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Glucosylceramide and Glucosylsphingosine Modulate Calcium Mobilization from Brain Microsomes via Different Mechanisms*

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We recently demonstrated that elevation of intracellular glucosylceramide (GlcCer) levels results in increased functional Ca^{2+} stores in cultured neurons, and suggested that this may be due to modulation of ryanodine receptors (RyaRs) by GlcCer (Korkotian, E., Schwarz, A., Pelled, D., Schwarzmann, G., Segal, M. and Futerman, A. H. (1999) *J. Biol. Chem.* 274, 21673–21678). We now systematically examine the effects of exogenously added GlcCer, other glycosphingolipids (GSLs) and their lyso-derivatives on Ca^{2+} release from rat brain microsomes. GlcCer had no direct effect on Ca^{2+} release, but rather augmented agonist-stimulated Ca^{2+} release via RyaRs, through a mechanism that may involve the redox sensor of the RyaR, but had no effect on Ca^{2+} release via inositol 1,4,5-trisphosphate receptors. Other GSLs and sphingolipids, including galactosylceramide, lactosylceramide, ceramide, sphingomyelin, sphingosine 1-phosphate, sphinganine 1-phosphate, and sphingosylphosphorylcholine had no effect on Ca^{2+} mobilization from rat brain microsomes, but both galactosylsphingosine (psychosine) and glucosylsphingosine stimulated Ca^{2+} release, although only galactosylsphingosine mediated Ca^{2+} release via the RyaR. Finally, we demonstrated that GlcCer levels were ~10-fold higher in microsomes prepared from the temporal lobe of a type 2 Gaucher disease patient compared with a control, and Ca^{2+} release via the RyaR was significantly elevated, which may be of relevance for explaining the pathophysiology of neuronopathic forms of Gaucher disease.

Sphingolipids (SLs)[1] act as structural components of cell membranes and as bioactive molecules, functioning as both first and second messengers (1, 2). Of the signaling pathways regulated by SLs, Ca^{2+} homeostasis has received wide attention due largely to observations that sphingosine, sphingosine 1-phosphate, and sphingosylphosphorylcholine modulate Ca^{2+}-homeostasis via the *edg* receptors, a class of plasma membrane G-protein-coupled receptors (GPCRs) (3–6). However, complex glyco-SLs (GSLs) and their lyso-derivatives (Fig. 1) have also been implicated in regulating Ca^{2+} homeostasis (7, 8). Of the lyso-GSLs, galactosylsphingosine (GalSph, psychosine) (9–13) has been shown to mobilize Ca^{2+} from intracellular stores, possibly via activation of the ryanodine receptor (RyaR), the major Ca^{2+}-release channel of the endoplasmic reticulum (ER), although this has never been unambiguously proven. Recent studies (reviewed in Ref. 14) have shown that complex GSLs, such as ganglioside GM1, can potentiate the activity of a nuclear envelope Na^{+}-Ca^{2+} exchanger (15), and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) can be modulated by gangliosides GM1 and GM3 (16, 17). Since GSLs and lyso-GSLs accumulate in the sphingolipidoses, in which neuronal function is often severely impaired (18, 19), and since altered Ca^{2+} homeostasis has been implicated in a number of neurodegenerative diseases (20, 21), determination of the molecular mechanisms by which GSLs or lyso-GSLs modulate intracellular Ca^{2+} signaling may be a prerequisite for determining the mechanism leading from GSL accumulation to neuronal cell dysfunction and/or death.

We recently demonstrated (22) that the simplest GSL, glucosylceramide (GlcCer), upon its accumulation in cultured neurons in a chemically induced model of type 2/3 Gaucher disease (the neuronopathic forms of Gaucher disease, Refs. 23 and 24), increases Ca^{2+} mobilization from intracellular stores, presumably via the RyaR. As a result, neurons with elevated GlcCer levels showed enhanced sensitivity to agents that induce cell death via Ca^{2+} mobilization (22, 25). In the current study we systematically examine the effects of GlcCer, other GSLs and their lyso-derivatives on Ca^{2+} release from isolated rat brain microsomes, and demonstrate that GlcCer and its lyso-derivative, glucosylsphingosine (GlcSph) (Fig. 1), both modulate Ca^{2+} release but via different molecular mechanisms, and by a different mechanism to that of galactosylsphingosine (GalSph), none of which involve GPCRs. Moreover, Ca^{2+} release was also enhanced in human brain microsomes obtained from a type 2 Gaucher disease patient, in which GlcCer levels are elevated ~10-fold.

