Sphingosine releases Ca\textsuperscript{2+} from intracellular stores via the ryanodine receptor in sea urchin egg homogenates

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Abstract

Various reports have demonstrated that the sphingolipids sphingosine and sphingosine-1-phosphate are able to induce Ca\textsuperscript{2+} release from intracellular stores in a similar way to second messengers. Here, we have used the sea urchin egg homogenate, a model system for the study of intracellular Ca\textsuperscript{2+} release mechanisms, to investigate the effect of these sphingolipids. While ceramide and sphingosine-1-phosphate did not display the ability to release Ca\textsuperscript{2+}, sphingosine stimulated transient Ca\textsuperscript{2+} release from thapsigargin-sensitive intracellular stores. This release was inhibited by ryanodine receptor blockers (high concentrations of ryanodine, Mg\textsuperscript{2+}, and procaine) but not by pre-treatment of homogenates with cADPR, 8-bromo-cADPR or blockers of other intracellular Ca\textsuperscript{2+} channels. However, sphingosine rendered the ryanodine receptor refractory to cADPR. We propose that, in the sea urchin egg, sphingosine is able to activate the ryanodine receptor via a mechanism distinct from that used by cADPR.

Keywords: Calcium signalling; Sea urchin egg; Sphingosine; Ryanodine receptors; cADPR

The transduction of extracellular stimuli to intracellular responses by means of Ca\textsuperscript{2+} signalling is a vitally important mechanism for a wide range of cellular processes. In using rises in intracellular Ca\textsuperscript{2+} concentrations, the cell plays a dangerous game as sustained high Ca\textsuperscript{2+} concentrations are lethal to the cell [1]. Therefore, the cell has developed intricate and highly regulated mechanisms for the induction and termination of stimulus-induced Ca\textsuperscript{2+} rises [2]. While many factors and proteins are involved in the influx and extrusion of Ca\textsuperscript{2+} across the plasma membrane, just a small number of channels, pumps, and factors regulate the movement of Ca\textsuperscript{2+} in and out of the intracellular organelles [2].

Second messengers regulate the release of Ca\textsuperscript{2+} from intracellular stores such as the endoplasmic reticulum (ER), the Golgi and vesicular compartments [3]. The sea urchin egg has long been used as a tool for the investigation of Ca\textsuperscript{2+} release from intracellular stores due to its ease of use in live Ca\textsuperscript{2+} imaging and homogenate experiments and experimental robustness. In this system, three second messengers have been well characterized to date and are; IP\textsubscript{3} (inositol-1,4,5-trisphosphate), a small phosphorylated inositol sugar produced by cleavage of membrane phospholipids which acts on IP\textsubscript{3} receptors in the ER [2]; cyclic adenosine diphosphate ribose (cADPR) [4], a cyclic derivative of NAD that activates ryanodine receptors: and nicotinic acid adenine dinucleotide phosphate (NAADP) [5,6], an NADP metabolite that acts on IP\textsubscript{3} receptors in the ER [2]; cyclic adenosine diphosphate ribose (cADPR) [4], a cyclic derivative of NAD that activates ryanodine receptors: and nicotinic acid adenine dinucleotide phosphate (NAADP) [5,6], an NADP metabolite that acts on an as yet unidentified receptor located on an acidic vesicular compartment that probably corresponds to lysosomes [7,8]. These three messengers are able to act in concert along with Ca\textsuperscript{2+} influx pathways to encode Ca\textsuperscript{2+} rises that, due to their exact spatial, temporal, and amplitude characteristics, are unique and activate only the desired targets in response to a specific stimulus.

