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Regulation of ryanodine receptors by sphingosylphosphorylcholine: involvement of both calmodulin-dependent and -independent mechanisms

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Abstract

Sphingosylphosphorylcholine (SPC), a lipid mediator with putative second messenger functions, has been reported to regulate ryanodine receptors (RyRs), Ca^{2+} channels of the sarco/endoplasmic reticulum. RyRs are also regulated by the ubiquitous Ca^{2+} sensor calmodulin (CaM), and we have previously shown that SPC disrupts the complex of CaM and the peptide corresponding to the CaM-binding domain of the skeletal muscle Ca^{2+} release channel (RyR1). Here we report that SPC also displaces Ca^{2+}-bound CaM from the intact RyR1, which we hypothesized might lead to channel activation by relieving the negative feedback Ca^{2+}CaM exerts on the channel. We could not demonstrate such channel activation as we have found that SPC has a direct, CaM-independent inhibitory effect on channel activity, confirmed by both single channel measurements and [^{3}H]ryanodine binding assays. In the presence of Ca^{2+}CaM, however, the addition of SPC did not reduce [^{3}H]ryanodine binding, which we could explain by assuming that the direct inhibitory action of the sphingolipid was negated by the simultaneous displacement of inhibitory Ca^{2+}CaM. Additional experiments revealed that RyRs are unlikely to be responsible for SPC-elicited Ca^{2+} release from brain microsomes, and that SPC does not exert detergent-like effects on sarcoplasmic reticulum vesicles. We conclude that regulation of RyRs by SPC involves both CaM-dependent and -independent mechanisms, thus, the sphingolipid might play a pysiological role in RyR regulation, but channel activation previously attributed to SPC is unlikely.

Keywords

ryanodine receptor; sphingosylphosphorylcholine; calmodulin; single channel measurements; calcium signaling

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Introduction

Sphingosylphosphorylcholine (SPC), a lipid mediator with diverse functions [1,2], displays the unique property of being capable to mobilize Ca^{2+} from internal stores acting both extracellularly through G protein-coupled receptors and intracellularly in a yet undefined manner [3,4]. Although there is a certain level of confusion in the literature, it is important to differentiate between these two modes of action [5]. Ghosh *et al.* were the first to report on the intracellular Ca^{2+} release mediating ability of sphingosine derivatives [6], and since then, two main proposals have been made on the intracellular target sites for SPC. Mao *et al.* suggested the involvement of SCaMPER, a sphingolipid Ca^{2+} release mediating protein from endoplasmic reticulum [7]. However, in a subsequent study Schnurbus *et al.* demonstrated that SCaMPER is unlikely to be a Ca^{2+} channel [8]. Another suggestion was that SPC activates ryanodine receptors (RyRs), Ca^{2+} channels of the endoplasmic reticulum. These receptors were proposed to play a role in SPC-induced Ca^{2+} release from brain microsomes [9] and from cardiac sarcoplasmic reticulum membranes [10]. In contrast, Uehara *et al.* demonstrated that the open probability of cardiac RyR ion channels decreases on application of SPC [11,12]; hence, the question of SPC's intracellular mechanism of action is still open.

RyRs are huge channel complexes composed of tetramers of 560 kDa monomers [13]. Among the three isoforms RyR1 is expressed primarily in skeletal muscle, RyR2 is the dominant isoform in cardiac muscle, and RyR3 is found in a wide variety of tissues. They display a characteristic bell-shaped, biphasic dependence on cytosolic Ca^{2+} concentration with micromolar Ca^{2+} causing channel activation and elevated Ca^{2+} concentrations resulting in channel inhibition. Besides Ca^{2+}, these receptors are regulated by several small molecules and proteins including the ubiquitous Ca^{2+} sensor calmodulin (CaM) [14]. Regulation of RyRs by CaM is complex, but the sensor clearly plays a role in the negative feedback of the Ca^{2+} signal, through inhibition of channel activity by Ca^{2+}CaM [15,16].