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[1] The abbreviations used are: SL, sphingolipid; DTT, dithiothreitol; GalCer, galactosylceramide; GalSph, galactosylsphingosine; GlcCer, glucosylceramide; GlcSph, glucosylsphingosine; GPCR, G-protein-coupled receptor; GSL, glycosphingolipid; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, inositol 1,4,5-trisphosphate receptor; LacCer, lactosylceramide; LC, long chain; RyaR, ryanodine receptor; ER, endoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; MOPS, 4-morpholinepropanesulfonic acid.

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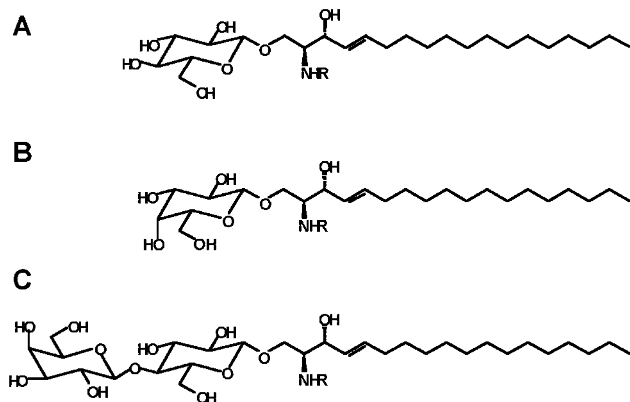

FIG. 1. **Lipids used in this study and their abbreviations.** *A*, GlcSph (*R* is H), C8-GlcCer (*R* is octanoyl), LC-GlcCer (*R* is natural long chain fatty acyl). *B*, GalSph (*R* is H), C8-GalCer (*R* is octanoyl), LC-GalCer (*R* is natural long chain fatty acyl). *C*, C8-LacCer (*R* is octanoyl), LC-LacCer (*R* is natural long chain fatty acyl).

EXPERIMENTAL PROCEDURES

Materials—C8-GlcCer (*N*-octanoyl-D-glucosylsphingosine), C8-galactosylceramide (C8-GalCer; *N*-octanoyl-D-galactosylsphingosine) and C8-lactosylceramide (C8-LacCer; *N*-octanoyl-D-lactosylsphingosine) werefrom Avanti Polar Lipids, Alabaster, AL. Natural long acyl-chain (LC)-LacCer (porcine), sphingosine 1-phosphate, sphinganine 1-phosphate and sphingosylphosphorylcholine were from Matreya, Pleasant Gap, PA. C8-Ceramide (C8-Cer; *N*-octanoyl-D-sphingosine), LC-GlcCer (from human Gaucher spleen), LC-GalCer (from bovine brain), GalSph, GlcSph, antipyrylazo III, A23187, heparin, inositol 1,4,5-trisphosphate ($InsP_3$), palmitoyl CoA, creatine phosphokinase, phosphocreatine, ATP, and NAD were from Sigma. GDPβS and pertussis toxin A protomer were from Calbiochem, Darmstadt, Germany. Aminopropyl (LC-NH_2, 100 mg) and weak cation exchanger (LC-WCX, 100 mg) columns were from Supelco (Bellefonte, PA). Ryanodine was from either Alomone Labs, Jerusalem, Israel, or from Sigma. [^{3}H]ryanodine (109 Ci/mmol) and [^{3}H]acetic anhydride (9.7 Ci/mmol) were from Amersham Biosciences.

