Inhibition of Sodium-Calcium Exchange by Ceramide and Sphingosine*

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Na+/Ca2+ exchange activity in Chinese hamster ovary cells expressing the bovine cardiac Na+/Ca2+ exchanger was inhibited by the short chain ceramide analogs N-acetylsphingosine and N-hexanoylsphingosine (5–15 μM). The sphingolipids reduced exchange-mediated Ba2+ influx by 50–70% and also inhibited the Ca2+ efflux mode of exchange activity. The biologically inactive ceramide analog N-acetylsphinganidine had only modest effects on exchange activity. Cells expressing the Δ(241–680) and Δ(680–685) deletion mutants of the Na+/Ca2+ exchanger were not inhibited by ceramide; these mutants show defects in both Na+-dependent and Ca2+-dependent regulatory behavior. Another mutant, which was defective only in Na+-dependent regulation, was as sensitive to ceramide inhibition as the wild-type exchanger. Inhibition of exchange activity by ceramide was time-dependent and was accelerated by depletion of internal Ca2+ stores. Sphingosine (2.5 μM) also inhibited the Ca2+ influx and efflux modes of exchange activity in cells expressing the wild-type exchanger; sphingosine did not affect Ba2+ influx in the Δ(241–680) mutant. The effects of the exogenous sphingolipids were reproduced by blocking cellular ceramide utilization pathways, suggesting that exchange activity is inhibited by increased levels of endogenous ceramide and/or sphingosine. We propose that sphingolipids impair Ca2+-dependent activation of the exchanger and that in cardiac myocytes, this process serves as a feedback mechanism that links exchange activity to the diastolic concentration of cytosolic Ca2+.

The Na+/Ca2+ exchanger is the principal Ca2+ efflux mechanism in cardiac myocytes and plays a critical role in regulating the force of cardiac muscle contraction (1). Its stoichiometry is thought to be 3 Na+/Ca2+ (2), although a recent report suggests that it may have a higher, or variable, stoichiometry (3). Exchange activity is regulated by Ca2+-dependent and Na+-dependent processes; cytosolic Ca2+ activates exchange activity by binding to high affinity regulatory sites located within a large (546 residues) hydrophilic domain situated between the fifth and sixth transmembrane segments of the exchanger (4, 5). Cytosolic Na+ is thought to induce the time-dependent formation of an inactive state (Na+-dependent inactivation) after it binds to the Na+ translocation sites on the exchanger (6). Na+-dependent inactivation can be antagonized by ATP-dependent synthesis of phosphatidylinositol 4,5-bisphosphate, by elevated concentrations of cytosolic Ca2+ and by certain mutations within the “regulatory” hydrophilic domain of the exchanger (6–8).

The physiological significance of these regulatory mechanisms is uncertain (9, 10). In intact cells, the Kd for Ca2+-dependent activation of the exchanger is ~50 nM (11–13), suggesting that the exchanger may be nearly fully activated under “resting” conditions. The low cytosolic Na+ concentration and the high levels of ATP and phosphatidyl 4,5-bisphosphate in healthy cells preclude a major role for Na+-dependent inactivation in regulating exchange activity under physiological conditions. Indeed, there is no direct evidence that Na+/Ca2+ exchange activity is in fact regulated in functioning cardiac myocytes. The present report addresses the possibility that sphingolipids such as ceramide and sphingosine serve as physiological regulators of Na+/Ca2+ exchange activity.

Ceramide is a central component of the sphingomyelin cycle, a stress-activated signaling pathway that participates in the induction of apoptosis and growth arrest. The activating or inhibiting effects of ceramide on a host of intracellular signaling pathways have been described in several recent reviews (14–16). Ceramide can also be converted to sphingosine and sphingosine phosphate, two other signaling lipids with important regulatory effects of their own.

Here we show that short chain ceramide analogs and sphingosine inhibit Na+/Ca2+ exchange activity in transfected Chinese hamster ovary (CHO) cells expressing the bovine or canine cardiac Na+/Ca2+ exchangers. Similar effects were noted when the cells were treated for 60 min with an inhibitor of endogenous ceramide metabolism. The differential effects of ceramide on exchange mutants defective in Na+- or Ca2+-dependent regulation suggest that ceramide blocks the conformational transitions associated with Ca2+-dependent activation of exchange activity. We propose that in functioning cardiac myocytes, sphingolipids and diastolic Ca2+ levels interact to control the distribution of exchangers between the Ca2+-activated and nonactivated forms.

**EXPERIMENTAL PROCEDURES**

Cells—CHO cells expressing Na+/Ca2+ exchange activity (CK1.4 cells) were prepared by transfecting the cells (CCL 61; American Type Culture Collection) with the expression vector pcDNA I/Neo (Invitrogen Corp., Carlsbad, CA) containing a cDNA insert coding for the bovine 1 The abbreviations used are: CHO, Chinese hamster ovary; C2-ceramide, N-acetylsphingosine; C6-ceramide, N-hexanoylsphingosine; C2-dihydrosphingosine, N-acetylsphinganine; PDMP, N-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PPMP, N-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; Na-PSS, sodium physiological salts solution; K-PSS, potassium physiological salts solution; Mops, 4-morpholinepropanesulfonic acid.