We have previously shown that SPC can selectively bind to CaM, and inhibits its activity on target enzymes phosphodiesterase and calcineurin [17]. Furthermore, we demonstrated that the sphingolipid disrupts the complex between CaM and the peptide representing the CaM-binding domain of RyR1 [18]. In this report, we describe the regulation of RyR1 by SPC focusing on the role of CaM. We found that SPC displaces CaM from the intact RyR1, and regulates these Ca^{2+} channels both in a CaM-dependent and -independent manner.

Materials and Methods

Lysophospholipids

D-*erythro*-sphingosylphosphorylcholine (SPC, cat. no. 860600), D-*erythro*-sphingosine-1-phosphate (S1P, cat. no. 860492), oleoyl-lysophosphatidylcholine (LPC, cat. no. 845875) and oleoyl-lysophosphatidic acid (LPA, cat. no. 857130) were purchased from Avanti Polar Lipids. Lipids were delivered from 10 mM methanolic stock solutions.

SR vesicle preparations and RyR purification

Heavy SR vesicles were isolated from rabbit hind limb and back muscle as previously described [19]. Endogenous CaM was removed by incubating SR vesicles for 30 min at 24 °C with 1 μM myosin light chain kinase-derived CaM binding peptide in the presence of 100 μM Ca^{2+} followed by centrifugation through a layer of 15% sucrose to remove complexed CaM and the peptide [15]. For purification of RyR1, SR vesicles were solubilized in CHAPS, purified by sucrose density gradient centrifugation, and reconstituted into phosphatidylcholine liposomes [20].

Single channel recordings

Single channel measurements were carried out as previously described [19] in planar lipid bilayers containing phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in the ratio of 5:3:2 (25 mg of total phospholipid/ml of *n*-decane). The side of the bilayer to which the proteoliposomes containing the purified RyR1s were added was defined as the cis (cytoplasmic) side. The trans (SR lumenal) side of the bilayer was defined as ground. Measurements were made with symmetrical 0.25 M KCl, 20 mM K-HEPES, pH 7.4, with 22 μM free Ca^{2+} in the cis chamber. CaM and SPC were added to the cis solution. For +CaM samples, proteoliposomes were preincubated with 1 μM CaM at room temperature for 30 min, and measurements were done in the presence of 100 nM cis CaM. Data were acquired using test potentials of ±35 mV and were sampled at 10 kHz and filtered at 2 kHz. Channel open probabilities (*Po*) were determined from at least 2 min of recordings for each condition.

[^{3}H]ryanodine binding

SR vesicles were incubated with 2.5 nM [^{3}H]ryanodine for 20 h at room temperature in 0.3 M sucrose, 0.15 M KCl, 20 mM K-Pipes, pH 7.0, 50 times diluted protease inhibitor cocktail (Sigma), 0.1 mg/ml BSA, either 5 mM reduced (GSH) or oxidized (GSSG) glutathione and 50 μM free Ca^{2+} (150 μM $CaCl_2$, 100 μM EGTA). Nonspecific binding was determined using heat-inactivated SR vesicles. Aliquots of the samples were diluted with 10 volumes of ice-cold water and placed on Whatman GF/B filters soaked with 2% polyethyleneimine. Filters were washed with three 5-ml volumes of ice-cold 0.1 M KCl, 1 mM K-Pipes, pH 7.0. Radioactivity remaining with the filters was determined by liquid scintillation counting to obtain bound [^{3}H]ryanodine.

[^{35}S]CaM binding

CaM was metabolically labeled with ^{35}S and purified according to Balshaw *et al.* [15]. SR vesicles were incubated at room temperature for 2 h with 84 nM [^{35}S]CaM in 0.3 M sucrose, 0.15 M KCl, 20 mM K-Pipes, pH 7.0, 50 times diluted protease inhibitor cocktail (Sigma), 0.1 mg/ml BSA, 5 mM GSH and 100 μM free Ca^{2+} (200 μM $CaCl_2$, 100 μM EGTA). Equilibrium [^{35}S]CaM binding was assayed by centrifugation in a Beckman Airfuge for 30 min at 90,000 *g*. Nonspecific binding was determined by measuring [^{35}S]CaM binding to heat-inactivated SR vesicles. Bound [^{35}S]CaM was determined by scintillation counting after solubilization of pellets in Tris-HCl buffer, pH 8.5, containing 2% SDS.

