Exogenous and endogenous ceramides elicit volume-sensitive chloride current in ventricular myocytes

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Aims
Because ceramide accumulates in several forms of cardiovascular disease and ceramide-induced apoptosis may involve the volume-sensitive \( I_{\text{Cl,swell}} \) current, we assessed whether ceramide activates \( I_{\text{Cl,swell}} \).

Methods
and results
\( I_{\text{Cl,swell}} \) was measured in rabbit ventricular myocytes by whole-cell patch clamp after isolating anion currents. Exogenous \( \text{C}_2 \)-ceramide (C2-Cer), a membrane-permeant short-chain ceramide, elicited an outwardly rectifying \( \text{Cl}^- \) current in both physiological and symmetrical \( \text{Cl}^- \) solutions that was fully inhibited by DCPIB, a specific \( I_{\text{Cl,swell}} \) blocker. In contrast, the metabolically inactive C2-H2-derivative (C2-H2Cer) failed to activate \( \text{Cl}^- \) current. Bacterial sphingomyelinase (SMase), which generates endogenous long-chain ceramides as confirmed by tandem mass spectrometry, also elicited an outwardly rectifying \( \text{Cl}^- \) current that was inhibited by DCPIB and tamoxifen, another \( I_{\text{Cl,swell}} \) blocker. Bacterial SMase-induced current was partially reversed by osmotic shrinkage and fully suppressed by ebselen, a scavenger of reactive oxygen species. Outward rectification with physiological and symmetrical \( \text{Cl}^- \) gradients, block by DCPIB and tamoxifen, and volume sensitivity are characteristics that identify \( I_{\text{Cl,swell}} \). Insensitivity to C2-H2Cer and block by ebselen suggest involvement of ceramide signalling rather than direct lipid-channel interaction.

Conclusion
Exogenous and endogenous ceramide elicited \( I_{\text{Cl,swell}} \) in ventricular myocytes. This may contribute to persistent activation of \( I_{\text{Cl,swell}} \) and aspects of altered myocyte function in cardiovascular diseases associated with ceramide accumulation.

Keywords
Cl channel • Ceramide • Sphingomyelinase • \( I_{\text{Cl,swell}} \) • VRAC

1. Introduction
Volume-sensitive \( \text{Cl}^- \) current, \( I_{\text{Cl,swell}} \), is elicited in cardiac myocytes by osmotic swelling, hydrostatic inflation, and \( \beta 1 \) integrin stretch, and in several models of cardiac disease. In turn, \( I_{\text{Cl,swell}} \) modulates cardiac electrical activity, cell volume, and apoptosis, and is implicated in ischaemic preconditioning.\(^1\)\(^-\)\(^3\) Regulation of \( I_{\text{Cl,swell}} \) is complex and involves a number of signalling pathways. Recently, reactive oxygen species (ROS) were identified as a downstream effector, and exogenous \( \text{H}_2\text{O}_2 \) elicits \( I_{\text{Cl,swell}} \) in cardiomyocytes\(^4\)^\(^-\)\(^6\) and other cells.\(^7\)-\(^9\)

Upstream signalling molecules include Src family kinases,\(^10\)-\(^12\) focal adhesion kinase,\(^12\),\(^13\) protein tyrosine kinase,\(^14\) angiotensin II (Ang II),\(^4\),\(^6\) epidermal growth factor receptor (EGFR) kinase,\(^11\) and phosphoinositide 3-kinase (PI3K).\(^5\),\(^6\) Protein kinase C (PKC) also is implicated, although its role is controversial because it appears to inhibit\(^15\) or activate \( I_{\text{Cl,swell}} \).\(^15\),\(^16\)