These three second messengers have been shown to exist and act also in other models, including mammalian cells.
where evidence suggests that a fourth mechanism may exist to add an extra level of complexity to the encoding of signals. Sphingosine (Sph) is a major sphingolipid that is involved in a large number of cellular functions in many cells [9]. It is the simplest, with respect to its structure, of the sphingolipid family, which includes ceramide (Cer), sphingosine-1-phosphate (S-1-P), and sphingomyelin, and is produced by the deacylation of Cer (by ceramidase) or the dephosphorylation of S-1-P (by sphingosine-1-phosphate phosphatase) [10]. The classical functions of these molecules are in the regulation of apoptosis, with Cer and Sph being anti-proliferative and pro-apoptotic while S-1-P is a proliferative and anti-apoptotic agent [10]. Therefore, the relative levels of each of these molecules in the cell (the “sphingolipid rheostat”) are able to determine cell fate, and this has led, among other lines of research, to intense study of these lipids in relation to cancer [11]. It has been shown that the actions of Sph are mediated by direct interactions with protein kinase C (PKC) and a number of other protein kinase families, meaning that the effects of Sph are wide ranging [12].

In addition to these functions, Sph has been shown to mediate intracellular Ca\(^{2+}\) release, in a number of cell types including T-cells, parotid and pancreatic acinar cells, skeletal muscle, neutrophils, and thyroid cells, and also Ca\(^{2+}\) influx in a number of models [13–18] however, in several of these models, it has been suggested that Sph metabolites are ultimately responsible for the activity [14,17]. Data in mammalian cells suggest that the Sph-sensitive Ca\(^{2+}\) store is the same as that sensitive to IP\(_3\), i.e., the ER. However, the molecular target of Sph for Ca\(^{2+}\) release is not known although a putative sphingosyl choline receptor has been identified [19,20]. These data have led to the hypothesis that Sph can act as a second messenger despite being located largely in intracellular membranes due to its hydrophobic tail. Indeed, it is thought that it is sufficiently soluble in aqueous media to shuttle between membranes crossing the cytosol [21]. Furthermore, the monophosphorylated derivative of sphingosine, S-1-P, has also been shown to induce Ca\(^{2+}\) release from stores in a number of cell models [22,23]. Again, the S-1-P-sensitive store is thapsigargin sensitive and interactions with the IP\(_3\) receptor have been proposed although the mechanism of this interaction is unclear as heparin is unable to block the effects of S-1-P [13,24].

In the present contribution, we have investigated the effect of Sph on Ca\(^{2+}\) release in sea urchin egg homogenates. Sph released Ca\(^{2+}\) in a dose-dependent manner from a thapsigargin-sensitive store and was extensively blocked by ryanodine, procaine, and Mg\(^{2+}\) but not by heparin or low concentrations of NAADP. Furthermore, cADPR-induced Ca\(^{2+}\) release was almost completely abrogated following Sph-induced Ca\(^{2+}\) release, suggesting that release occurs via the ryanodine receptor (RyR). Surprisingly, though, pre-treatment with cADPR had little effect on subsequent Sph-induced Ca\(^{2+}\) release and \(^{32}\)PcADPR binding was unaffected by Sph.

### Materials and methods

**Preparation of sea urchin egg homogenates and fluorimetry.** Lytechinus pictus (Painted Urchin) were collected from the Pacific during the breeding season (Marinus Scientific, CA, USA). Homogenates were prepared using a protocol largely similar to that used by Dargie et al. [25,26].

Ca\(^{2+}\) loading was achieved by sequential dilution of 50% homogenate in intracellular medium (IM) consisting of 250 mM potassium gluconate, 250 mM N-methylglucamine, 25 mM Hepes, pH 7.2, 1 mM MgCl\(_2\), 1 mM ATP, 10 mM phosphocreatine, 10 U/ml creatine phosphokinase, and 3 \(\mu\)M fluo-3. Dilutions were carried out at 17 °C and the final homogenate concentration was 2.5%. Fluorimetry was performed at 17 °C using a stirred cuvette containing 750 μl of homogenate in a Perkin-Elmer LS-50B fluorimeter. All experiments are referred to an appropriate control for the vehicle in which the Sph was dissolved (DMSO).

**Radioligand binding.** \(^{32}\)PcADPR was prepared as previously described [27]. For binding assays, \(^{32}\)PcADPR (200 pM) was incubated with 200 μg sea urchin egg homogenate in IM buffer for 20 min at 4 °C with 100 μM Sph or DMSO. Alternatively, 200 μg of homogenate was pre-incubated at 17 °C for 30 min with 100 μM Sph or DMSO before being added to the binding reaction. Reactions were terminated by the addition of 500 μg of γ-globulins and 15% polyethylene glycol. Reactions were vortexed and centrifuged at 20,000g for 5 min at 4 °C. The supernatant was aspirated and the pellet was washed once in 1 ml IM buffer before the addition of 1 ml scintillation fluid and standard scintillation counting.