Membrane permeability assay

Skeletal SR vesicles were incubated for 4 h at 0 °C in a large volume (1 mg of protein/ml) of incubation medium (0.3 M sucrose, 150 mM KCl, 20 mM K-Pipes, pH 7.0, 50 times diluted protease inhibitor cocktail (Sigma), 0.1 mg/ml BSA, 10 mM glucose, 2 mM $MgCl_2$), sedimented by centrifugation for 30 min at 35,000 rpm in a Beckman 42.1 rotor, and resuspended in a small volume (20 mg of protein/ml) of incubation medium. Vesicles were incubated overnight at 4 °C in the presence of 0.1 mCi/ml [^{14}C]glucose. The vesicles were then diluted 100-fold into an unlabeled release medium supplemented with the appropriate lysophospholipid. Efflux of [^{14}C]glucose was terminated by placing aliquots (0.2 ml) on 0.45 μm HAWP Millipore filters followed by rapid rinsing with unlabeled release medium. The radioactivity retained on the filters was determined by scintillation counting.

Results

Monomeric SPC induces long channel closings of purified RyR1 independently of CaM

The effects of SPC on the skeletal muscle ryanodine receptor (RyR1) both in the presence and absence of CaM were studied with the method of single channel recordings in planar lipid bilayers. Proteoliposomes containing purified RyR1 were fused with the lipid bilayer and the resulting channel activities were recorded.

Fig. 1A shows a representative single channel recording in the absence (upper panel) and after the addition of 1 μM SPC (lower panel) to the cis chamber with 22 μM free Ca^{2+} in the cis chamber. Addition of SPC decreased single channel open probability (P_o) from 0.51 to 0.16. The lipid induced long channel closings without noticeably altering the duration of open events. In Fig. 1B, the effects of the lipid on RyR1 were measured in the presence of 100 nM CaM in the cis chamber. To assure CaM binding to RyR1, proteoliposomes were preincubated with 1 μM CaM for 30 min. Addition of 1 μM SPC reduced P_o from 0.15 to 0.05 by inducing long channel closings, similarly to those observed in the absence of CaM. The open probability of the channel was decreased by SPC with an IC_{50} of 1.3± 0.3 μM and 0.7 ± 0.2 μM in the absence and presence of 100 nM CaM, respectively (Fig. 1C). Analysis of single channel recordings showed that, both in the absence and presence of CaM, the open and closed times of Ca^{2+}-activated channels could be fitted by the sum of two exponentials (data not shown), in agreement with a previous report [21]. SPC significantly increased lifetimes of the second closed state (an increase from 3.21 ± 2.09 ms to 452 ± 140 ms and from 16.53 ± 6.21 ms to 619 ± 242 ms in the absence and presence of 100 nM CaM, respectively, Table 1).

Micellar SPC affects [^{3}H]ryanodine binding in a CaM-dependent and -independent manner

We have previously shown that clusters of SPC are required for an efficient interaction with CaM, thus, to see an effect on the Ca^{2+} sensor, SPC has to be applied at concentrations near its critical micelle concentration (CMC, ~30 μM) [17]. We could not study the effects of micellar SPC in single channel measurements, because the sensitive lipid bilayer broke at SPC concentrations above 10 μM. To explore the effect of SPC on RyR activity at concentrations expected to disrupt the CaM-RyR interaction, [^{3}H]ryanodine binding assays were conducted. This ligand binding assay is a good indicator of channel activity, since the plant alkaloid ryanodine has been shown to bind with higher affinity to the open than the closed channel state [22].

Measurements with skeletal SR vesicles at 50 μM free Ca^{2+}, under both reducing (5 mM GSH) and oxidizing (5 mM GSSG) conditions were carried out. Ca^{2+} concentration was optimized to saturate CaM, but not to inhibit RyR activity directly [15]. An oxidizing environment is expected to yield higher ryanodine but lower CaM binding, while CaM is expected to be inhibitory under both conditions [15].

