by the data shown in Fig 3. In this experiment, the transients were completely blocked with high SPH, as seen before. Then the cells were washed free of external SPH by several rinses over a 20-minute period with 0.2 mmol/L fatty acid–free BSA. After washout, the cells exhibited higher diastolic Ca\(^{2+}\) levels and exhibited both paced and spontaneous Ca\(^{2+}\) oscillations. The addition of BSA/Tyrode's solution by itself did not elicit spontaneous activity in nonpaced cells, and BSA was not required to reverse the SPH effect (not shown). The observed spontaneous activity and elevated diastolic Ca\(^{2+}\) seen in Fig 3 were similar to the effects of S1P on the myocytes (data not shown). Although S1P may be responsible for these effects, it is also possible that the oscillations represent spontaneous Ca\(^{2+}\) release from an overloaded SR.

**Effect of SPH Is Protein Kinase Independent**

Since SPH has been shown to inhibit several protein kinases, including PKC\(^{1,5,40}\) we tested the effects of SPH on the cultured myocytes under conditions that would either inactivate or enhance protein kinase activity. Fig 4A shows the typical response of control cells to SPH. These control cells were cultured for the same period as those shown in subsequent panels. As seen in Fig 4B, 30 minutes of preincubation with the general protein kinase inhibitor H7 did not prevent the rapid and complete blockage of the cardiac Ca\(^{2+}\) transients by SPH. In related experiments,
endogenous PKC levels were downregulated by 24 hours of pretreatment with 100 nmol/L phorbol 12,13-dibutyrate (PDBu) (Fig 4C). This protocol lowers endogenous PKC activity levels by >85%.[4] PKC-downregulated cells responded to SPH in a manner indistinguishable from that of control cells (Fig 4A) cultured for the same period but without the presence of PDBu. Finally, activation of PKC by acute (6.5-minute) PDBu (100 nmol/L) pretreatment did not alter the SPH-mediated inhibition of the transients (Fig 4D).

**Effects of SPH Analogues and Dihydrosphingosine**

To demonstrate the specificity of the effect of SPH on myocyte transients, we tested the abilities of other SPH analogues to affect cardiac Ca²⁺ transients. Fig 5A shows that 50 μmol/L N-acetyl SPH (C2 ceramide) slightly reduced the transients but was unable to completely block the transients in a SPH-like manner. N-Acetyl SPH is a synthetic cell-permeant ceramide that differs from SPH by the acylation of its primary amine (refer to chemical structure on the figure). The longer-chain-length naturally occurring ceramide, N-stearyl-p-SPH, was even less able to reduce the magnitude of the Ca²⁺ transients (Fig 5B) and did not prevent the complete inhibition produced by the subsequent addition of SPH. Neither N-stearyl-p-SPH nor N-acetyl-p-SPH was capable of significantly reducing the rate of caffeine-induced Ca²⁺ release from isolated canine cardiac SR membranes (data not shown). In contrast to the lack of significant effect of the N-stearyl and N-acetyl analogues of SPH, dihydrosphingosine (DHSPH) reduced the transients in an SPH-like manner, but by compared with SPH, DHSPH was slower than SPH in blocking the Ca²⁺ transients and did not always completely block them (Fig 5C). As seen in Fig 5C, DHSPH reduced the magnitude of the transients by about half. SPH is distinguished from DHSPH by the lack of the double bond in the hydrocarbon tail. The data in Fig 5C were from an experiment using eDHSPH. tDHSPH was equally effective in blocking the Ca²⁺ transients of neonatal cardiac cells (data not shown).

In a limited number of experiments, we also tested the effects of SIP on the Ca²⁺ transients and found that this metabolite of SPH also lacked the ability to block the transients in an SPH-like manner (not shown). SPC failed to block the electrically evoked Ca²⁺ transients (not shown); however, SPC produced a rapid and instantaneous release of Ca²⁺. SPC has been shown to mobilize Ca²⁺ from intracellular stores,[11,18,21] and we have recently shown that it can release Ca²⁺ from isolated skeletal SR membranes.[26] The results obtained by using a variety of SPH analogues suggest that nonspecific lipid effects do not account for the SPH action on cardiac Ca²⁺.