### Results and discussion

The sea urchin egg and the sea urchin egg homogenate have long been used for the study of Ca\(^{2+}\) signalling [26]. While the intact egg has been pivotal for those studying the signalling and physiology of fertilization and cell division, the homogenate has proved invaluable as a model for the study of second messenger-induced Ca\(^{2+}\) release [28]. Several reports have suggested that Sph is able to stimulate Ca\(^{2+}\) release from intracellular stores in mammalian cells and we therefore attempted to confirm this finding in the sea urchin egg homogenate [12].

Application of micromolar concentrations of Sph to sea urchin egg homogenates induced a dose-dependent Ca\(^{2+}\) transient (Figs. 1A and B). Although Sph released Ca\(^{2+}\) on all experimental days, large variability in the extent and kinetic parameters of release was observed. Furthermore, loss of activity was noted when Sph was kept at room temperature for longer than 1 h, possibly due to oxidation. The increase in fluo-3 fluorescence observed was indeed due to Ca\(^{2+}\) release since addition of identical amounts of Sph to fluo-3 in IM buffer alone did not elicit fluorescence changes (data not shown). The Sph-induced Ca\(^{2+}\) transient in homogenates was markedly different from that elicited by NAADP or cADPR since the rising phase of the release was significantly slower resulting in a long time to peak (7.6 ± 1.9 min at 100 μM Sph compared to ~1 min for cADPR or NAADP) and the re-uptake was also significantly slower. A second addition of 100 μM after the trace had returned to baseline induced a Ca\(^{2+}\) transient essentially identical to the first (second response 106.5 ± 30.8% of the first; \(n = 5\); Fig. 1A). Ca\(^{2+}\)-re-uptake was not observed after the second addition of Sph. Sph
therefore appears to activate Ca\(^{2+}\)-release and may interact with Ca\(^{2+}\)-uptake mechanisms as well.

In the intact sea urchin egg, the formation of the fertilization envelope is due to the cortical reaction which is a highly Ca\(^{2+}\)-dependent process [29]. Indeed, a rise in the intracellular Ca\(^{2+}\) concentration in the egg is sufficient to activate this process even in the absence of sperm–egg fusion [29]. To investigate whether Sph could elicit a Ca\(^{2+}\)-rise also in the intact egg, we therefore incubated Sph (200 \(\mu\)M) with intact dejellied sea urchin eggs and used the formation of the fertilization envelope as a read out. After 20 min incubation with Sph, 17.6\% of eggs (27/153 eggs) had raised the envelope compared to 4\% (4/100 eggs) for the vehicle control (DMSO), suggesting that extracellular Sph is able to raise Ca\(^{2+}\) concentrations in the intact egg and that the effects observed in the homogenate are not artefactual.

In the homogenate system, the apparent EC\(_{50}\) (70.9 ± 3.5 \(\mu\)M; Fig. 1B) of this release correlates with the effective concentrations observed in mammalian cells (low micromolar). Furthermore, the concentrations inducing the minimal detectable response (30 \(\mu\)M) and maximal response (140 \(\mu\)M) are less than one log unit apart, suggesting a steep Hill slope as was seen previously in Jurkat T-cells ([13]; Fig. 1B).

Previous work in rat pancreatic cells has suggested that Sph-induced Ca\(^{2+}\)-release is dependent on the metabolism of Sph to another compound, possibly sphingosylcholine or S-1-P [14,17]. In the sea urchin egg homogenate, we did not observe a lag phase of several minutes as reported in pancreatic cells. However, we did observe a long rising phase of the release which could be indicative of metabolism to an active compound. In order to verify that the Ca\(^{2+}\) transient was not due to a Sph metabolite, experiments were performed at 7 °C to reduce enzymatic activity. Under these conditions, the transient observed in response to 100 \(\mu\)M Sph was grossly similar in both extent and rate to that at 17 °C, suggesting that Sph itself is likely to be responsible for the release observed (Fig. 1C). Furthermore, neither S-1-P (Fig. 1D) nor Cer (Fig. 1E) was able to induce Ca\(^{2+}\)-release at 100 \(\mu\)M, strengthening the idea that the effects seen are not due to metabolism of Sph. Nonetheless, pre-treatment with S-1-P partially antagonized Sph release (Table 1), suggesting a possible competitive receptor interaction between the two sphingolipids.