In the absence of CaM, SPC inhibited [^{3}H]ryanodine binding with an apparent IC_{50} of 26.1 ± 0.8 μM (Fig. 2A) and 25.5 ± 4.0 μM (Fig. 2C) under reducing and oxidizing conditions, respectively. These values are approximately 20-fold higher than the ones obtained in the single channel measurements and may have been caused by the different SPC to membrane lipid ratios in the two techniques. The effect of SPC was selective compared to structurally similar lysophospholipid mediators such as lysophosphatidylcholine (LPC) and lysophosphatidic acid (LPA), but a minor inhibitory effect could also be observed in case of sphingosine-1-phosphate (S1P) (Figs. 2B and D).

In the presence of CaM, on the other hand, SPC did not significantly alter [^{3}H]ryanodine binding. A possible explanation for this finding is that SPC exerts two opposing effects on

the Ca^{2+}CaM-bound RyR. Displacement of inhibitory Ca^{2+}CaM from RyR1 has an activating effect, whereas direct interaction with RyR1 is inhibitory, negating the effects of SPC. [^{35}S]CaM binding studies (see below) and the fact that S1P (which does not interact with CaM [17,18]) further decreased [^{3}H]ryanodine binding in the presence of CaM (Fig. 2D), favour this explanation.

SPC displaces CaM from skeletal muscle SR vesicles

[^{35}S]CaM binding assays revealed that SPC displaces Ca^{2+}-saturated CaM from skeletal SR vesicles enriched in RyR1 with an IC_{50} of 14.2 ± 0.8 μM (Fig. 3A). This value compares well with the results of peptide binding assays (IC_{50} = 19.4 ± 1.4 μM) [18]. S1P and LPA were ineffective, while LPC also decreased [^{35}S]CaM binding to SR vesicles (Fig.s 3B). This effect is probably an artefact, as LPC also decreased nonspecific binding, and it was without action in peptide binding experiments [18].

SPC does not break the permeability barrier of SR vesicle preparations

To rule out the possibility that SPC's action on SR vesicles is an artefact of membrane permeabilization, we examined the sphingolipid's effect on the permeability barrier of preparations used in the study. SR vesicles were loaded with [^{14}C]glucose, and release was determined after dilution in an unlabeled medium. We found that neither 50 μM SPC, S1P, LPC or LPA increased glucose permeability significantly, while 1% CHAPS detergent released a large amount of stored glucose (Fig. 4). Thus, the lysophospholipids used in our measurements do not disrupt the permeability barrier.

Discussion

The effects of the lipid mediator SPC on the RyRs have been previously studied. One group of investigators reported an activation of these receptors by the sphingolipid [9,10], while another one observed channel inhibition [11,12]. Neither group examined the role of CaM in this process. Since we formerly showed that SPC is a potent inhibitor of CaM-dependent enzymatic activities [17], and selectively dissociates the complex between CaM and the CaM-binding domain of RyR1 at concentrations near its CMC (~30 μM) [18], we studied the interaction between RyR1 and SPC, focusing on the role of CaM in modulating this interaction. According to our initial hypothesis, we expected that SPC, by dissociating CaM from the RyR1, would relieve negative feedback inhibition of Ca^{2+}CaM on the channel. By leading to channel opening, SPC would mobilize intracellular Ca^{2+} [6].

In this report, we show that SPC indeed displaces CaM from the intact channel (Fig. 3). The functional consequences of CaM dissociation were explored in single channel measurements and a ligand ([^{3}H]ryanodine) binding assay. Single channel recordings on purified RyR1 (Fig. 1) revealed that SPC at concentrations well below its CMC decreases the open channel probability of RyRs, and induces the appearance of long-lived closed states. These data are in good agreement with former reports that low concentrations of SPC decrease the open probability of the cardiac ryanodine receptor (RyR2), and RyR2 inhibition is characterized by the appearance of long-lived closed channel states [11,12]. As expected, this inhibitory effect was independent of CaM, since we previously demonstrated that CaM only binds efficiently to the micellar form of SPC. We were unable to study the effects of SPC on RyR1 above the lipid's CMC in single channel measurements, because SPC concentrations greater than 10 μM resulted in disruption of the sensitive reconstituted bilayer.