**SPH Blocks the Cardiac L-type Ca²⁺ Channel**

An important potential mechanism of SPH action on Ca²⁺ transients was investigated by examining the ability of the sphingolipid bases to affect whole-cell I_{Ca,L}. Fig 6A shows the time-dependent inhibition of L-type channel conductance after 25 μmol/L SPH addition. Addition of SPH led to nearly complete inhibition of L-type channel
conductance, with a time course similar to the inhibitory action of SPH on the contractile Ca\textsuperscript{2+} transients. Moreover, partial reversibility of the I\textsubscript{Ca} was observed after SPH washout (Fig 6A). SPH shifted the peak of the current-voltage relation of the L-type channel in a concentration-dependent fashion by \( \approx \)15 mV in the positive direction (data not shown). Fig 6B shows another patch-clamped cell before and after addition of the SPH analogue N-acetyl-d-SPH. Compared with SPH, N-acetyl-d-SPH was only marginally able to reduce I\textsubscript{Ca}. The subsequent addition of 25 \( \mu \)mol/L SPH to the bathing solution produced its typical inhibition of I\textsubscript{Ca}. In contrast to the profound SPH effect on L-type channel conductance, SPC was less able to modulate I\textsubscript{Ca} of patch-clamped myocytes (not shown). Curiously, the effect of SPC on the L-type channel was inhibitory, whereas it elicited intact myocyte Ca\textsuperscript{2+} transients in the absence of electrical stimulation (data not shown). Both panels of Fig 6 show that inhibition by SPH of the L-type channel was partially (\( \approx \)40%) reversible after SPH washout. It is significant that the Ca\textsuperscript{2+} transients also recovered after SPH washout (Fig 3).

In agreement with the data for contractile Ca\textsuperscript{2+} transients, H7 was unable to block the SPH-induced inhibition of L-type Ca\textsuperscript{2+} channel currents. In cells pretreated with 100 \( \mu \)mol/L H7 internally via the patch pipette, SPH (10 to 25 \( \mu \)mol/L) was able to reduce L-type channel conductance by 89\% (n=4). Addition of H7 to the bath solution gave identical results (89\% SPH inhibition). When SPH was applied to the cell by slow perfusion, rather than by direct application, H7 was also unable to block the SPH-induced changes in L-type channel conductance; however, the magnitude of the SPH inhibition was not as great (40\% inhibition when H7 was applied in the patch

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**Fig 5.** Tracings showing the effects of sphingosine (SPH) analogues on Ca\textsuperscript{2+} transients of neonatal ventricular myocytes. A, The SPH analogue N-acetyl SPH (C2) was used as a control to determine whether the inhibition by SPH of the Ca\textsuperscript{2+} transients was due to a nonspecific lipid effect. Although some small reductions in the transients were seen, the inability of C2 to block the transients in an SPH-like manner suggests that the effect of SPH was specific. B, A similar lack of effect was seen with N-stearoyl SPH (ceramide). C, Erythro dihydrosphingosine (eDHSPH) was tested for its potential effects on the Ca\textsuperscript{2+} transients. Fifty-micromolar aliquots of eDHSPH were added at the indicated times. The transients were blocked initially by 33\% after the addition of 50 \( \mu \)mol/L eDHSPH and by 70\% after the addition of 100 \( \mu \)mol/L total eDHSPH. At 190 seconds, the pacing was turned off, and 5 mmol/L caffeine (Caf) was added.

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**Fig 6.** Plots and tracings showing the effects of sphingosine (SPH) on the Ca\textsuperscript{2+} current (I\textsubscript{Ca}) of adult rat ventricular myocytes. Whole-cell patch-clamp currents were measured in adult dissociated ventricular myocytes before and after the application of various sphingolipids. A, Ca\textsuperscript{2+} currents recorded from adult rat ventricular myocytes before and after application of 25 \( \mu \)mol/L SPH. The partial recovery of I\textsubscript{Ca} is also shown after washout of SPH from the bathing medium. B, Lack of effect of N-acetyl SPH (C2) on I\textsubscript{Ca}. Ca\textsuperscript{2+} currents were recorded before and after the addition of 25 \( \mu \)mol/L C2. The subsequent addition of 25 \( \mu \)mol/L SPH blocked the channel. Holding potentials were \(-50\) mV throughout, and test depolarizations were to 0 mV. The direct-application method for the addition of SPH was used in these experiments.
pipette and 34±8% when H7 was applied externally). In control experiments, the extent of L-type channel inhibition by H7 varied from 3% to 55% (average, 21.6%), but in none of our experiments did H7 block the subsequent SPH effects. Although most of the electrophysiological experiments were performed on adult dissociated ventricular myocytes, we have confirmed that SPH inhibits I_{Ca} of cultured neonatal rat ventricular cells as well.