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cardiac Na+/Ca2+ exchange (17). The Δ(421–680) deletion mutant was prepared and expressed in CHO cells (CK138 cells) as described (18). For XIPA cells, the basic residues in the XIP region of the exchanger (21RPLLRYKYYKRYRAGQKR) were replaced by alanines (21AALLFYAVVAYAAAGAAQA). For this purpose, RevII and BoI restriction sites were introduced into the cDNA for the bovine exchanger (p17; Ref. 19) by site-directed mutagenesis at positions 1016 and 1079, respectively; an oligonucleotide cassette with the desired sequence was ligated into the cDNA after cleavage with RevII and BoI to create the XIPA mutant. cDNAs for the Δ(680–865) mutant of the canine exchanger, as well as the wild-type canine exchanger, was kindly provided by Drs. Kenneth D. Philipson and Deborah A. Nicoll, UCLA School of Medicine; the full-length cDNAs were ligated into the pCDS2A expression vector (Invitrogen) and used for transfection of CHO cells following the procedures described previously (17). The cells were grown in Iscove's modified Dulbecco's medium containing 10% fetal calf serum and antibiotics as described (17).

Materials and Solutions—Na-PSS contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM glucose, and 20 mM HEPES adjusted to pH 7.4 (37 °C) with Tris. K-PSS had the same composition as Na-PSS except for 240 mM KCl. A modified Dulbecco's medium containing 10% fetal calf serum and antibiotics as described (17) was diluted 7-fold with K-PSS to yield 20/120 Na/K-PSS. Fura-2/acetoxymethyl ester and 0.25 mM bongkrekic acid (3 mM NaCl) was added to the cuvettes after the 30-min loading period, the cells were centrifuged for several minutes, washed with Na-PSS, and resuspended in 4–5 ml of Na-PSS. For fura-2-based assay of Ba2+ influx, the cells were centrifuged and resuspended in 4–5 ml of Na-PSS containing 20 mM Mops and 0.3 mM EGTA. Gramicidin (3 μg/ml) was added to the cuvettes and yields a greater initial inhibition of Ba2+ influx, except that 0.1% bovine serum albumin was present in the medium instead of 1% bovine serum albumin. PPMP (30 μM) was added from a 1000-fold concentrated stock solution in dimethyl sulfoxide; for the control cells, an equal volume of dimethyl sulfoxide alone was added. After 30 min of incubation, 3 μM fur-2/acetoxymethyl ester was added, and after an additional 30 min of incubation, the cells were centrifuged, washed, and preincubated for 2 min in Na-PSS with the additions specified in individual experiments. PPMP was not present in the wash or the 2-min preincubation solutions.

RESULTS

Ceramide Analogs Inhibit Exchange-mediated Ba2+ Influx—The data in Fig. 1 show that 15 μM N-acetylphosphosine (C2-ceramide), a short chain fatty acid analog of ceramide, inhibited Na+/Ca2+ exchange activity in transfected CHO cells expressing the bovine cardiac Na+/Ca2+ exchanger (CK1.4 cells). Exchange activity was assayed as the rate of Ba2+ influx in cells treated with gramicidin in a medium containing 20 mM NaCl and 120 mM KCl; the gramicidin was added to equalize the Na+ concentrations across the plasma membrane (20). As shown in Fig. 1, the initial rate of Ba2+ influx was inhibited by 38% (p < 0.02) when 15 μM C2-ceramide was included in the assay medium. The degree of inhibition increased with time, as shown by the downward curvature of the trace in Fig. 1. By 200–300 s, the rate of Ba2+ influx in the presence of C2-ceramide was 72% less (p < 0.001) than that of control cells over the same interval (Fig. 1B).

Ceramide Analogs Inhibit Exchange-mediated Ba2+ Influx—The traces in Fig. 3 depict the
effects of C2-ceramide on Na\(^+\)-dependent Ca\(^{2+}\) efflux. In this experiment, cells were placed in either Na- or K-PSS, and thapsigargin (1 \(\mu\)M) was added 60 s later. Following the addition of thapsigargin, an increase in [Ca\(^{2+}\)] was observed because of the release of Ca\(^{2+}\) from internal stores. The traces in panel A for control cells show that the rise in [Ca\(^{2+}\)] was reduced in Na-PSS compared with K-PSS; as discussed in detail elsewhere (21), this difference is due to Ca\(^{2+}\) efflux by the Na\(^+/\)Ca\(^{2+}\) exchanger. When 15 \(\mu\)M C2-ceramide was included in the assay medium, the difference between Na- and K-PSS was substantially reduced, indicating that exchange-mediated Ca\(^{2+}\) efflux was inhibited by the presence of ceramide. The peak rise in [Ca\(^{2+}\)] in K-PSS was reduced in the ceramide-treated cells compared with untreated cells, and a small, gradual rise in [Ca\(^{2+}\)] was observed during the 30 s prior to the addition of thapsigargin (Fig. 3B). The rise in [Ca\(^{2+}\)] evoked by ceramide was clearly evident during a 3-min incubation period (data not shown), suggesting that ceramide itself induced a slow release of Ca\(^{2+}\) from the stores.