To overcome this limitation, [^{3}H]ryanodine binding experiments were carried out (Fig. 2), which confirmed inhibition of receptor activity in the absence of CaM. However, if Ca^{2+}CaM was present, inhibition could not be observed. This phenomenon could be

explained by assuming that two antagonistic molecular events are occurring at the same time: 1) a direct inhibition of channel activity by the sphingolipid, as suggested by single channel recordings and 2) displacement of inhibitory Ca^{2+}CaM by SPC, as suggested by [^{35}S]CaM binding assays.

Taken together, our data are consistent with the findings of Uehara *et al.* [11,12] in that SPC directly inhibits RyRs. Our current results are also consistent with our former data suggesting that SPC can dissociate the complex between RyRs and CaM, and we have found that the presence of CaM can modify the direct inhibitory effect of SPC on the channel. On the other hand, an involvement of RyRs in SPC-induced Ca^{2+} release from brain microsomes has been reported [9]. Hence, we studied RyR activity similarly as these authors had, isolating microsomes from bovine brain cerebrum by differential centrifugation, loading these with Ca^{2+} in presence of an ATP-regenerating system, and measuring Ca^{2+} release using a fluorescent Ca^{2+} indicator. We found that while 50 μM SPC, LPC and LPA each released a large amount of Ca^{2+} from brain microsomes, classical channel activators such as 1 μM ryanodine and 5 mM caffeine only had minor effects (data not shown). Surprisingly, Dettbarn *et al.* [9] were also incapable of triggering Ca^{2+} release by ryanodine or caffeine. Their major argument for SPC's action on RyRs was that ruthenium red, an inhibitor of the RyRs, inhibited SPC-induced Ca^{2+} release. However, in two subsequent studies [10,23], SPC-induced Ca^{2+} release was much less sensitive to ruthenium red inhibition. Taken together, the sphingolipid's mechanism of action on cerebral microsomes remains elusive, but a significant contribution of RyRs to the phenomenon can most likely be excluded.

In conclusion, we have shown that SPC regulates RyR1 in a diverse manner. Below the CMC, SPC directly inhibits the skeletal muscle Ca^{2+} release channel, while above the CMC, besides a direct action on the channel, the sphingolipid also displaces inhibitory Ca^{2+}CaM from the RyR1, further modifying channel activity. Here we note that we use micelles as the simplest alternative for the *in vitro* presentation of an SPC cluster to CaM, which might arise *in vivo* upon the activation of an SPC producing enzyme. Net activation of the channel never occurs, so SPC's effect on the RyR cannot explain how the sphingolipid liberates Ca^{2+} from the endoplasmic reticulum. Although this question remains open, we have clarified the role of SPC in RyR regulation. The relevance of the findings in the present study regarding the *in vivo* role of SPC in regulating the skeletal muscle RyR and the receptor's interaction with CaM remains to be shown, but it is tempting to speculate that the sphingolipid might locally modify channel function in living cells.

Acknowledgments

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Abbreviations

CaM	calmodulin
CMC	critical micelle concentration
LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine
RyR	ryanodine receptor
S1P	sphingosine-1-phosphate

