Effect of sphingosylphosphorylcholine on the single channel gating properties of the cardiac ryanodine receptor

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Abstract The effects of the lysosphingolipid, sphingosylphosphorylcholine (SPC), on the cardiac ryanodine receptor (RyR) were examined. The open probability of cardiac RyR incorporated in lipid bilayers was decreased by cytoplasmic, but not lumenal side application of micromolar concentrations of SPC. Modification of channel function was characterized by the appearance of a long-lived closed state in addition to the brief channel closings observed in the presence and absence of SPC. Open channel kinetics and ion conduction properties, however, were not altered by this compound. These results suggest that SPC, a putative second messenger derived from sphingomyelin, may regulate Ca^{2+} release from the sarcoplasmic reticulum by modifying the gating kinetics of the RyR.

Key words: Heart; Ca^{2+} release; Ryanodine receptor; Sphingolipid; Single channel; Lipid bilayer

1. Introduction

Ca^{2+} release from the sarcoplasmic reticulum through the cardiac ryanodine receptor (RyR) has a central role in the excitation-contraction coupling mechanism of the heart [1,2]. During normal excitation-contraction coupling, the open probability of the RyR is regulated by the increase of cytosolic Ca^{2+} concentration produced by Ca^{2+} influx through the sarcolemmal L-type Ca^{2+} channels. In addition to Ca^{2+}, Mg^{2+} and ATP, potential endogenous modulators of the RyR have been identified [1–4]. Furthermore, it seems likely that endogenous modulators that target the RyR such as calmodulin [5], FK506 binding protein [6,7], arachidonic acid [8] and spermine [9] exist in the cardiac cell.

Sphingosylphosphorylcholine (SPC), a metabolite of the ubiquitous sarcolemmal constituent sphingomyelin, has been implicated as an important lipid second messenger in various cellular functions [10]. Betto et al. [11] recently showed that SPC activates ryanodine-sensitive Ca^{2+} release from sarcoplasmic reticulum vesicles isolated from heart muscle and in cardiac myocytes. However, their results could not distinguish whether SPC-induced Ca^{2+} release is due to a direct interaction with the RyR. For this reason, we examined the effect of SPC on single channel properties of reconstituted cardiac RyR in lipid bilayers.

The endogenous SPC level in the rabbit cardiac muscle has been estimated to be 16 μM [11]. This report shows that lower concentrations of SPC significantly modify the gating kinetics of the RyR so that long-lived closures are induced without changing open channel kinetics. Given these data, SPC should be considered a potential endogenous modulator of RyR function.

2. Materials and methods

2.1. Preparation of SR membranes and single channel current recording

Junctional sarcoplasmic reticulum (SR) membrane vesicles were prepared from rabbit ventricular muscles [12,13] as detailed by Uehara et al. (see [8], section on JSR membrane vesicle preparation). Single channel currents were measured after incorporation of membrane vesicles into planar lipid bilayers in which the *trans* chamber was grounded. Membrane potential was controlled with an amplifier using Ag/AgCl electrodes and KCl-agar bridges. Junction potentials were measured and the appropriate corrections were made. The orientation of the ryanodine receptor in the bilayer was established by the sidedness of ATP, Ca^{2+}, and Mg^{2+} sensitivity of the incorporated channels. During vesicle incorporation and single channel recording, the chambers contained symmetrical solutions composed of 210 mM CsOH, and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pCa 5, pH 7.4). The free calcium of all solutions was determined using the method described by Fabiato and Fabiato [14] and corrected by Tsien and Rink [15]. Experiments were performed at room temperature.