Ceramide and Mutant Exchangers—The exchange protein consists of multiple transmembrane segments with a large hydrophilic domain of 546 amino acids on the cytosolic membrane surface between the fifth and sixth transmembrane segments (4, 5). The hydrophilic domain is thought to be essential for the regulation of exchange activity because its elimination by proteolysis or mutagenesis eliminates normal regulatory behavior (23). The data in Figs. 4 and 5 show the effects of ceramide in cells expressing several different mutations involving the hydrophilic domain. The traces in Fig. 4A show that C2-ceramide had only a modest effect on exchange activity in cells expressing a mutant, \(\Delta\)241–680, in which 440 amino acids were deleted from the exchanger’s hydrophilic domain. The initial rate of Ba\(^{2+}\) influx was reduced by 28% in the presence of C2-ceramide (p = 0.05, paired t test), a value similar to that seen with the wild-type exchanger when treated with the biologically inactive C2-dihydroceramide (Fig. 2). As with C2-dihydroceramide, little or no inhibition was observed during the later portions of the time course of Ba\(^{2+}\) uptake.

A 20-residue region at the N-terminal end of the hydrophilic domain plays an important role in exchanger regulation (24–26). This region contains positively charged and hydrophobic residues in a pattern that suggests an amphipathic helix. A peptide corresponding to this region inhibits Na\(^+/\)Ca\(^{2+}\) exchange activity when applied to the cytosolic membrane surface, and this has therefore been designated the XIP region for exchange inhibitory peptide (25). Site-directed mutagenesis studies have shown that substitution of key positively charged residues in this region with uncharged amino acids abrogates sodium-dependent inactivation, suggesting an important contribution of this region to regulatory behavior. In Fig. 4, the traces in Panel B show the effect of ceramide on cells expressing a mutant exchanger, designated XIPA, in which all the positively charged residues in this region have been substituted with alanines. The data show that C2-ceramide strongly inhibits exchange activity in this mutant. Traces a and b in Fig. 4B show Ba\(^{2+}\) influx in a Na\(^+\)-free assay medium; under these conditions, exchange activity is absent, and Ba\(^{2+}\) enters cells through passive leak pathways. After correcting for passive Ba\(^{2+}\) entry, the initial rate of Ba\(^{2+}\) influx was found to be inhibited by 68% (p < 0.001) in the presence of C2-ceramide. The biologically inactive C2-dihydroceramide (15 \(\mu\)M) had no effect on exchange activity in this mutant (data not shown).

The behavior of cells expressing a small deletion, \(\Delta\)680–685, within the hydrophilic domain of the canine cardiac exchanger is presented in Fig. 5A. The activity of this mutant is not regulated by cytosolic Ca\(^{2+}\) and shows little or no Na\(^+\)-dependent inactivation (27). The extent of Ba\(^{2+}\) influx in these cells was reduced compared with wild-type cells (see below), and so 5 mM BaCl\(_2\) was used in these experiments to increase the signal obtained. As shown in Fig. 5 (A and C), exchange activity was not significantly inhibited by 15 \(\mu\)M C2-ceramide (11% inhibition; p > 0.3). Traces a and b in Fig. 5A show the
cells were pretreated with thapsigargin and assayed for Ba\(^{2+}\) influx in the presence of 15 mM Ba\(^{2+}\). Cells expressing the canine wild-type exchanger were pretreated with thapsigargin in K-PSS/EGTA and assayed for Ba\(^{2+}\) influx in Na-free assay medium (K-PSS/EGTA + 1 \mu g/ml gramicidin) (n = 9).

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**Fig. 4. Effect of C2-ceramide in cells expressing \(\Delta(241-680)\) and XIPA mutations of the exchanger.** A, cells expressing the \(\Delta(241-680)\) exchanger mutant were pretreated with thapsigargin and assayed for Ba\(^{2+}\) influx with or without 15 \mu M C2-ceramide as described in Fig. 2 (n = 10). B, as in A, but with XIPA cells (see text; n = 9–10). For traces a and b, cells were pretreated with 1 \mu M thapsigargin in K-PSS/EGTA and assayed for Ba\(^{2+}\) influx in Na-free assay medium (K-PSS/EGTA + 1 \mu g/ml gramicidin) (n = 9). Rates in panel C were calculated from the slopes of the traces in panels A and B between 64 and 82 s.