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>Response (% of 100 (\mu)M Sph control release)</th>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>MgCl(_2) (20 nM)</td>
<td>-8.1 ± 10.7</td>
</tr>
<tr>
<td>Heparin (300 (\mu)g/ml)</td>
<td>75.2 ± 2.5</td>
</tr>
<tr>
<td>NAADP (5 nM)</td>
<td>94.6 ± 11.9</td>
</tr>
<tr>
<td>8-Br-cADPR (100 (\mu)M)</td>
<td>76.9 ± 5.4</td>
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<tr>
<td>cADPR (500 (\mu)M)</td>
<td>85.9 ± 15.6</td>
</tr>
<tr>
<td>S-1-P (100 (\mu)M)</td>
<td>64.9 ± 5.2</td>
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</table>

Data are expressed as means ± SEM of at least four determinations.
Pre-treatment for 1 min with 20 mM MgCl₂ caused a complete inhibition of the Sph response (Table 1). This evidence would suggest that the increase in Ca²⁺ observed occurs via a Ca²⁺ channel and not via the inhibition of pumps or via an increase in membrane leak.

Release was from intracellular stores expressing the SERCA pump as release was completely blocked by pre-treatment with thapsigargin (10 μM), suggesting that Sph released Ca²⁺ from the endoplasmic reticulum (Fig. 1F). As reported previously, NAADP-induced Ca²⁺ release was insensitive to thapsigargin (data not shown, [30]). Furthermore, pre-treatment of homogenates with GPN (glycyl-phenylalanine 2-naphthylamide), which causes osmotic lysis of lysosomes and blocks NAADP-induced Ca²⁺ release [8], had no effect on Sph-induced Ca²⁺ release (data not shown).

These data led us to investigate whether Sph was able to induce Ca²⁺ release via one of the already characterized release mechanisms. Ryanodine is the classical blocker of the RyR and at high concentrations (>100 μM) exerts its action by locking the receptor in a closed conformation leaving it refractory to stimulation by cADPR, Ca²⁺, and other RyR modulators. Ryanodine (200 μM) completely inhibited release by 100 μM Sph (104.6 ± 10.8% inhibition; Fig. 2A). Furthermore, the local anaesthetic and RyR inhibitor, procaine (1 mM), was able to inhibit Sph-induced Ca²⁺ release by 75.7 ± 8.9% (Fig. 2B), indicating that the pharmacology of Sph-induced Ca²⁺-release overlaps with that of the RyR. Surprisingly, though, pre-treatment of homogenates with the cADPR antagonist 8-Br-cADPR (100 μM), a concentration able to inhibit cADPR-induced Ca²⁺ release (data not shown; [31]), had only a small effect on Sph-induced Ca²⁺ release (Table 1).

Heparin (300 μg/ml), the IP₃ receptor antagonist, inhibited Sph-induced Ca²⁺ release by 25 ± 2.5%, suggesting that the IP₃ receptors are not key to Sph-induced release (Table 1). Furthermore, pre-treatment of homogenates with 5 nM NAADP, a concentration sufficient to completely inhibit NAADP-induced Ca²⁺ release [32,33], was unable to inhibit Sph-induced Ca²⁺ release significantly (Table 1). Several reports have shown that redundancy exists between intracellular Ca²⁺ release mechanisms in that physiological responses cannot be blocked by the addition of an inhibitor of one mechanism alone but that they can be blocked by the addition of inhibitors of two mechanisms simultaneously [34,35]. This suggests that mechanisms may be able to compensate for each other. In order to discount this possibility, we pre-treated homogenates with a cocktail of heparin (300 μg/ml), 8-Br-cADPR (100 μM), and NAADP (5 nM) for 5 min, and then added Sph (100 μM; Fig. 2C). Release was not significantly affected by the pre-treatment with this inhibitor cocktail.