2.2. Data acquisition and analysis

The data were low-pass filtered at 2 kHz (fc), digitized at 8 kHz and analyzed using pCLAMP software (Axon Instruments, Burlingame, CA). The minimum detectable event duration (equal to $0.179 \times 2/fc$, i.e. the dead time of the system) was 179 μs. Slope conductances (Table 1) were calculated from the *I-V* curves (Fig. 2A) in which the unitary amplitudes were obtained by calculating the peaks of all-points histograms. Fundamental gating properties such as open probability, opening frequency, mean open and closed time (Table 1) were obtained from idealized current traces. Distributions of dwell time durations (Fig. 3) were fitted by the maximum log likelihood method [16]. Quantitative values of the gating parameters were obtained from 1 min of steady-state recording unless otherwise stated.

Burst analysis was conducted using Patch Analyst Pro (MT Corporation, Lake Oswego, OR) and pCLAMP software. The critical gap time was defined from a crossing point between the exponentially fitted curves of the two long-lived components in the closed time histogram for SPC-modified channels.

2.3. Chemicals

Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) used to form the planar lipid membrane were purchased from Avanti Polar Lipids (Alabaster, AL). Sphingosylphosphorylcholine (SPC) was from Biomol Research laboratory Inc. (La Jolla, CA). All other chemicals were from Sigma Chemical (St. Louis, MO).

2.4. Statistical methods

Data are expressed as mean ± S.E.M. Statistical analysis of the data was performed with Student's *t*-test. Mean values were considered significantly different when the *P* value was less than 0.05.

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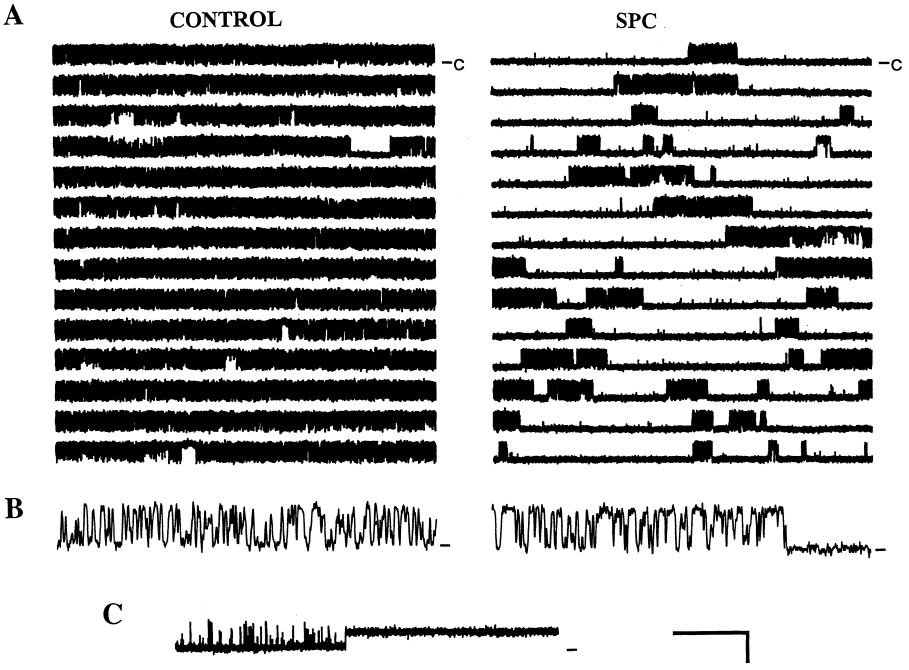

Fig. 1. Effect of SPC on RyR single channel current. A: Current traces before (left panel) and after (right panel) the *cis* side application of the agent. Note that long-lived closures were clearly induced by SPC application. B: Current traces in the control (left panel) and the SPC-modified (right panel) channel taken from A but shown at a faster time base. C: Identification of the RyR. In this experiment, *cis* Ca^{2+} concentration was 1 μM. The calibration bars of time and current amplitude represent 400 ms and 20 pA in A and C, and 25 ms and 9 pA in B, respectively. −C: closed state.