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**Fig. 5. Effect of C2-ceramide in cells expressing \(\Delta(680–685)\) mutation of the exchanger (A) and the canine wild-type exchanger (B).** A, cells expressing the canine \(\Delta(680–685)\) exchanger mutant were pretreated with thapsigargin and assayed for Ba\(^{2+}\) influx with (n = 10) or without (n = 18) 15 \mu M C2-ceramide; conditions were as described in Fig. 2, except that the Ba\(^{2+}\) concentration was 5 mM. B, as in A., but with cells expressing the canine wild-type exchanger (n = 10–11). For traces a and b in both panels, the cells were pretreated with 1 \mu M thapsigargin in K-PSS and assayed for Ba\(^{2+}\) influx (5 mM Ba\(^{2+}\)) in sodium-free assay medium (K-PSS + 1 \mu g/ml gramicidin; n = 9–13). C, initial rates of Ba\(^{2+}\) influx, given as the slopes of the traces in panels A and B between 64 and 82 s.
preincubated in EGTA \((p > 0.3)\). This was not simply due to saturation of the fura-2 or to Ba\(^{2+}\) uptake achieving a steady state, because other traces with C2-ceramide (e.g. in Fig. 2) did not display a downward curvature over a similar range of fura-2 ratios. We suggest that \([Ca^{2+}]_i\) probably continued to decline following the addition of Ba\(^{2+}\) in these experiments, leading to gradual decline in the Ca\(^{2+}\)-dependent activation of exchange activity in the ceramide-treated cells.

**Sphingosine Inhibits Exchange Activity**—The ceramide analogs used in this study are short chain acyl derivatives of sphingosine. The data in Fig. 7 demonstrate that sphingosine itself (2.5 \(\mu\)M) inhibited exchange-mediated Ba\(^{2+}\) influx in cells expressing the wild-type exchanger but not in cells expressing the \(\Delta(241–680)\) mutant. At a concentration of 2.5 \(\mu\)M, sphingosine did not make the cells leaky to Ba\(^{2+}\), as shown in Fig. 7A (trace a). At higher concentrations, however, a substantial Ba\(^{2+}\) leak was induced, and so we were restricted to sphingosine concentrations \(\leq 2.5 \mu\)M. At this concentration, sphingosine inhibited the initial rate of exchange-mediated Ba\(^{2+}\) influx by 68\%, after correcting for the passive rates of Ba\(^{2+}\) influx. The data in Fig. 7D show that sphingosine partially inhibited the Ca\(^{2+}\)-efflux mode of exchange activity. The difference between the traces for Tg-induced Ca\(^{2+}\) release in K-PSS versus Na-PSS was reduced compared with the control cells (Fig. 7C), indicating a reduction in Na\(^{+}\)-dependent Ca\(^{2+}\) efflux. Note that sphingosine also induced a substantial leak of Ca\(^{2+}\) from internal stores, as shown by the increase in the fura-2 signal prior to the addition of Tg; this probably accounts for the more rapid rise and shorter duration of the Tg-evoked \([Ca^{2+}]_i\), transient in the presence of sphingosine compared with controls.

**Endogenous Sphingolipids and Exchange Activity**—DIC-Threeo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) and its shorter chain analog PDMP inhibit glucosyler-ramide synthase and sphingomyelin synthase in CHO cells (28) and other cell types (reviewed in Ref. 29). Because they block important pathways of ceramide utilization, they have been shown to inhibit the synthesis of both the short chain and other cell types (reviewed in Ref. 29). Because they block important pathways of ceramide utilization, they have been shown to inhibit the synthesis of both the short chain and long chain ceramide analogs, we incubated CK1.4 cells with 30 \(\mu\)M C2-ceramide, as indicated (n = 6–7). For traces a and b in panel A, the cells were preincubated for 2 min with 10 \(\mu\)M ionomycin in K-PSS + 0.3 mM EGTA, and assayed for Ba\(^{2+}\) influx in K-PSS + 0.3 mM EGTA with (a) or without (b) 15 \(\mu\)M C2-ceramide \((n = 3)\). C, initial rates of Ba\(^{2+}\) influx \((65–95\) s\) for cells preincubated in 0.3 mM Ca, 0.3 mM EGTA or assayed in the absence of Na\(^{+}\) (K-PSS).

To examine this issue, we pre-incubated the PPMP-treated and control cells for 2 min with ionomycin in Na-PSS containing either 0.3 mM EGTA or 0.3 mM CaCl\(_2\), exactly as described above for Fig. 6. As shown in Fig. 8C (traces a and b), in Table I, the initial values for \([Ca^{2+}]_i\) for the PPMP-treated and control cells after exposure to ionomycin + 0.3 mM CaCl\(_2\). Importantly, the rate of Ba\(^{2+}\) influx under these conditions was inhibited by 42\% in the PPMP-treated cells, indicating that the PPMP treatment inhibited exchange activity by a mechanism that was independent of changes in \([Ca^{2+}]_i\).