Many of the signalling cascades that activate \( I_{\text{Cl,swell}} \) overlap those involved in sphingolipid signalling.\(^17\)-\(^19\) raising the possibility that certain sphingolipids might regulate \( I_{\text{Cl,swell}} \). Sphingolipids are a class of phospholipids defined by the presence of an amide-linked fatty
acid, a free hydroxyl group at position 3, and a trans double-bond between carbons 4 and 5. Initially, sphingolipids were considered membrane structural components without further function. More recently, sphingolipids, specifically ceramide and sphingosine, were recognized as bioactive molecules that participate in a number of signalling cascades and mediate apoptosis, mitogenesis, and other cellular processes. Alterations in sphingolipid metabolism are implicated in cardiovascular diseases, including congestive heart failure, athero-sclerosis, and ischaemia/reperfusion injury.18,20 Sphingosine kinase, which phosphorylates the ceramide metabolite sphingosine, mediates Ang II-induced PI-3K activation21 and EGRF upregulation22 in vascular smooth muscle cells. Exogenous ceramide elicits ROS production via NADPH oxidase in bovine coronary artery cells23 and the mitochondrial electron transport chain in rat liver24 and heart.25 Moreover, I_{Cl swell} is postulated to control the ceramide-induced apoptotic volume decrease (AVD) in cardiomyocytes,26 but effects of ceramide on I_{Cl swell} were not assessed.

This study tested whether ceramide activates I_{Cl swell} in ventricular myocytes. Under isosmotic conditions, exogenous, short-chain ceramide elicited an outwardly rectifying Cl^{-} current in both physiological and symmetrical Cl^{-} gradients that was suppressed by DCPIB, a highly selective I_{Cl swell} blocker. Bacterial sphingomyelinase (SMase), which generates endogenous long-chain ceramides, also elicited an outwardly rectifying Cl^{-} current that was inhibited by DCPIB and tamoxifen, a second I_{Cl swell} blocker. Finally, osmotic shrinkage partially reversed and the ROS scavenger ebselen fully reversed SMase-induced current. These data suggest that ceramide evokes I_{Cl swell} in cardiac myocytes. This may contribute to the persistant activation of I_{Cl swell} in cardiovascular diseases marked by ceramide accumulation.

2. Methods

This study conforms to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, 1996) and was approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (AM10290).

2.1 Cell isolation and experimental solutions

Ventricular myocytes were isolated from adult New Zealand white rabbits (~3 kg) by an enzymatic dissociation procedure.13 Complete cell isolation methods are given in Supplementary material online.

Bath and pipette solutions were designed to isolate Cl^{-} current. Isosmotic bath solution (1 T: 300 mOsm/kg; T, times isosmotic) contained (in mM): 90 N-methyl-D-glucamine-Cl, 3 MgCl2, 10 HEPES, 10 glucose, 5 CsCl, 0.5 CdCl2, 70 mannitol (pH 7.4, adjusted with CsOH). Hyperosmotic bath solution (1.5 T, 450 mOsm/kg) had the same composition except for an additional 150 mM mannitol, and hypo-osmotic bath solution (0.7 T, 230 mOsm/kg) contained no mannitol. For experiments with SMase, 6 mM MgCl2 was added to augment enzymatic activity, with mannitol concentrations adjusted accordingly. Pipette solution contained (in mM): 110 Cs-Aspartate, 20 TEA-Cl, 5 Mg-ATP, 0.1 Tris–GTP, 0.15 CaCl2, 8 Cs2-EGTA, 10 HEPES (pH 7.1, adjusted with CsOH). To make symmetrical Cl^{-} pipette solution, 82 mM CsCl replaced an equal amount of Cs-Aspartate. Osmolarity was verified by freezing-point depression.

Stock solutions of α-erythro-C2-Ceramide (C2-Cer; 5 mM; Biomol), α-erythro-C2-dihydriceramide (C2-H2Cer; 5 mM; Biomol), ebselen (15 mM, Calbiochem), DCPIB (20 mM; Tocris) in DMSO, and tamoxifen (20 mM; Sigma-Aldrich) in ethanol were frozen (~20 °C) in aliquots until use. Stock solutions of Mg^{2+}-dependent, neutral bacterial SMase, also known as SMase C, from Bacillus cereus (50 U/mL, in H2O; Sigma-Aldrich) were stored in aliquots at 4 °C until use.

Endogenous synthetic short-chain C2-Cer was employed because it is membrane-permeant and is soluble in serum-free experimental solutions without forming micelles. In contrast, bacterial SMase generates native long-chain ceramides from membrane SMase and may better represent ceramide accumulation in physiological and pathophysiological settings.