Our data would suggest that the ryanodine receptor is the main effector of sphingosine-induced Ca²⁺-release in the sea urchin egg homogenate. Yet, it is peculiar that the competitive antagonist of cADPR did not display a profound inhibition on this behaviour. To elucidate this, we investigated heterologous desensitization between cADPR and Sph. This phenomenon manifests itself by the inability of a substance to release Ca²⁺ after an initial stimulation with a maximal

![Fig. 2](image-url). (A) Inhibition of Sph-induced Ca²⁺-release by ryanodine. The control shows the release by Sph in the absence of ryanodine pre-treatment. (B) Inhibition of Sph-induced Ca²⁺-release by procaine. (C) Lack of inhibition of Sph-induced Ca²⁺-release by an inhibitor cocktail containing heparin (300 μg/ml), 8-Br-cADPR (100 μM), and NAADP (5 nM). (D) Inhibition of cADPR-induced Ca²⁺-release by pre-treatment with Sph. Traces are representative of at least three independent experiments. RFU, relative fluorescent units.
dose with another substance. Usually, the presence of such phenomenon has been considered indicative of an interaction of the two substances with the same receptor. We first induced Ca\(^{2+}\) release with cADPR (500 nM) and, when the trace returned to baseline, we added Sph (100 μM). Ca\(^{2+}\) release by Sph after release by cADPR was only slightly reduced compared to control (~14% reduction; Table 1). In control experiments with two sequential additions of cADPR we always observed >90% inhibition of the second response (data not shown; [32,36]). Strikingly, when similar experiments were performed with addition of Sph (100 μM) followed by subsequent addition of cADPR (500 nM), we observed a large inhibition of the cADPR response (Fig. 2D; 73.3 ± 6.8% inhibition, n = 6).

Finally, when radioligand binding experiments were carried out using \(^{32}\)PcADPR, 100 μM Sph was unable to compete for the cADPR binding site on sea urchin egg membranes. This was true for homogenates pre-treated with Sph for 30 min and homogenates where Sph was co-incubated with \(^{32}\)PcADPR (specific binding after Sph pretreatment 91.6 ± 3.9% of control, specific binding after Sph co-incubation 98.8 ± 2.5% of control).

Our data therefore indicate that Sph releases Ca\(^{2+}\) via the RyR, and that this effect is not mediated by the same mechanism used by cADPR. At present, it is difficult to reconcile collectively that (i) Sph release does not display homologous desensitization; (ii) that Sph release is unaffected by prior stimulation of the receptor by cADPR; and (iii) that cADPR is unable to activate the receptor after Sph. It has been shown that cADPR-induced Ca\(^{2+}\) release requires calmodulin [37] and that desensitization is mediated by calmodulin dissociating from the receptor complex [36]. It is possible, therefore, that Sph release does not require calmodulin but induces its dissociation. Although this possibility is plausible, it would be expected that cADPR binding should be significantly affected by the dissociation of calmodulin [27], while this was not observed. It may be that other, as yet unidentified proteins are required for cADPR-induced Ca\(^{2+}\) release and desensitization which are affected by Sph. Intriguingly, in mammalian cardiac and skeletal muscle, Sph has been shown to inhibit \[^{3}H\]ryanodine binding and RyR Ca\(^{2+}\)-release activity [38,39], and this may represent a late evolutionary development in the RyR or a difference between RyR subtypes.

Taken together, these data suggest that Sph can act as a Ca\(^{2+}\) release agent in both the intact sea urchin egg and in sea urchin egg homogenates. Release is from a thapsigargin-sensitive store that most likely relates to the endoplasmic reticulum. Last, Sph appears to release Ca\(^{2+}\) via the RyR by a mechanism distinct from that sensitive to cADPR. It would be interesting to speculate that sphingosine itself or similar sphingolipids may participate in the physiology of echinoderms. Our contribution demonstrates that if another sphingolipid is responsible for these effects, it is not S-1-P or ceramide, the two main lipids thought to be involved in Ca\(^{2+}\) signalling in mammalian cells.

References