Relative Effects of SPH on the L-type Channel Versus the SR

Considering that SPH has effects on both L-type channel conductance and SR Ca^{2+} release and that the former triggers the latter under physiological conditions,^{42} the ability of SPH to block myocyte Ca^{2+} transients could be due to one or both of these sites of action. To distinguish the two sites of action, we examined the ability of caffeine to induce Ca^{2+} release from intracellular SR stores. It has been clearly demonstrated^{42-45} that electrically evoked Ca^{2+} transients of the type presented in the present study result primarily from release of Ca^{2+} from the SR even when the cells are bathed with Ca^{2+}. Fig 7A shows an experiment using a cultured myocyte in which the cell was stimulated to produce Ca^{2+} transients, after which time the electrical pacing was discontinued and the normal Tyrode’s solution was replaced by two washes with Ca^{2+}-free Tyrode’s solution. The cell was then challenged with 5 mmol/L caffeine and then subsequently with 20 mmol/L caffeine to elicit Ca^{2+} release from the SR. As seen in Fig 7A, a substantial Ca^{2+} transient was produced by the addition of 5 mmol/L caffeine. The lack of extracellular Ca^{2+} and the cessation of electrical pacing ensured that any Ca^{2+} transient observed under these conditions was due to SR Ca^{2+} release and not a result of transsarcolemmal Ca^{2+} fluxes. In a parallel experiment (Fig 7B), SPH was added before caffeine. As seen in the figure, 5 mmol/L caffeine was not able to induce SR Ca^{2+} release after SPH treatment. SR Ca^{2+} release was only elicited on the subsequent addition of an additional 20 mmol/L caffeine (total, 25 mmol/L caffeine). Thus, SPH effectively raised the threshold for caffeine-induced Ca^{2+} release. Moreover, the experiment in Fig 7B demonstrates that SPH does not appreciably alter the state of SR loading, since very high caffeine levels can ultimately release SR Ca^{2+} in the presence of SPH. Further, Fig 7B demonstrates that SPH by itself does not have the ability to elicit a Ca^{2+} transient. However, SPH was unable to completely block the caffeine response at a concentration of SPH (50 μmol/L) that abolished electrically induced Ca^{2+} transients, suggesting that the L-type channel may be more sensitive to SPH than is the ryanodine receptor.

To estimate how much of the effect of SPH on electrically stimulated Ca^{2+} transients was due to I_{Ca} inhibition and how much was due to the inhibition of CICR exerted at the level of the SR Ca^{2+} release channel, we performed a few experiments simultaneously monitoring Ca^{2+} transients with fluo 3 and I_{Ca} in adult ventricular myocytes under voltage-clamp conditions. As demonstrated in Fig 8, the reduction by SPH of I_{Ca} was associated with a reduced rate and magnitude of the Ca^{2+} transients. With ventricular myocytes, most of the Ca^{2+} transient is due to Ca^{2+} release from the SR,^{43,44} with only a minor component, estimated at <10%, due to Ca^{2+} influx through the L-type channel.^{46} The first derivative of the Ca^{2+} transient represents the rate of increase of [Ca^{2+}] in the myoplasm due to both processes, but the rate of Ca^{2+} release from the SR will make for the larger contribution to the d[Ca^{2+}]/dt tracing. Thus, the amplitude of this tracing represents a reasonable approximation of the relative rate of SR Ca^{2+} release.^{45} Since I_{Ca} is proportional to the amount of trigger Ca^{2+} entering via the DHPR, the rate of SR Ca^{2+} release should be graded according to the peak I_{Ca} amplitude.^{42} Close examination of Fig 8 shows that 30 seconds after addition, SPH appeared to inhibit I_{Ca} more than the rate or extent of SR Ca^{2+} release, but at 60 seconds the effects on all parameters appeared more similar. Notwithstanding nonlinearities in the fluorescence response of fluo 3 to Ca^{2+}, which might preclude precise quantification of particularly large changes in [Ca^{2+}], in a total

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**Figure 7.** Tracings showing the effect of sphingosine (SPH) on caffeine (Caf)-induced Ca^{2+} transients from neonatal ventricular myocytes measured with indo 1. A, Ca^{2+} transients were recorded from cultured myocytes under electrical pacing conditions for 20 seconds, after which time the stimulus was turned off, and the normal Tyrode’s solution was replaced by Ca^{2+}-free Tyrode’s solution. The subsequent addition of 5 mmol/L Caf and then 20 mmol/L Caf allowed for the release of sarcoplasmic reticulum (SR) Ca^{2+}. Note that bovine serum albumin (BSA) had no effect on the transients. B, The effect of SPH on the ability of caffeine to release SR Ca^{2+} is shown. Fifty-micromolar SPH (added at 37 seconds) prevented 5 mmol/L Caf-induced Ca^{2+} release but not Ca^{2+} release elicited by the addition of 20 mmol/L Caf.
of five experiments in which simultaneous measurements were made, SR Ca\(^{2+}\) release was inhibited on average to the same extent as \(I_{\text{Ca}}\). These data support the notion that the L-type channel is likely the primary site of action of SPH in these experiments.