3. Results

3.1. Single channel gating modulated by SPC

The left panel of Fig. 1A shows representative current traces recorded under control conditions at the holding potential +30 mV. The concentration of *cis* Ca^{2+} was 10 μM. Fig. 1B is a selected current trace displayed with higher temporal resolution. Open events are shown as upward deflections separated by brief channel closings. The right panel of Fig. 1A shows current traces 1 min after *cis* side addition of 1 μM SPC. Application of SPC to the *cis* chamber corresponds to that of the cytoplasmic side of the reconstituted channel molecule. SPC decreased channel open probability and led to the appearance of intermittent channel openings. The fast time base sweeps of the SPC-modified channel in Fig. 1B showed fast open and closed transitions, similar to the channel under control conditions. Channel gating was unaltered by the vehicle solution containing ethanol, albumin and phosphate buffer without SPC in control experiments (data not shown, $n=4$).

As shown in Fig. 1C, single channel currents were consistently locked in an open state by the *cis* addition of 10 μM ryanodine, as was observed by Rousseau et al. [17]. This sensitivity to the plant alkaloid confirms that the channels were the RyR.

3.2. Single channel conductance and fundamental gating properties

Single channel conductance properties were examined in control and SPC-modified channels. The slope conductance calculated from the *I-V* curves as shown in Fig. 2A was 369 ± 12 pS ($n=5$) and 358 ± 13 pS ($n=5$) in the control and the 1 μM SPC-modified channel, respectively (Table 1). Thus, the conductance properties were not significantly different between the control and the SPC-modified channel ($P > 0.05$).

Single channel kinetic properties including open probabilities and opening frequency were also compared in control and 1 μM SPC-modified channels. Results of this analysis are summarized in Table 1 ($n=5$). Fig. 2B shows diaries of open probability for 30 s before (upper panel) and after the application of 1 μM SPC (lower panel). The open probability was 0.56 ± 0.06 ($n=5$) in the control channel and was significantly decreased to 0.13 ± 0.03 ($n=5$) in 1 μM SPC-modified channels. When the SPC concentration was increased from 1 μM to 5 μM, the open probability was further decreased to 0.078 ± 0.02 ($n=4$) (data not shown). The opening frequency also significantly decreased in 1 μM SPC-modified channels (Table 1). The mean open times in control and SPC-modified channels were not significantly different. On the other hand, the mean closed time increased significantly from 1.68 ± 0.16 ms ($n=5$) in control conditions to 11.67 ± 1.76 ms in SPC-modified channels (Table 1). Taken together, the data indicate that SPC decreases the rate of channel opening without affecting ion permeation properties of the RyR.

Table 1
Single channel properties

	Control	SPC
Open probability (P_o)	0.56 ± 0.06	0.13 ± 0.03*
Opening frequency (Hz)	264 ± 20	75 ± 14*
Mean open time (ms)	2.29 ± 0.33	1.66 ± 0.25^{NS}
Mean closed time (ms)	1.68 ± 0.16	11.67 ± 1.76*
Slope conductance (pS)	369 ± 12	358 ± 13^{NS}

*$P < 0.05$; NS, not significant.