This experiment also shows that the initial rates of Ba\(^{2+}\) influx for the control and PPMP-treated cells were stimulated 3.6- to 4.1-fold, respectively, in the cells incubated with 0.3 mM CaCl\(_2\) compared with those incubated with EGTA (Fig. 8D and Table I). Thus, PPMP treatment did not block the activation of the exchanger by Ca\(^{2+}\), despite the lower overall exchange activity. As in the experiments with C2-ceramide (Fig. 6), the rate of Ba\(^{2+}\) influx for the PPMP-treated cells incubated with 0.3 mM CaCl\(_2\) gradually declined following the addition of Ba\(^{2+}\) and by 200–300 s became identical to that for the EGTA-treated cells (110\%, \(p = 0.17\)). (Note that with this batch of cells, the initial values of \([Ca^{2+}]_i\), following treatment with ionomycin + 0.3 mM CaCl\(_2\), were lower than for the experiment in Fig. 6; the reasons for this variability are not known.)

In two of the experiments shown in Fig. 8 (A and trace b, Cont in C), the slopes of the traces for Ba\(^{2+}\) influx in control cells increased following the addition of Ba\(^{2+}\). In both cases, this upward curvature was not observed in the PPMP-treated cells. This behavior is essentially identical to that observed with C2-ceramide, as discussed in connection with Fig. 6.

Some additional observations with PPMP-treated cells are listed below, although detailed data will not be presented in most instances: (a) The inhibitory effects of PPMP required time to develop. For example, after 30 min of incubation with 30 \(\mu\)M PPMP, inhibition of exchange activity was poor compared with cells incubated for 60 min. This behavior is consistent with a time-dependent accumulation of endogenous ceramide and is not compatible with a direct pharmacological effect of PPMP on exchange activity. Indeed, 30 \(\mu\)M PPMP added directly to the cuvette, without preincubation, did not inhibit exchange activity. (b) The data shown in Fig. 8 (A and B) were obtained with cells that had been treated with thapsigargin before assaying for Na\(^+/Ca^{2+}\) exchange activity. Without thap-
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FIG. 7. Sphingosine inhibits Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange activity. A, fura-2 loaded CK1.4 cells were preincubated for 2 min in Na-PSS + 0.3 mM EGTA with 1 \(\mu\)M thapsigargin and then assayed for exchange activity as described in the legend to Fig. 1, with (SPH) or without (b) sphingosine. (n = 11–12). For traces a and b, the cells were preincubated in K-PSS with thapsigargin and assayed for Ba\textsuperscript{2+} influx in K-PSS + gramicidin with (a) or without (b) sphingosine. (n = 5–6). B, as in A, except that cells expressing the \(\Delta(241-680)\) deletion mutant were used (n = 4–6). C, cells were preincubated for 5 min. in Na-PSS + 1 mM CaCl\textsubscript{2} and placed in cuvettes containing either Na-PSS or K-PSS as indicated. Thapsigargin (1 \(\mu\)M) was added after 60 s (n = 6–7). Vertical arrow, Tg. D, conditions were identical to C, except that 2.5 \(\mu\)M sphingosine was included in the medium in the cuvettes (n = 8).

FIG. 8. Endogenous sphingolipids inhibit exchange activity. A, CK1.4 cells were preincubated for 60 min with 30 \(\mu\)M PPMP and loaded with fura-2 as described under “Experimental Procedures.” The cells were then preincubated for an additional 2 min in Na-PSS + 0.3 mM EGTA with 1 \(\mu\)M thapsigargin and assayed for exchange activity as described in the legend to Fig. 1 (n = 7). B, as in A, except that cells expressing the \(\Delta(241-680)\) mutant were used (n = 9–10). C, CK1.4 cells were preincubated with 30 \(\mu\)M PPMP and loaded with fura-2 as described under “Experimental Procedures.” The cells were then incubated for 2 min in Na-PSS + 10 \(\mu\)M ionomycin containing either 0.3 mM CaCl\textsubscript{2} (traces a) or 0.3 mM EGTA (traces b) and assayed for exchange activity as described in the legend to Fig. 1 (n = 7). D, initial rates of Ba\textsuperscript{2+} influx for 34–64 s) for the data in A–C, as indicated.