**Discussion**

The commonly accepted mechanism for EC coupling in cardiac myocytes is CICR, in which Ca\(^{2+}\) entering the cell via the voltage-regulated T-tubule L-type Ca\(^{2+}\) channels serves as trigger Ca\(^{2+}\) to induce the release of activator Ca\(^{2+}\) from the SR.\(^{22-24}\) Our data demonstrate that the endogenous second messenger, SPH, can inhibit CICR both by reducing the entry of trigger Ca\(^{2+}\) and by inhibiting SR Ca\(^{2+}\) release through the RyR. Analysis of the data such as presented in Fig 8 suggests that the action of SPH on electrically evoked contractions is largely attributable to its inhibitory effects on the L-type channel, thus reducing the Ca\(^{2+}\) trigger. An additional effect could be due to its ability to selectively render the RyR/Ca\(^{2+}\) release channel less sensitive to this Ca\(^{2+}\) trigger. We have recently demonstrated that SPH can raise the threshold for CICR in isolated cardiac SR\(^{27}\) and that the CICR mechanism of skipped cardiac myocytes is attenuated by SPH.\(^{28}\) The ability of SPH to inhibit SR Ca\(^{2+}\) release in intact cells is also shown in the present study by the ability of SPH to block electrically evoked Ca\(^{2+}\) transients and by the ability of SPH to raise the threshold for caffeine-induced Ca\(^{2+}\) in Ca\(^{2+}\)-free media where transsarcolemmal Ca\(^{2+}\) fluxes cannot occur. However, concentrations of SPH (50 \(\mu\)mol/L) that would otherwise completely block the Ca\(^{2+}\) transients greatly attenuated but did not usually prevent 25 mmol/L caffeine–induced Ca\(^{2+}\) release.

SPH is a naturally occurring compound endogenous to the cardiac muscle.\(^{27,47}\) Although we reported that whole-cell SPH levels could be quite high (\(~\approx 20 \mu\)mol/g), free cytoplasmic SPH concentrations are \(\approx 0.5 \mu\)mol/L and represent only 1% to 2% of total cellular SPH. Although fluorescently labeled SPH has been shown to rapidly equilibrate across biological membranes and enter the cell, its intracellular location is likely membrane-associated.\(^{48}\) Therefore, the 50 \(\mu\)mol/L SPH used to completely block myocyte Ca\(^{2+}\) transients in the present study likely represented 0.5 to 1.0 \(\mu\)mol/L free cytoplasmic content of the sphingolipid. This is in the range over which we have previously shown SPH able to partially, but not completely, block isolated SR Ca\(^{2+}\) release.\(^{27}\) Since SPH is likely produced by the T-tubule membrane where the L-type channel (DHPR) is located, the level of endogenously produced SPH seen by the DHPR could be substantially higher than that seen by the RyR. Thus, it is possible that physiological levels of SPH in the cytosol may be too low to block the RyR directly but yet high enough in the surface membrane to substantially affect L-type channel conductance.

SPH is a potent inhibitor of protein kinases, including PKC and Ca\(^{2+}\)/calmodulin-dependent kinase (CaMK), and it has been proposed that sphingolipids contribute to kinase-dependent cell signaling.\(^{2,49-51}\) Our data indicate that the ability of SPH to control cardiac cell Ca\(^{2+}\) is independent of many protein kinases. This conclusion comes from the observation that the general protein kinase inhibitor H7 did not prevent the ability of SPH to modulate cardiac myocyte Ca\(^{2+}\) transients or L-type channel currents. These data are in agreement with our previous work showing that H7 did not prevent SPH inhibition of isolated SR Ca\(^{2+}\) release.\(^{2,27}\) Since the concentrations of H7 used in the present study (25 to 100 \(\mu\)mol/L) can inhibit PKC, protein kinase A, CaMK, and cGMP-dependent kinases,\(^{52,53}\) it is possible that none of these kinases mediate the effects of SPH on our cells. Downregulation of PKC by 24 hours of pretreatment with the phorbol ester PDBu also did not prevent the effects of SPH on whole-cell Ca\(^{2+}\) transients, and stimulation of PKC by acute PDBu treatment did not reduce the potency of the action of SPH on transients. However, it is possible that other less well described kinases could be responsible for SPH action. However, isolated SR experiments demonstrate that SPH inhibits ryanodine binding in the absence of ATP,\(^{27}\) thus excluding the involvement of protein kinases.