Brain Microsomes—Wistar rats, obtained from the Weizmann Institute Breeding Center, were sacrificed, their brains removed, separated into cerebral cortex and cerebellum, rapidly frozen in liquid N_2, and stored at −80 °C. Microsomes (from 10–12 gm of tissue) were prepared essentially as described (26) with some modifications. Tissue was suspended at a ratio of 1:4 (w/v) in ice cold 0.32 M sucrose, 20 mM HEPES-KOH, pH 7.0, containing 0.4 mM phenylmethylsulfonyl fluoride, leupeptin (0.8 μg/ml), and aprotinin (1.4 TIU) (buffer A), and homogenized at 4 °C using 8 up and down strokes of a rotating Potter-Elvehjem homogenizer. After centrifugation (700 × g_{av}, 10 min), the resulting pellet (P1) was gently resuspended in one-fourth of the original volume of buffer A, centrifuged (700 × g_{av}, 10 min), and the two supernatants pooled (S1). Mitochondria were removed by centrifugation (8,000 × g_{av}, 45 min) of S1 and the resulting supernatant (S2) centrifuged (115,000 × g_{av}, 90 min) to obtain a microsomal pellet (P3), which was resuspended in 0.4–0.8 ml of buffer A. Protein was determined (27), and the microsomes subsequently flash-frozen in liquid N_2. Microsomes were stored at −80 °C and used for up to six months after their preparation, during which time there was no change in their activity with respect to Ca^{2+} release and uptake.

Human brain microsomes were prepared exactly as described for rat brain microsomes. Microsomes were prepared from a control human brain of a young adult and from the brain of a type 2 Gaucher patient who died at 1 year of age.

Spectrophotometric Assay of Ca^{2+} Uptake and Release—Ca^{2+} uptake and release was measured by a spectrophotometric assay using the Ca^{2+}-sensitive dye, antipyrylazo III (26, 28, 30), with some modifications. Brain microsomes (330 μg in 8–15 μl of buffer A) were added to 0.95 ml of 8 mM NaMOPS, pH 7.0, 40 mM KCl, 62.5 mM K_2HPO_4, and 250 μM antipyrylazo III, in a plastic cuvette containing a magnetic stir bar, to which 1 mM MgATP, 40 μg/ml creatine phosphokinase, and 5 mM phosphocreatine, pH 7.0, were added. Ca^{2+} uptake and release were measured in a Cary spectrophotometer (Varian Australia Pty Ltd.) at 37 °C by subtracting the absorbance at A_{790} from A_{710} at 2-s intervals.

The effect of GSLs and lyso-GSLs was tested by their addition either prior to or after Ca^{2+} loading (see for example, Fig. 2). C8-GSLs and lyso-GSLs were dissolved in absolute ethanol, and LC-GSLs were dissolved in ethanol/dodecane (98:2, v/v) (31, 32). The final ethanol or ethanol/dodecane concentration did not exceed 2% (v/v) in the cuvette. Sphingosine-1-phosphate and sphinganine-1-phosphate were added as a complex with defatted bovine serum albumin (4:1, mol/mol). Sphingosylphosphorylcholine was dissolved in dimethyl sulfoxide. Pertussis toxin A protomer (1 μg/ml) was added in a solution containing NAD (25 μM), dithiothreitol (1 mM), and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (0.002%, w/v). In all experiments, the effect of solvents and buffers alone was tested.

The amount of Ca^{2+} released from microsomes was expressed as a percent of total Ca^{2+} in the microsomes, which was obtained by summing Ca^{2+} taken up during the Ca^{2+}-loading period together with endogenous Ca^{2+} from the microsomal preparation (measured separately after addition of a Ca^{2+} ionophore, A23187 (2 μM), without Ca^{2+} loading). The rate of Ca^{2+} uptake into microsomes was calculated by measuring the linear portion of the slope after addition of Ca^{2+}, agonist, or lipid. Occasionally, spontaneous quantal Ca^{2+} release (calcium sparks) was observed. Spontaneous Ca^{2+} release was considered to be a spark when $A_{710} - A_{790}$ increased by >0.002 over the baseline, with the baseline defined as the $A_{710} - A_{790}$ value measured immediately before the spark.

[^{3}H]Ryanodine Binding—Rat brain cortical microsomes were resuspended in binding buffer (20 mM HEPES, pH 7.4, 1 M KCl, 550 μM ATP, 100 μM $CaCl_2$) to give a final protein concentration of 1 mg/ml. [^{3}H]Ryanodine-binding was performed as described (33) for 1 h at 37 °C, in a final volume of 200 μl of binding buffer containing 50 μl of microsomes and 0.1–30 nM [^{3}H]ryanodine. Nonspecific binding was determined by preincubation with 50 μM ryanodine. The reaction was terminated by addition of 5 ml of ice-cold wash buffer (20 mM HEPES, pH 7.4, 1 M KCl, 100 μM $CaCl_2$), filtration through GF/C glass fiber filters (Whatman) in a Millipore filtration device (Millipore, Bedford, MA), followed by two additional 5-ml washes. The dissociation constant (K_D) and the maximum number of receptor sites (B_{max}) were derived by Scatchard analysis.