SPC	sphingosylphosphorylcholine
SR	sarcoplasmic reticulum

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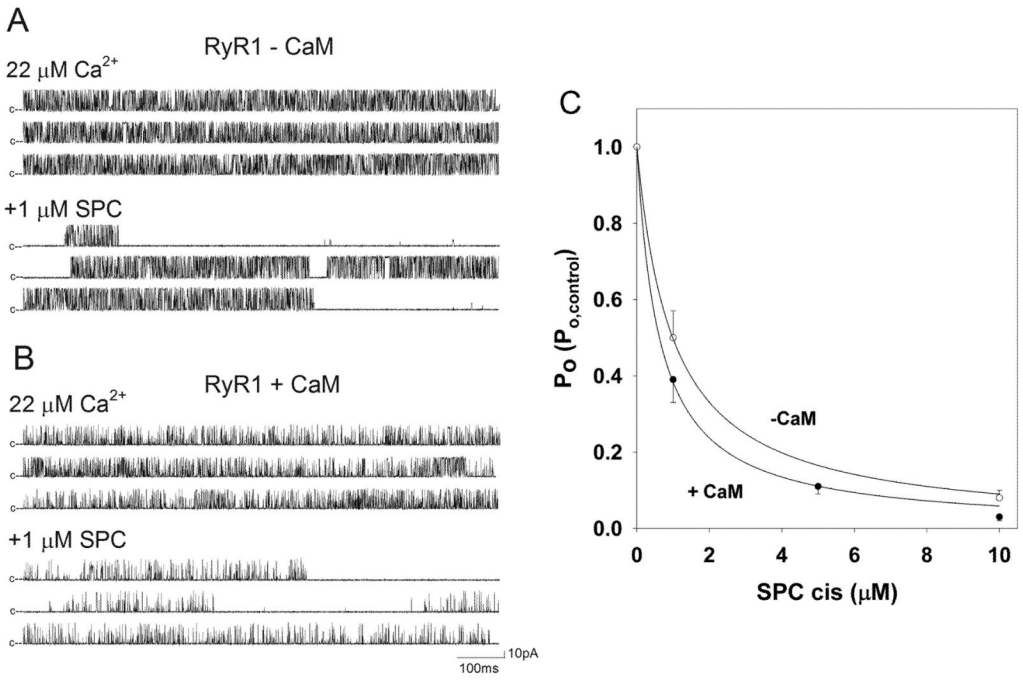

Fig. 1.

Effects of SPC on single channel activities of purified RyR1 in absence and presence of 100 nM CaM. (A) Single channel currents were recorded at +35 mV before (top 3 traces) and after the addition of 1 μM SPC cis (bottom 3 traces) in symmetrical 0.25 M KCl in presence of 22 μM free Ca^{2+} cis and absence of CaM. The closed states are indicated by c--. (B) As in (A) in presence of 100 nM CaM. Proteoliposomes were incubated with 1 μM CaM for 30 min before the addition to the cis chamber of the lipid bilayer system, and channels were recorded in presence of 100 nM CaM in the cis chamber. (C) Dependence of purified RyR1 channel activity on SPC concentration in absence and presence of CaM. Relative open channel probability ($P_o/P_{o,control}$) was obtained from single channel recordings similar to those shown in panels A and B. Solid lines were obtained according to the equation $P_o = P_{o,control}\ (1 + [SPC]/K_i)^{-1}$, where K_i is the inhibition constant and P_o and $P_{o,control}$ are single channel open probabilities in presence and absence of SPC. Inhibition constants in the absence (n=6) and presence (n=10) of 100 nM CaM were 1.3 ± 0.3 μM and 0.7 ± 0.2 μM, respectively.