DISCUSSION

The results presented here demonstrate that short chain ceramide analogs inhibited both the Ca\textsuperscript{2+} influx and Ba\textsuperscript{2+} efflux modes of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange activity (Figs. 1–5). A biologically inactive ceramide analog, in which the essential double bond in the sphingosine moiety is hydrogenated, had only minor effects on exchange activity (Fig. 2). Moreover, certain mutant exchangers that are defective in their regulatory behavior were not inhibited by ceramide (Figs. 4 and 5). These data suggest that the effects of ceramide are not simply due to a generalized membrane perturbation but that ceramide acts in a biologically relevant manner and specifically targets the mechanisms that regulate exchange activity. Recent results...
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**Table I**

| Cells | Post-PPMP treatment | PPMP | \(\text{Ca}^{2+}\) influx | Inhibition |
|-------|---------------------|------|--------------------------|-----------|
| CK1.4 | 1 mM CaCl\(_2\) | – | 60.4 ± 2.6 | 12 ± 2.1 | 64 | <0.001 |
|       | + | 38.2 ± 2.5 | 0.45 ± 0.06 |           |           |
| CK1.4 | Thapsigargin + 0.3 mM EGTA | – | 44.7 ± 1.2 | 1.1 ± 0.1 | 49 | <0.001 |
|       | + | 23.8 ± 1.8 | 0.57 ± 0.08 |           |           |
| CK1.4 | Ionomycin + 0.3 mM CaCl\(_2\) | – | 56.0 ± 2.1 | 2.9 ± 0.3 | 42 | <0.01 |
|       | + | 63.9 ± 9.0 | 1.6 ± 0.1 |           |           |
| Δ(241–680) | 1 mM CaCl\(_2\) | – | 28.0 ± 2.2 | 0.82 ± 0.04 | 50 | <0.001 |
|       | + | 15.9 ± 1.8 | 0.41 ± 0.04 |           |           |
| Δ(241–680) | Thapsigargin + 0.3 mM EGTA | – | 54.2 ± 8.9 | 1.9 ± 0.2 | 35 | 0.015 |
|       | + | 29.7 ± 4.8 | 1.3 ± 0.1 |           |           |

**Effect of PPMP treatment on Na\(^+\)/Ca\(^{2+}\) exchange activity**

Cells were incubated for 60 min with or without 30 μM PPMP and loaded with fura-2 as described under “Experimental Procedures.” They were then centrifuged, washed, and incubated (in the absence of PPMP) for an additional 2 min in 100 μl of Na-FSS plus the additions shown under “Post-PPMP treatment.” The concentrations of thapsigargin and ionomycin were 1 and 10 μM, respectively. The cells were then transferred to cuvettes to assay for Ba\(^{2+}\) influx. [Ca\(^{2+}\)]\(_i\) values were determined during the 10-s interval prior to Ba\(^{2+}\) addition. The significance level for differences in the rates of Ba\(^{2+}\) influx (\(p\)) were determined by Student’s t test (2-tailed).

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indicate that 15 μM C2-ceramide also inhibits Na\(^+\)/Ca\(^{2+}\) exchange currents in cardiac myocytes but does not inhibit either Na\(^+\) or Ca\(^{2+}\) channel activity.\(^2\)

Ceramide is a central component of the sphingomyelin signaling pathway and plays a critical role in the activation of the caspase cascade that leads to apoptosis (14–16). In cardiac myocytes, ceramide and sphingosine levels increase following ischemia/reperfusion (36, 37) and after exposure of the cells to tumor necrosis factor α (38). Ceramide induces a multitude of cellular responses, including induction of the stress-activated protein kinase pathway, activation or inhibition of various individual protein kinases, activation of protein phosphatase 2A, inhibition of mitochondrial respiration, activation of cytochrome c release from mitochondria, and inhibition of phospholipase D (reviewed in Refs. 14–16). Moreover, ceramide can be rapidly converted to ceramide-1-phosphate, sphingosine, and sphingosine-1-phosphate, signaling lipids that have multiple effects of their own. The rapid time course of sphingolipid-induced inhibition of exchange activity in the present study suggests that these agents exert their effects directly on the exchanger rather than through one of the above signal transduction pathways. This conclusion is strongly supported by the recent finding that both sphingosine and C2-ceramide inhibit exchange activity when applied to excised patches from cardiac myocytes.\(^3\)

Short chain ceramide analogs sometimes induce effects that are not mimicked by increases in endogenous ceramide (reviewed in Ref. 39). Moreover, the short chain analogs may themselves bring about alterations in endogenous ceramide and/or sphingosine levels (35, 40, 41). To address these issues, we preincubated the cells for 60 min with PPMP, an agent that blocks conversion of ceramide to glucosylceramide and has been used to elevate endogenous ceramide levels in several different cell types (30–33). As shown in Fig. 8, treatment with PPMP mimicked the effects of the short chain ceramide analogs on Na\(^+\)/Ca\(^{2+}\) exchange activity. We conclude that exchange activity is inhibited by increased endogenous ceramide and/or sphingosine as well as by the exogenous analogs.