2.2 Electrophysiological recordings

Ventricular myocytes were scattered on a glass-bottomed chamber and on an inverted light microscope (Nikon) with Hoffman modulation optics and a high-resolution video camera to visualize cells. Cells were superfused with bath solution at 2–3 μL/min at 22–23 °C. Pipettes were pulled from 7740 thin-walled borosilicate tubing (Sutter) and fire-polished to a final tip diameter of ~3 μm with a resistance in bath solution of 2–4 MΩ. Whole-cell currents were recorded using an Axopatch 200B amplifier and Digidata 1322A (Axon). A 3 M KCl agar bridge served as ground. Seal resistances of 2–20 GΩ were obtained typically, and membrane capacitance was measured routinely. Membrane potential was corrected for measured liquid junction potential in all experiments, and myocytes were dialysed with pipette solution for 8–10 min prior to the start of recording. Voltage clamp protocols and data acquisition were controlled by pClamp 8.2. Successive 500 ms steps were from a holding potential of −60 mV to test potentials from −100 to 60 mV in 10 mV increments. Membrane currents were low-pass-filtered at 2 kHz and digitized at 5 kHz. Representative traces were low-pass-filtered at 500 Hz for presentation, and displayed I–V curves are from corresponding current traces. Currents were not leak-corrected. To minimize variability, experiments used cells as their own controls.

2.3 Lipid analysis by tandem mass spectrometry

Cells in 1 T bath solution were treated with bacterial SMase (0.03 U/mL, 15 min), or left untreated. Lipids were extracted and assayed as described,27,28 with slight modification. Sphingosine, sphinganine, sphingosine-1-phosphate sphinganine-1-phosphate, and ceramide-1-phosphate were quantified via reversed-phase HPLC ESI-MS/MS using a Discovery C18 column attached to a Shimadzu HPLC (20AD series) and mass spectrometric analysis using a 4000 Q-Trap (Applied Biosystems).27 Ceramides, sphingomyelins, and monohexosyl ceramides were quantified via normal-phase HPLC ESI-MS/MS using an amino column (Sigma).28 Complete methods for lipid analysis are given in Supplementary material online.

2.4 Statistics

Summary data are reported as mean ± SEM; n denotes the number of cells. Mean currents are expressed as current density (pA/pF), and selected paired comparisons are expressed as a percentage or as intervention-induced difference currents. Statistical analysis was executed using SigmaStat 3.11 (Systat). Except as noted, a one-way or one-way repeated measures ANOVA was performed followed by a Student–Newman–Keuls test. P < 0.05 was taken as significant. Non-linear curve fits were done in SigmaPlot 10.0 (Systat).

3. Results

3.1 Exogenous ceramide activates a Cl\(^{-}\) current resembling I_{Cl swell}

C2-Cer (2 μM, 10–12 min), a membrane-permeant, short-chain ceramide analogue, activated an outwardly rectifying Cl\(^{-}\) current with a reversal potential near the Cl\(^{-}\) equilibrium potential (E_{Cl}). −43 mV (figure 1). Current at 60 mV increased by 0.70 ± 0.09 pA/pF (n = 15,
from 0.94 ± 0.13 to 1.57 ± 0.22 pA/pF, and a C2-Cer-induced current was observed in >90% of cells tested. Addition of DCPIB (10 μM, 12–15 min), a highly selective I\textsubscript{Cl,swell} blocker, inhibited C2-Cer-induced Cl\textsuperscript{−} current by 76 ± 8% (n = 6, P < 0.001) in the continued presence of C2-Cer, and there was no significant difference between the DCPIB-inhibited and control currents. Furthermore, C2-Cer-induced current was steeply concentration-dependent with an EC\textsubscript{50} of 0.41 μM and a Hill coefficient of 3.6. The physiological range for native ceramide in many cell types is 1–5 μM,\textsuperscript{29} although local concentrations under some conditions may be greater\textsuperscript{19}; because C2-Cer is a short-chain synthetic ceramide, its concentration dependence may not match that of native ceramides.