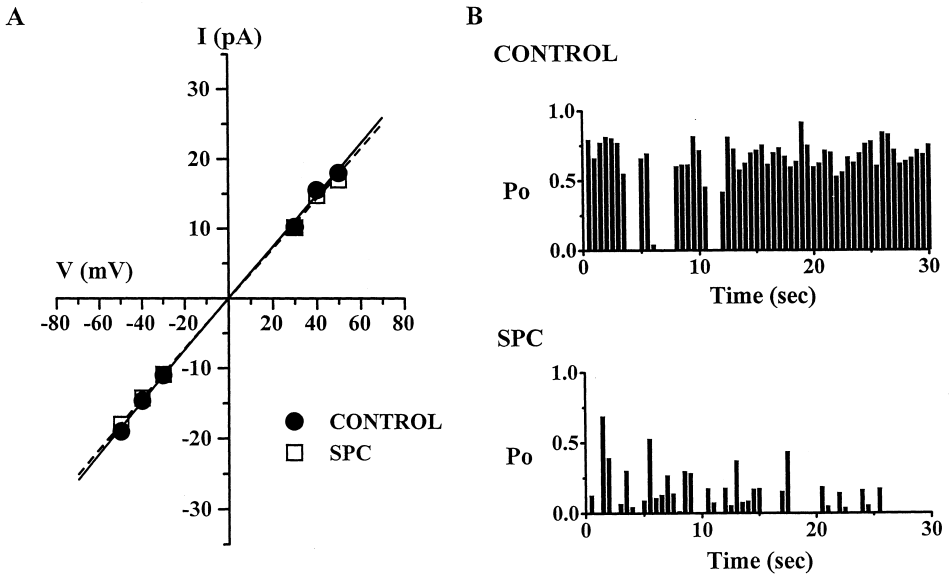

Fig. 2. Effect of SPC on conductance and gating properties of the RyR. A: Current-voltage (*I-V*) relationships for control and SPC-modified channels. Single channel conductance was not altered by the SPC application. B: Diaries of the open probability for a control (upper panel) and SPC-modified channel (lower panel). Note that SPC greatly decreased the open probability in this channel.

These *cis* experiments were repeated using RyR proteins isolated from CHAPS-solubilized SR membranes prepared according to Uehara et al. [8]. In these experiments, 1 μM SPC significantly decreased the open probability from 0.75 ± 0.03 to 0.38 ± 0.01 ($n = 4$, $P < 0.05$) with *cis* Ca^{2+} at 100 μM. These effects are similar to those shown in Fig. 1.

When 1–5 μM SPC was added to the *trans* chamber, corresponding to the SR lumenal side of the RyR, no changes were observed in single channel gating kinetics. The open probability was 0.73 ± 0.12 and 0.66 ± 0.08 in the absence and presence of 5μM *trans* SPC, respectively, with 100 μM *cis* Ca^{2+}. These values were not significantly different ($P > 0.05$, $n = 4$). However, subsequent addition of 5 μM SPC to the *cis* chamber produced a significant decrease in open probability to 0.13 ± 0.03 ($P < 0.05$, $n = 4$) and induced the same SPC-modified gating pattern as shown in the right panel of Fig. 1A. Thus, only cytoplasmic application of SPC caused a prominent modification in RyR gating.

3.3. Dwell time histograms

Open and closed time analysis of the rabbit cardiac RyR was conducted to define, in more detail, the *cis* effect of 1 μM SPC on channel gating kinetics. Fig. 3A shows the closed time distribution histograms derived from RyR channels in the presence and absence of 1 μM SPC. The time constants and the relative areas of each dwell state component (broken curves) were obtained by multiple exponential fitting (solid curves). Data from control channels ($n = 7$) were best fit with two exponential components that had time constants of 0.23 ± 0.09 ms and 0.91 ± 0.08 ms. The relative areas of the fast and slow components were $94 \pm 11\%$ and $6 \pm 2\%$, respectively (Fig. 3B). Control channels also exhibited a low incidence of longer-lived closed events. These closed events, however, were not fitted by an additional exponential component because they were scattered over an extremely wide range of closed time durations (up to several seconds) which never formed a clear peak in the histogram. On the other hand, SPC-modified channels showed a third exponential component in the closed-time histograms corresponding to a long-lived closed state (lower panel of Fig. 3A). The calculated time constants for drug-modified channels were 0.25 ± 0.11 ms, 1.32 ± 0.50 ms and 388 ± 130 ms (Fig. 3B) with relative areas of $87 \pm 15\%$, $10 \pm 3\%$ and $3 \pm 1\%$, respectively. The two short-lived closed state components in SPC-modified channels were not significantly different ($P > 0.05$) from the closed state components in control channels. Thus, SPC appears to modify the gating kinetics of RyR by inducing a long-lived closed state without modifying normal opening rates.