Ceramide and sphingosine did not inhibit exchange activity in certain regulatory-deficient mutants, and we therefore conclude that the sphingolipids target one of the mechanisms that regulate exchange activity. The best characterized regulatory mechanisms involve two time-dependent processes that promote inactive states of the exchanger (6, 7). The first process is called “Na\(^+\)-dependent inactivation” and is observed in excised patches as an exponential decay of current to a steady-state value following application of cytosolic Na\(^+\). Na\(^+\)-dependent inactivation is counteracted by the presence of phosphatidylinositol 4,5-bisphosphate (42), by high concentrations of cytosolic Ca\(^{2+}\) (6), and by mutations involving key basic residues or tyrosines in the XIP region (24, 43). The second regulatory process involves the interaction of Ca\(^{2+}\) with high affinity regulatory sites within the central hydrophilic domain of the exchanger (7, 44); the binding of Ca\(^{2+}\) to these regulatory sites appears to be required for all modes of exchanger operation (1, 9).

Na\(^+\)-dependent inactivation does not appear to be involved in the effects of sphingolipids, because the XIPA mutant was as sensitive to inhibition by ceramide as the wild type (Fig. 4B); this mutant does not display Na\(^+\)-dependent inactivation because of the alteration of critical basic residues in the XIP region. Moreover, ceramide inhibited wild-type exchange activity equally well at high (95 mM) and low (9 mM) Na\(^+\) concentrations (data not shown). Because Na\(^+\)-dependent inactivation requires high concentrations of cytosolic Na\(^+\) (6), these results provide another indication that ceramide does not promote this inactivation process.

The findings with the various exchanger mutants and the effects of alterations in Ca\(^{2+}\) homeostasis suggest that sphingolipids interfere with the regulatory activation of the exchanger by Ca\(^{2+}\). C2-ceramide did not inhibit the activities of the deletion mutants Δ(680–685) and Δ(241–680) (Figs. 4 and 5), which are defective in both Ca\(^{2+}\)-dependent activation and Na\(^+\)-dependent inactivation. As mentioned above, C2-ceramide did inhibit the activity of the XIPA mutant (Fig. 4B), in which regulatory Ca\(^{2+}\) activation is intact, but Na\(^+\)-dependent inactivation does not occur (24, 45). Thus, ceramide inhibited the activity of exchangers that display Ca\(^{2+}\)-dependent activation but not in exchanger mutants in which this regulatory mechanism was defective.

Experimental conditions that altered Ca\(^{2+}\) homeostatic processes also affected the response of exchange activity to ceramide. For example, the effects of ceramide developed slowly in

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\(^2\) N. Shepherd, personal communication.

\(^3\) D. Hilgemann, personal communication.
cells with filled Ca$^{2+}$ stores, as shown by the time-dependent decline in the rate of Ba$^{2+}$ influx in the presence of C2-ceramide (Fig. 1A). When internal Ca$^{2+}$ stores were depleted by prior treatment of the cells with thapsigargin or ionomycin, the development of the inhibition of ceramide was accelerated, and the traces for Ba$^{2+}$ influx no longer displayed a downward curvature (Figs. 2 and 6A). The results suggest that there is a link between the filling state of intracellular Ca$^{2+}$ stores and the susceptibility of the exchanger to ceramide inhibition. The basis for this observation is not known; perhaps filled Ca$^{2+}$ stores generate local gradients of elevated [Ca$^{2+}$], in the vicinity of the exchanger, and this antagonizes ceramide inhibition. For PPMP-treated cells, inhibition of exchange activity in the absence of thapsigargin was immediate (Table I) and did not increase with time following Ba$^{2+}$ addition (data not shown). This observation is entirely consistent with the observations described above, because internal Ca$^{2+}$ stores in the PPMP-treated cells were already depleted, presumably because of an inhibition of Ca$^{2+}$ influx (see discussion under "Results").

In several experiments, control cells showed a time-dependent increase in the rate of Ba$^{2+}$ influx, as shown by the upward curvature of the traces (Figs. 4B, 6A, 7A, and 8, A and C; trace b, Cont.). This behavior was not seen with the Δ(241–680) mutant (Figs. 4B, 7B, and 8B), but it was observed with the XIPA mutant (Fig. 4B). We have previously suggested that the acceleration in Ba$^{2+}$ influx is due to the auto-activation of the exchanger by cytosolic Ba$^{2+}$ through its interaction with the Ca$^{2+}$ regulatory sites (11). In the presence of ceramide or sphingosine or in PPMP-treated cells, the corresponding traces for Ba$^{2+}$ influx remained linear throughout the entire time course, indicating that the sphingolipids blocked this process.