No change in membrane capacitance was observed in individual cells treated with C2-Cer. Under control conditions, background current usually displayed modest outward rectification, and its amplitude varied from cell to cell. Such variation was noted previously and likely reflects partial activation of I\textsubscript{Cl,swell} under control conditions.

Figure 1 C2-Cer elicited a Cl\textsuperscript{−} current that resembled I\textsubscript{Cl,swell}. (A) Families of currents under control conditions (Ctrl), after C2-Cer exposure (2 μM, 10 min), and after addition of DCPIB (+DCPIB; 10 μM) in continued presence of C2-Cer. Holding potential, −60 mV; test potentials, −100 to 60 mV. (B) Current−voltage (I−V) relationships for A. (C) Normalized currents at 60 mV. C2-Cer increased Cl\textsuperscript{−} current by 0.70 ± 0.09 pA/pF (n = 14, P < 0.001). C2-Cer-induced current was inhibited by 76 ± 8% (n = 6, P < 0.001) by the I\textsubscript{Cl,swell}-specific inhibitor DCPIB; current after DCPIB was not different than control. (D) C2-Cer-induced currents at 0.2 (n = 3), 0.36 (n = 3), 0.6 (n = 3), 2 (n = 14), and 20 μM (n = 4) and fit (solid line) to EC\textsubscript{50} of 0.41 μM and Hill coefficient of 3.6.

Figure 2 C2-Cer (2 μM, 10 min) activated outwardly rectifying Cl\textsuperscript{−} current in symmetrical Cl\textsuperscript{−}. (A) Families of currents and (B) I−V relationships. C2-Cer-induced current reversed near 0 mV. (C) C2-Cer-induced current at 60 mV was 1.30 ± 0.35 pA/pF (n = 6, P < 0.01). Outward rectification in symmetrical Cl\textsuperscript{−} and block by DCPIB (Figure 1) indicate C2-Cer activated I\textsubscript{Cl,swell}.

Ceramide activates volume-sensitive Cl\textsuperscript{−} current.
C2-H2Cer suggests $I_{\text{Cl,swell}}$ was elicited via normal ceramide pathways rather than by a non-specific mechanism.

### 3.2 Endogenous ceramide generation is sufficient to activate $I_{\text{Cl,swell}}$

Bacterial SMase is a neutral, Mg$^{2+}$-dependent enzyme that acts specifically at the plasmalemma to convert sphingomyelin to long-chain ceramides that are native to the cell. Bacterial SMase (0.03 U/mL, 15–18 min), like exogenous C2-Cer, evoked an outwardly rectifying Cl$^{-}$ current in >90% of cells tested, and current at 60 mV increased by 1.01 ± 0.05 pA/pF ($n = 75$, $P < 0.001$), from 1.22 ± 0.07 to 2.23 ± 0.10 pA/pF (Figure 4A and 8). SMase-induced current was reversible with 20 min of washout in control bath solution in each of the cells tested ($n = 3$, $P < 0.05$) (Figure 4C). No change in membrane capacitance was observed with bacterial SMase treatment. As expected and confirmed by tandem mass spectrometry, bacterial SMase increased myocyte ceramides and decreased sphingomyelins under the same experimental conditions (Figure 4D and E).

Two blockers of $I_{\text{Cl,swell}}$ inhibited bacterial SMase-induced current. DCPIB suppressed 78 ± 6% (10 μM, $n = 7$, $P < 0.01$) of the current, and the remaining current was not significantly different than control.

Figure 3 C2-H2Cer, a metabolically inactive C2-Cer analogue, did not alter membrane current, but C2-Cer elicited $I_{\text{Cl,swell}}$ in the same cell. (A) Typical currents at 60 mV. (B) Cl$^{-}$ current densities in control and with C2-H2Cer and C2-Cer (both: 2 μM, 10 min). C2-H2Cer was ineffective ($-4 ± 5\%$, $n = 6$, NS), whereas C2-Cer subsequently activated current in all four cells tested ($P < 0.01$). The data suggest C2-Cer elicited $I_{\text{Cl,swell}}$ via its normal pathway rather than by non-specific mechanisms.