Fig. 3C shows open time distribution histograms for RyR in the presence and absence of 1 μM SPC. Under control conditions, a minimum of three kinetic components with time constants of 0.34 ± 0.32 ms, 1.86 ± 0.70 ms and 6.14 ± 2.04 ms ($n = 7$) were required to fit the data with relative areas of $70.7 \pm 8.01\%$, $27.6 \pm 9.0\%$ and $1.7 \pm 0.2\%$, respectively (Fig. 3D). Similarly, in SPC-modified channels ($n = 7$), three kinetic components with time constants of 0.24 ± 0.34 ms, 1.69 ± 1.03 ms and 5.99 ± 3.02 ms were needed to fit the data. The areas of these components were $74.4 \pm 10\%$, $24.6 \pm 5.0\%$ and $1.0 \pm 2.0\%$ ($n = 7$), respectively (Fig. 3D). None of these values were significantly different from those calculated for control channels. Thus, SPC did not affect the open state properties of RyR channels.

3.4. Burst properties of the SPC-modified channel

The pattern of SPC-induced gating consisted of grouped openings separated by long-lived closings, as shown in the right panel of Fig. 1A. To analyze this gating pattern, a burst analysis was conducted using a critical burst gap time of 10 ms that was determined by the crossing point method described in Section 2. In Fig. 4A, the upper trace shows an idealized digital burst pattern generated from the lower current tracing recorded with a 1 μM SPC-modified channel. Corresponding opening events in both current and idealized traces showed a high coincidence. The calculated burst durations for SPC-modified channels were plotted in a histogram (Fig. 4B, right) and fitted with an exponential function (time