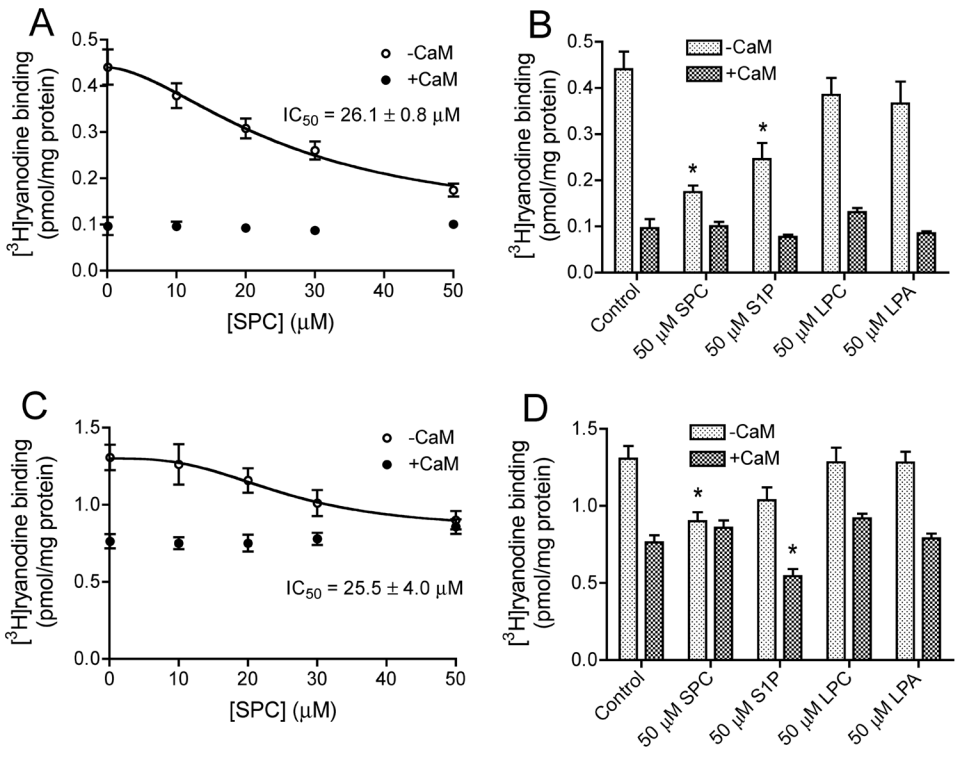

Fig. 2.

Effect of SPC on [^{3}H]ryanodine binding to skeletal SR vesicles. Free Ca^{2+} concentration was set to 50 μM and either 5 mM GSH (A,B) or 5 mM GSSG (C,D) was added. Open circles and light bars depict [^{3}H]ryanodine binding in the absence of CaM, while closed circles and dark bars depict [^{3}H]ryanodine binding in the presence of 0.1 μM (A,B) and 1 μM (C,D) CaM. In the absence of CaM, the dose-response for SPC yielded an apparent IC_{50} of 26.1 ± 0.8 μM and 25.5 ± 4.0 μM and a Hill-slope of −1.7 ± 0.04 and −2.9 ± 0.2 under reducing (A) and oxidizing (C) conditions, respectively. Data points depict mean ± S.E. (n=3), a sigmoidal dose-response curve with variable slope was fitted to the data, and asterisks denote significant differences at $p<0.05$ compared to control.

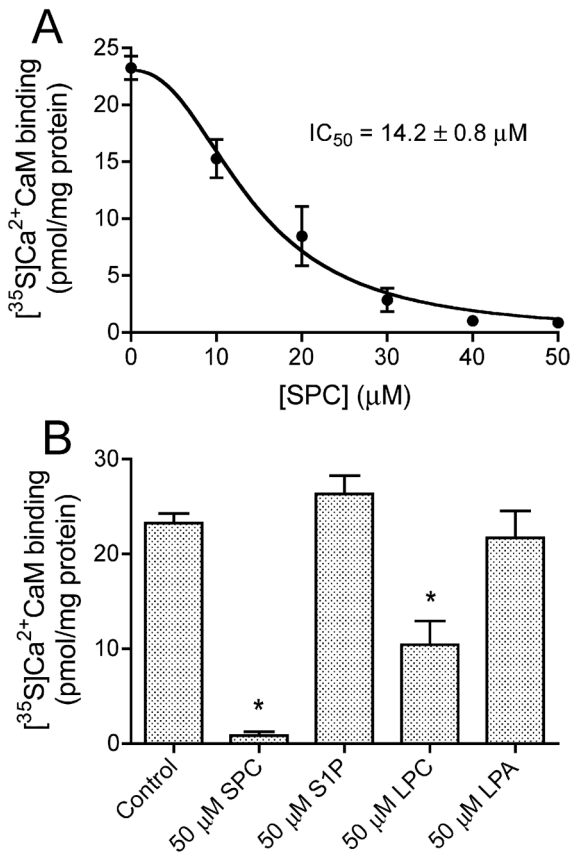

Fig. 3.

Displacement of [^{35}S]CaM from RyR1 by SPC. (A) SPC dissociates Ca^{2+}-saturated [^{35}S]CaM from skeletal SR vesicles rich in RyR1 with an IC_{50} of 14.2 ± 0.8 μM and a Hill-slope of 2.3 ± 0.7. Data points depict mean ± S.E. (n=3), a sigmoidal dose-response curve with variable slope was fitted to the data. (B) Among the tested lysophospholipids, SPC and LPC significantly decrease [^{35}S]CaM binding to skeletal SR vesicles. Data points depict mean ± S.E. (n=3), asterisks denote significant differences at $p<0.05$ compared to control. Note that the effect of LPC is probably an artefact as LPC also decreased nonspecific binding, and it was without action in peptide binding assays [18].