The inability of other sphingolipids like N-acetyl-D-SPH or N-stearoyl-D-SPH to reproduce the effect of SPH is evidence that SPH does not act via nonspecific membrane effects, particularly since these sphingolipids are more lipophilic than SPH. Compared with SPH itself, the analogues N-acetyl-D-SPH and N-stearoyl-D-SPH were not effective modulators of either myocyte cellular Ca\(^{2+}\) transients or L-type channel currents. Using isolated canine SR, we have also seen the relative lack of effects of N-acylated SPH derivatives on the rate of Ca\(^{2+}\) release (data not shown), similar to the findings on whole-cell Ca\(^{2+}\) transients seen in Fig 5A and the \(I_{\text{Ca}}\) measurements of Fig 6B. Since these sphingolipids differ from SPH by the acylation of the primary amine of sphingosine, it is possible that the ionizabile amino group of SPH is required for effective action on cardiac muscle Ca\(^{2+}\) channels. The ionizable amino group of
SPH is required for its inhibitory actions on PKCα and phospholipid A2.20 In addition, the reversibility of the effects of SPH on L-type channel conductance and the Ca2+ transients suggests that SPH does not produce nonspecific membrane effects that might alter the parameters tested in the present study. The finding that DHSPH was ≈50% as effective as SPH in blocking the Ca2+ transients suggests that the presence of the double bond on the hydrocarbon tail of SPH is important but not critical in determining SPH function. Our data are similar to the finding that DHSPH stimulates SPH-activated protein kinase ≈50% as well as SPH itself.27 Since both the three and erythro stereoisomers of DHSPH were effective in blocking the transients, the position of the third hydroxyl relative to the primary amine is not critical in determining SPH action.

In preliminary experiments, we have localized to T-tubule membranes the major enzyme responsible for sphingolipid synthesis, the neutral form of sphingomyelinase.20 Using high-performance liquid chromatography for analyses of isolated skeletal muscle membranes,27 we have determined that T tubules and not SR membranes are a major source of endogenous SPH, suggesting that the complete metabolic machinery for SPH synthesis is likely in the T-tubule membranes. It is significant that ceramides like N-stearoyl-D-SPH, which are the immediate precursors of SPH, are not able to block either the Ca2+ transients or L-type channel conductances of cardiac myocytes, nor are the ceramides able to block SR Ca2+ release in an SPH-like manner. Thus, although ceramides have important physiological actions in other cells (see Reference 50 for review), they do not appear to affect cardiac Ca2+. Also, it can be tentatively concluded that the SPH actions reported in the present study are not dependent on its enzymatic conversion to SIP. This conclusion is supported by the observations that (1) SIP does not block Ca2+ transients, (2) the effects of SPH on intact cell Ca2+ transients are nearly immediate and not temperature dependent in the 22°C to 37°C range (data not shown), and (3) SPH can block SR Ca2+ release in cell-free systems (presumably lacking the SPH kinase) and that action is immediate.

The sphingomyelin signal transduction pathway has received much attention as a novel system involved in the activation of a variety of cell types, including EL4 thymoma cells,25 HL60 cells,26 and GH3 pituitary cells,27 and has been postulated to participate in NFkB transcription factor activation28 and apoptosis.59 Ceramide is considered the principle second messenger responsible for most of these processes. However, our data indicate that SPH, which is also a component of the sphingomyelin pathway,26,27 might be important in Ca2+ signaling and contractility, especially in muscle cells. Moreover, in muscle, SPH may be more important in this regard than other components of the signal transduction pathway, such as ceramide.

In conclusion, the present data indicate that the second messenger, SPH, is capable of strongly influencing intracellular Ca2+ levels in cardiac ventricular myocytes. This effect may be due to the inhibition by SPH of both Ca2+ entry through the voltage-dependent Ca2+ channel and its inhibition of CICR. Since SPH is endogenous to the cardiac cell, we propose that it may be an important second messenger, which when generated by cytokines, growth factors, or other stimuli, could influence cardiac contractility. Since SPH is likely produced in the same location (ie, the T tubule) where the DPHR is located, it is possible that SPH is a natural ligand for the DPHR. We are currently investigating this possibility in our laboratories.

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