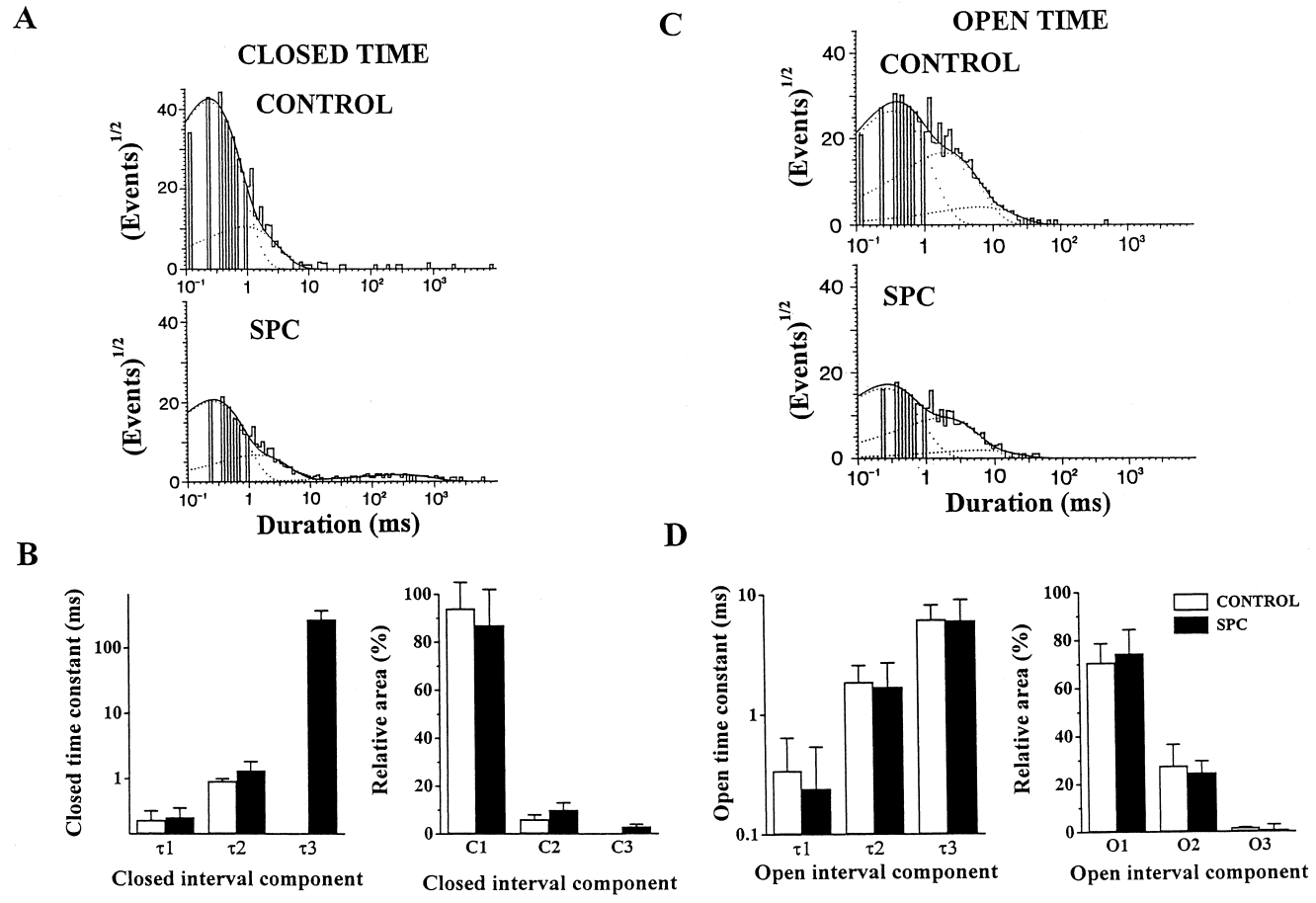

Fig. 3. Effect of SPC on the opening and closing kinetics of the RyR. A: Closed time distribution histograms before (upper panel) and after SPC application (lower panel). B: The time constants (left panel) and relative areas (right panel) of closed state components are shown for control (open) and SPC-modified channels (filled). C: Open time distribution histograms before (upper panel) and after SPC application (lower panel). D: The time constants (left panel) and relative areas (right panel) of the open state components calculated for control and the SPC-modified channels. SPC produced the third closed state component without altering the time constants or the relative areas in open state components.

constant = 132 ± 18 ms). RyR open probability calculated only within the burst periods was 0.66 ± 0.13, similar to the open probability under control conditions. As with the dwell time analysis above, these data suggest that SPC induces a long-lived closed state without affecting faster kinetic components of channel gating.

A similar analysis could not be performed in control channels since a long-lived closed state could not be identified (see above). This point was reinforced by performing a burst analysis on the control data using the same critical gap time of 10 ms calculated for SPC-modified channels. The calculated burst durations, shown in Fig. 4B, left panel, were widely distributed, yielding no obvious kinetic information. Thus, bursting behavior appears to result specifically from SPC interactions with the RyR.

4. Discussion

The present work demonstrates that SPC application to the cytoplasmic side of the cardiac RyR incorporated in lipid bilayers caused a dramatic modification of channel gating properties. SPC-induced gating modulation was characterized by a decrease in open probability and the emergence of groups of opening separated by long-lived closures. The apparent gating modulation in the SPC-modified cardiac RyR resembles that in tetracaine-modified skeletal RyR (Fig. 3B in Xu et al. [18]). Neither mean open time nor single channel conductance of the RyR was altered by SPC. Instead, the decrease in open probability reflected a significant increase in mean closed time (Table 1). These data suggest that SPC alters the opening kinetics of the RyR without blocking the open channel. The increase in mean closed time may also underlie the appearance of a SPC-induced long-lived closed state (time constant = 388 ms) which could be the determining factor in the formation of the characteristic gating pattern of SPC-modified channels. The dwell time of the long-lived closure period may represent the blocked state of the channel where SPC is bound to the RyR; however, this point requires further investigation.