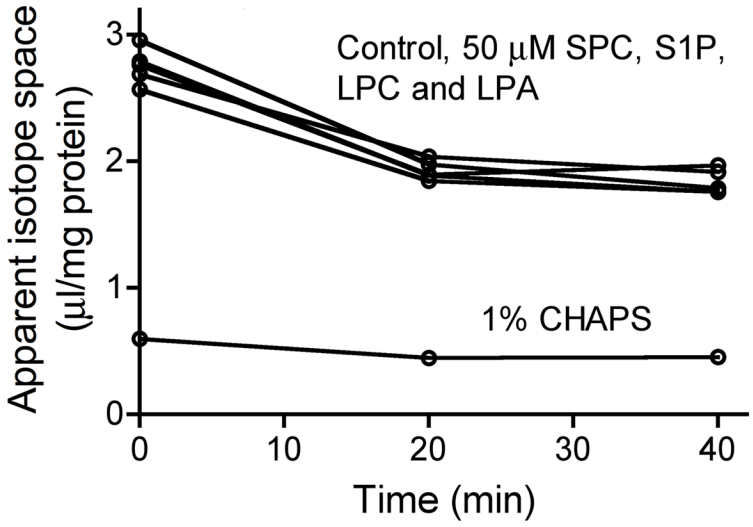

Fig. 4.

Effect of SPC on the membrane permeability of skeletal SR vesicle preparations. Vesicles were loaded with [^{14}C]glucose and retained radioactivity was measured after dilution into unlabeled release medium.

Table 1

Effect of SPC on purified RyR1 channel parameters in the absence and presence of CaM. Channel parameters were obtained from 2 min continuous recordings as described in Experimental Procedures. P_o refers to open channel probability. Dwell-time data were fitted by the maximum likelihood method to the probability density function: $f(t) = \Sigma A_i\ (1/\tau_i) \exp(-t/\tau_i)$, where A_i and τ_i are the relative areas of the distributions and time constants of the *i*th state, respectively [24]. Both the open and closed time histograms could be fitted by the sum of two exponentials.

Channel parameter	Additions to cis bilayer chamber			
	22 μM Ca^{2+}		22 μM Ca^{2+} and 100 nM CaM	
	−1 μM SPC	+1 μM SPC	−1 μM SPC	+1 μM SPC
No. of events	19,874 ± 7337	9767 ± 1884	20,544 ± 4074	10,392 ±2633*
P_o	0.52 ± 0.16	0.24 ± 0.10	0.39 ± 0.12	0.18 ± 0.09*
A_{o1}	0.87 ± 0.12	0.81 ± 0.18	0.91 ± 0.08	0.94 ± 0.05
A_{o2}	0.13 ± 0.12	0.19 ± 0.18	0.09 ± 0.08	0.06 ± 0.05
τ_{o1} (ms)	0.56 ± 0.53	0.24 ± 0.19	0.26 ± 0.22	0.16 ± 0.12
τ_{o2} (ms)	2.93 ± 2.22	5.09 ± 3.32	1.48 ± 0.85	3.12 ± 2.10
A_{c1}	0.98 ± 0.01	0.994 ± 0.003	0.85 ± 0.09	0.96 ±0.03
A_{c2}	0.02 ± 0.01	0.006 ± 0.003	0.15 ± 0.09	0.04 ± 0.03
τ_{c1} (ms)	0.05 ± 0.02	1.07 ± 0.68	0.77 ± 0.47	2.81 ± 1.66
τ_{c2} (ms)	3.21 ± 2.09	452 ± 140*	16.53 ± 6.21	619 ± 242*

*Values significantly different from the − SPC control ($P < 0.05$; n = 4 and 6 in case of −CaM and +CaM samples, respectively).

[1]Sphingosylphosphorylcholine (SPC) directly inhibits ryanodine receptor 1 (RyR1).

[2]SPC also displaces calmodulin (CaM) from the intact RyR1.

[3]The sphingolipids effect on RyR1 is further modified by the removal of CaM.

[4]Thus, SPC regulates channel activity in both a CaM-dependent and -independent manner.