Figure 4 Bacterial SMase reversibly activated $I_{\text{Cl,swell}}$. (A) I–V relationships for Cl$^{-}$ current elicited by SMase (0.03 U/mL, 15–18 min) and inhibition by DCPIB (10 μM). (B) SMase increased Cl$^{-}$ current by 1.1 ± 0.1 pA/pF at 60 mV ($n = 30$), and DCPIB (10 or 30 μM) suppressed 78 ± 6% ($n = 7$) or 81 ± 6% ($n = 4$), respectively ($P < 0.01$ for both). (C) Effect of SMase reversed on washout (18–20 min, $n = 3$, $P < 0.05$). (D and E) Exposure to SMase (20 min) generated endogenous long-chain ceramides and depleted a substantial fraction of sarcolemmal sphingomyelins ($n = 6$; *$P < 0.05$). For ceramides and sphingomyelins, each lipid species was compared with control using a three-way ANOVA based on two separate experimental data sets, each analysed in triplicate.
Increasing DCPIB to 30 μM did not reduce the SMase-induced current further (81 ± 6%; n = 4, P = 0.86 vs. 10 μM DCPIB). Tamoxifen (10 μM, 5–8 min) also was effective in blocking the SMase-induced Cl\textsuperscript{−}\textsuperscript{2} current (Figure 5); it decreased current by 116 ± 16% at 60 mV (n = 5, P < 0.01). Block of SMase-induced current by DCPIB and tamoxifen confirms its attribution to I\textsubscript{Cl,swell}.

The volume-sensitivity of Cl\textsuperscript{−}\textsuperscript{2} current elicited by bacterial SMase was tested by exposure to hyperosmotic (1.5 T) bathing solution in the continued presence of SMase (Figure 6). Cell shrinkage for 15 min inhibited SMase-induced current by 43 ± 8% (n = 6, P < 0.01), from 2.46 ± 0.43 pA/pF at 60 mV to 1.56 ± 0.33 pA/pF at 60 mV. There was no difference in Cl\textsuperscript{−}\textsuperscript{2} currents under control conditions (1.59 ± 0.55 pA/pF) and after the addition of ebselen (P = 0.87). This demonstrates that the SMase-induced Cl\textsuperscript{−}\textsuperscript{2} current is mediated by ROS.

### 3.3 ROS mediate bacterial SMase-induced activation of I\textsubscript{Cl,swell}

Previously, we demonstrated that H\textsubscript{2}O\textsubscript{2} is a downstream mediator of I\textsubscript{Cl,swell} activation and exogenous H\textsubscript{2}O\textsubscript{2} elicits I\textsubscript{Cl,swell} even under hyperosmotic conditions. As shown in Figure 7, ebselen (20 μM, 5 min), a cell-permeable glutathione peroxidase mimetic that converts H\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O, inhibited SMase-induced Cl\textsuperscript{−}\textsuperscript{2} current by 124 ± 39% (n = 5, P < 0.01) from 2.46 ± 0.43 pA/pF to 1.56 ± 0.33 pA/pF at 60 mV. There was no difference in Cl\textsuperscript{−}\textsuperscript{2} currents under control conditions (1.59 ± 0.55 pA/pF) and after the addition of ebselen (P = 0.87). This demonstrates that the SMase-induced Cl\textsuperscript{−}\textsuperscript{2} current is mediated by ROS.

### 3.4 Differences in time course of activation due to exogenous and endogenous ceramides

Figure 8 compares the time course of activation of I\textsubscript{Cl,swell} by C\textsubscript{2}-Cer and bacterial SMase. The C\textsubscript{2}-Cer-induced difference current was fit by a single exponential function with a time constant of 6.4 ± 1.6 min (R\textsuperscript{2} = 0.93, n = 11), equivalent to a t\textsubscript{1/2} of 4.8 ± 1.2 min. In contrast, SMase-induced difference current was fit by a sigmoid function with a t\textsubscript{1/2} of 9.3 ± 0.6 min (R\textsuperscript{2} = 0.99, n = 10). The magnitude of the current turned on at 60 mV by C\textsubscript{2}-Cer, bacterial SMase, and osmotic swelling (i.e. test—control) also were compared. The C\textsubscript{2}-Cer-induced current (0.70 ± 0.09 pA/pF; n = 14) was significantly different than that evoked by bacterial SMase (1.01 ± 0.05 pA/pF; n = 75, P < 0.02) or by hypo-osmotic cell swelling in 0.7 T bath solution (1.22 ± 0.17 pA/pF; data not shown; n = 6, P < 0.05), whereas the SMase- and swelling-induced currents were indistinguishable (P = 0.24).
4. Discussion