These considerations suggest that ceramide/sphingosine impairs the regulatory activation of exchange activity by Ca$^{2+}$. Its precise mechanism of action is not known, however. It does not seem likely that these hydrophobic lipids would interact directly with high affinity Ca$^{2+}$ regulatory sites, because these are located in the hydrophilic domain of the exchanger. A more plausible possibility is that they interact with the transmembrane segments of the exchanger and stabilize the inactive conformation that is attained upon dissociation of Ca$^{2+}$ from its regulatory binding sites (Fig. 9). In this way, the sphingolipids might increase the $K_d$ for Ca$^{2+}$ activation, slow the conformational transitions involved, or block Ca$^{2+}$ activation of the exchange altogether in a subpopulation of exchangers. In any event, it is clear that ceramide does not completely block Ca$^{2+}$ activation, because exchange activity was stimulated by increasing [Ca$^{2+}$], in the ionomycin experiments, both in the presence of C2-ceramide (Fig. 6C) and in the PPMP-treated cells (Fig. 8D and Table I).

In the experiments with ionomycin-treated cells (Figs. 6 and 8C), we sought to determine whether an increase in [Ca$^{2+}$]i would antagonize the inhibitory effects of ceramide or PPMP treatment, as expected if a competitive effect were involved. In both cases, however, the degree of inhibition was similar at high and low [Ca$^{2+}$]i. The highest value of [Ca$^{2+}$]i attained in these experiments was only ~110 nM, however, and it will be important to re-examine this issue in an experimental system (e.g. excised patches) that permits a broader range of [Ca$^{2+}$]i values to be studied. These experiments also showed that for the ceramide- or PPMP-treated cells at the elevated [Ca$^{2+}$]i, the rate of Ba$^{2+}$ influx declined gradually following Ba$^{2+}$ addition and eventually became equal to that seen at the lower [Ca$^{2+}$]i (Figs. 6B and 8C). This behavior probably reflects the gradual reduction in regulatory activation of exchange activity because the initially high level of [Ca$^{2+}$]i declined following Ba$^{2+}$ addition. In the corresponding experiments with control cells, the rate of Ba$^{2+}$ influx did not decline in this manner, consistent with our hypothesis that the ability of Ca$^{2+}$ and/or Ba$^{2+}$ to activate exchange activity is impaired by the sphingolipids.

What are the physiological implications of our results? Ceramide and sphingosine are elevated in cardiac myocytes during stress. The resulting inhibition of exchange activity could be viewed as a protective measure to preserve Ca$^{2+}$ stores and maintain contractile strength under stressful conditions. Alternatively, when cytosolic [Na$^+$] is elevated, as in ischemia, inhibition of exchange activity could be a means of protecting the cell against Ca$^{2+}$ overload by reducing exchange-mediated Ca$^{2+}$ influx. A more interesting possibility, however, is that endogenous ceramide/sphingosine antagonize exchange activity could be a means of protecting the cell against Ca$^{2+}$ overload by reducing exchange-mediated Ca$^{2+}$ influx. A more interesting possibility, however, is that under nonpathological conditions endogenous sphingolipids and the exchanger work coordinately as a Ca$^{2+}$-dependent feedback mechanism to control the distribution of exchangers between active and inactive states, in the manner described below.

The high affinity of the exchanger for regulatory Ca$^{2+}$ ($K_d$ ~50 nM) (11–13) would seem to provide no opportunity for meaningful regulation of exchange activity within a physiological range of [Ca$^{2+}$], values. In a functioning cardiac myocyte, the small fraction of exchangers that become inactive because of dissociation of regulatory Ca$^{2+}$ during diastole ([Ca$^{2+}$]i ~100 nM) would be rapidly reactivated by the ensuing rise in [Ca$^{2+}$i during the next systole. However, if endogenous ceramide/sphingosine were to interfere with Ca$^{2+}$-dependent activation of the exchanger by any of the mechanisms suggested above (Fig. 9), a fraction of the inactive exchangers would be retained in the inactive state ($I_2$ in Fig. 9) despite the rise in [Ca$^{2+}$].

Over multiple contraction/relaxation cycles, the distribution of exchangers between active and inactive states would be determined by two principal factors: diastolic [Ca$^{2+}$], and endogenous levels of ceramide/sphingosine. A fall in diastolic [Ca$^{2+}$] would increase the population of inactive exchangers, thereby reducing exchange-mediated Ca$^{2+}$ influx and eventually restoring diastolic [Ca$^{2+}$], to its normal level. An increase in endogenous ceramide or sphingosine, e.g., during stress, would have the same effect and establish a new steady-state relation between diastolic [Ca$^{2+}$], and exchange activity.

This hypothesis, although speculative, provides a welcome framework for understanding the physiological role of Ca$^{2+}$-dependent activation of exchange activity in light of the high

![Fig. 9. Proposed mechanism of ceramide inhibition.](https://example.com/fig9.png)
affinity of the exchanger for regulatory Ca\textsuperscript{2+} (11–13). In considering the physiological role of sphingolipid-exchanger interactions, it will be essential to define more precisely its effects on Ca\textsuperscript{2+}-dependent activation and the influence of internal Ca\textsuperscript{2+} stores on this process. Work toward this end is currently in progress.

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