The mechanism by which SPC alters the gating kinetics of the RyR remains to be determined. Possible mechanisms of action could include a direct ligand-receptor binding between the SPC and the RyR molecule, a masking of negative surface charges on the RyR molecule by the positive charges of SPC, or a change in membrane structure due to the incorporation of SPC molecules into the lipid bilayer. The absence of sphingolipid-induced effects on RyR single channel currents with

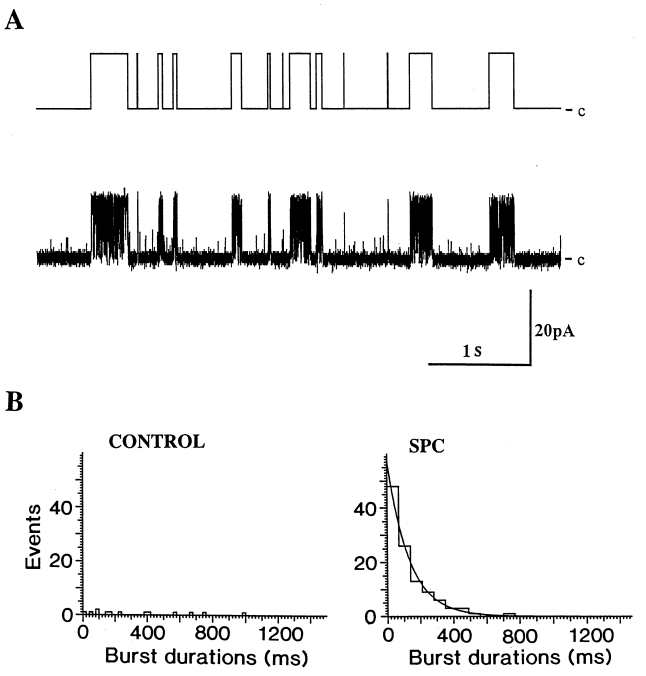

Fig. 4. Burst analysis. A: Idealized current trace showing calculated burst periods of a SPC-modified channel (upper trace). The corresponding raw data trace is shown below. The raw trace was well reconstituted by the digital burst pattern. −C: closed state. B: Burst duration histogram where the critical gap time was set at 10 ms. The left and right panels show histograms for control and SPC-modified channels, respectively. The solid curve in the right panel is a best-fit exponential function.

trans side addition of SPC suggests that a non-specific action on membrane structure is unlikely. In addition, RyR proteins prepared with and without CHAPS solubilization of SR microsomes [8] exhibited similar SPC-induced modification of channel gating kinetics. Thus, indirect interactions via other proteins such as calmodulin and FK-506 binding protein probably do not account for the effects of SPC on the RyR. The precise mechanism of action remains to be determined; however, the present data suggest that a direct interaction between the sphingolipid and a site(s) on the cytosolic face of the RyR protein is likely.

Betto et al. [11] showed that SPC induced activation of Ca^{2+} release from cardiac sarcoplasmic reticulum in $^{45}Ca^{2+}$ flux studies. Based on the present results, SPC would be anticipated to decrease, rather than increase, Ca^{2+} flux through the RyR. However, given the different experimental models in the present study and in Betto et al. [11], the contradictory nature of these two studies can only be resolved by examining the effects of SPC on the RyR over a much broader range of conditions.

A sphingolipid Ca^{2+} release-mediating channel of the endoplasmic reticulum (SCaMPER) was recently discovered in basophilic leukemia cells by Kindman et al. [19] and in endothelial cells by Kim et al. [20]. This channel protein has been cloned and functionally expressed by Mao et al. [21]. Betto et al. [11] claimed that SCaMPER channel is also expressed in heart muscle; however, it seems unlikely that the present results would be explained by this novel channel because currents were ryanodine-sensitive.

Sphingomyelin is a component of the sarcolemmal membrane [22]. Metabolism of sphingomyelin could expose the cytoplasmic side of the RyR to SPC and thereby regulate Ca^{2+} release from the SR. Whether such a regulatory pathway exists in situ remains to be determined; however, the concentrations of SPC that affected RyR gating kinetics were lower than endogenous tissue concentrations measured in cardiac muscle so that such a regulatory mechanism should, at least, be considered feasible.

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