Exogenous C2-Cer and endogenous long-chain ceramides generated by bacterial SMase activated currents that reversed near \( E_{Cl} \) exhibited outward rectification in physiological and symmetrical \( Cl^- \) gradients, were partially inhibited by hyperosmotic shrinkage, and were suppressed by the ROS scavenger ebselen. These biophysical features matched those of volume-sensitive \( I_{Cl,swell} \).1,3,30 ROS are required for \( I_{Cl,swell} \).4–9 Additionally, block by DCPIB and tamoxifen strongly implicated \( I_{Cl,swell} \). Tamoxifen may suppress \( I_{Cl,swell} \) by scavenging ROS and inhibiting mitochondrial complex I,32 whereas the mechanism of block by DCPIB is unknown. Although several independent lines of evidence support the conclusion that ceramides activate \( I_{Cl,swell} \), we cannot rigorously exclude the possibility that short-chain and native ceramides form plasmalemmal pores that fortuitously share multiple characteristics with \( I_{Cl,swell} \). C2-Cer and C16-Cer produce pores with very high conductances, up to 200 nS, in mitochondrial outer membranes and lipid bilayers,33 but the resulting currents are far too large to explain those described here.

Swelling in 0.7 T gives nearly full activation of \( I_{Cl,swell} \) in ventricular myocytes,34 and the magnitude of the current elicited by bacterial SMase and hypo-osmotic swelling were not distinguishable. In contrast, 2 \( \mu M \) C2-Cer evoked a significantly smaller current (\(-70\%\) of SMase- and 60\% of 0.7 T-induced currents) that activated more rapidly, and increasing C2-Cer from 2 to 20 \( \mu M \) did not elicit additional current. These differences may reflect, in part, that C2-Cer must permeate the sarcolemma to reach its target(s) and that SMase must first hydrolyse sarcolemmal sphingomyelin to native long-chain ceramides, which also must reach target(s). It also is possible that synthetic short-chain and native long-chain ceramides work via distinct pathways or differ in their efficacy to stimulate processes causing \( I_{Cl,swell} \) activation. That hyperosmotic shrinkage in 1.5 T only partially inhibited SMase-induced current may suggest that it acts at multiple sites and one is downstream from the site controlled by shrinkage. Insensitivity of \( I_{Cl,swell} \) to osmotic shrinkage when elicited by a downstream effector is not unique. We previously showed that \( H_2O_2 \)-induced \( I_{Cl,swell} \) is insensitive to osmotic shrinkage.6 Effects of sphingolipids on sarcolemmal channel function have been explored only recently. Prolonged (>10 h) C2-Cer and bacterial SMase exposure downregulates hERG K+ channels via a pathway involving ROS,35,36 and CFTR is inhibited more rapidly (<60 min).37 These effects appear to be PKA- and PKC-independent. d’Anglemont de Tassigny et al.26 found that \( I_{Cl,swell} \) is required for the AVD in cardiomyocytes and hypothesized that \( I_{Cl,swell} \) is activated in C2-Cer-induced apoptosis. Although outwardly rectifying \( Cl^- \) currents were

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Figure 7 Bacterial SMase-induced \( Cl^- \) current was inhibited by ebselen. (A) Currents before and after treatment with SMase (0.03 U/mL, 15–18 min) and after the addition of ebselen (20 \( \mu M \), 5 min). (B) I–V relationships. (C) Ebselen, a glutathione peroxidase mimetic that scavenges \( H_2O_2 \), fully blocked SMase-induced \( Cl^- \) current at 60 mV (\( n = 5 \), \( P < 0.01 \)). These data suggest the SMase-induced \( Cl^- \) current is elicited by \( H_2O_2 \), a downstream mediator of \( I_{Cl,swell} \).6

Figure 8 Time course of \( I_{Cl,swell} \) activation by C2-Cer and bacterial SMase. C2-Cer data were fit by an exponential function with a time constant of 6.4 ± 1.6 min (\( R^2 = 0.98 \), \( n = 11 \)), equivalent to a \( t_{1/2} \) of 4.8 ± 1.2 min. SMase data were fit by a sigmoid function with a \( t_{1/2} = 9.3 ± 0.6 \) min (\( R^2 = 0.99 \), \( n = 10 \)). Soluble C2-Cer may reach the site of activation of \( I_{Cl,swell} \) more quickly than long-chain endogenous ceramides that must first be produced by SMase. Alternatively, ceramides with different chain lengths may activate different sites in the signalling cascade.
observed during doxorubicin-induced apoptosis, these authors did not establish a link between ceramide and $I_{\text{Cl,swell}}$ activation.

Modification of direct interactions between membrane lipids and channel proteins has been invoked to explain altered gating of Kv channels.38,39 and CFTR inhibition.40 after SMase D treatment. SMase D depletes membrane sphingomyelin without stimulating ceramide signalling; it produces choline and ceramide-1-phosphate, whereas bacterial SMase (SMase C) generates phosphorylcholine and ceramide. Such depletion of membrane lipids is not likely to explain the present results, however. C2-Cer and bacterial SMase both activated $I_{\text{Cl,swell}}$ whereas C2-Cer will favour, if anything, an increase in sphingolipids rather than depletion.

The lack of an effect of metabolically inactive C2-H2Cer supports the hypothesis that both C2-Cer and endogenous ceramides generated by bacterial SMase act via one or more ceramide signalling cascades rather than by a non-specific mechanism.31 Furthermore, block of SMase-induced current by ebselen strongly suggests ROS, most likely H2O2, are an intermediate. Amplification by a signalling cascade may contribute to the strong concentration dependence of current activation. In cardiomyocytes, ROS produced by NADPH oxidase14-6 and mitochondria41 are essential downstream effectors of $I_{\text{Cl,swell}}$ activation by osmotic swelling, integrin stretch, and growth factors, and exogenous H2O2 elicits $I_{\text{Cl,swell}}$ in cardiomyocytes and other tissues.7,8 Ceramides also produce ROS. For example, apoptosis triggered by ceramide is accompanied by mitochondrial ROS production,10-5 and ceramide is involved in NADPH oxidase activation in rat mesangial and bovine coronary artery smooth muscle cells.23,42

Native ceramides generated by bacterial SMase may not be the ultimate sphingolipid mediator of $I_{\text{Cl,swell}}$. Both ceramide and its metabolite, S1P, are potent lipid second messengers, often with opposing effects on signalling and a cell’s fate via the ceramide/S1P rheostat.17,19 Native ceramides generated by bacterial SMase may not be the ultimate sphingolipid mediator of $I_{\text{Cl,swell}}$. Both ceramide and its metabolite, S1P, are potent lipid second messengers, often with opposing effects on signalling and a cell’s fate via the ceramide/S1P rheostat.17,19 In contrast, metabolites are unlikely to be required to explain the action of synthetic C2-Cer because it does not undergo metabolism by the cellular ceramide pathway.43

$I_{\text{Cl,swell}}$ is persistently activated in models of dilated cardiomyopathy1 and is involved in the AVD7,8 that precedes premature cell death in normal development, ischaemia, or heart failure. The sphingomyelin/ceramide pathway is activated in vivo during ischaemia/reperfusion,20,44,45 and heart failure.7,46,47 and the oxidation of sphingolipids is implicated in atherosclerotic plaque formation.18 The data presented here show a link between intracellular ceramide accumulation and $I_{\text{Cl,swell}}$ activation that may be important for understanding these cardiovascular disease states. Because $I_{\text{Cl,swell}}$ outwardly rectifies, its activation tends to shorten action potential duration and depolarize resting membrane potential. Nevertheless, effects on other ion channels must be assessed to evaluate the consequences of ceramide accumulation on cardiac electrophysiology.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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