GlcCer and GlcSph Analysis—Lipids were extracted (34) from the same human temporal lobe microsomes used for Ca^{2+} analysis. GlcCer and GlcSph were eluted in one fraction by aminopropyl solid phase chromatography using a LC-NH_2 cartridge as described (34). GSLs and lyso-GSLs were separated by weak cation exchange solid phase extraction using a LC-WCX cartridge, and GSLs subsequently deacylated by alkaline hydrolysis (1 M KOH in methanol, 100 °C, 24 h). The resulting lyso-GSLs were acetylated using 5 mM acetic anhydride containing [^{3}H]acetic anhydride (2 μCi) and NaOH (4 mM) in chloroform/methanol (1:1, v/v), as were the lyso-GSLs obtained in the initial fractionation. A detailed account of this method will appear elsewhere.[2]

RESULTS

Ca^{2+} mobilization from rat brain microsomes was analyzed using the Ca^{2+}-sensitive dye, antipyrylazo III. This dye has been used to measure Ca^{2+} release from muscle (28, 35, 36), which contains high levels of RyaRs (37), and from canine brain (30). By using a rigorous homogenization procedure and by optimizing the recovery of microsomal membranes with respect to Ca^{2+} uptake, we were able to use this dye to measure Ca^{2+} release in rat brain microsomes, from which significantly lower levels of RyaRs can be recovered. Upon its addition to the cuvette, Ca^{2+} accumulated in microsomes and could be released by palmitoyl CoA (Fig. 2*A*), a RyaR agonist (38, 39), to a similar extent to that previously reported in canine brain (30).

Palmitoyl CoA-induced Ca^{2+} release was enhanced upon preincubation with C8-GlcCer (10 μM) by ~3-fold (Fig. 2*B*), and could be blocked by preincubation with 350 μM ryanodine (Fig. 2*C*), a concentration similar to or lower than that used previously to block RyaR-mediated Ca^{2+} release measured using antipyrylazo III (26, 28, 40). In contrast, C8-GlcCer did not induce Ca^{2+} release by itself (Fig. 2*D*), demonstrating that GlcCer is not an agonist of the RyaR, but rather modulates its

[2] J. Bodennec, S. Trajkovic-Bodennec, and A. H. Futerman, in press.

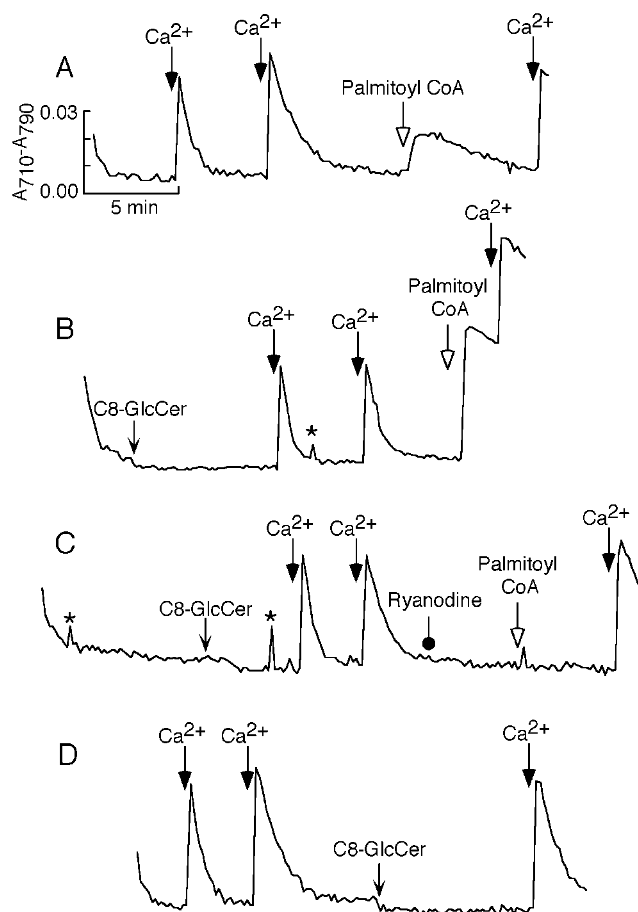

FIG. 2. **Effect of C8-GlcCer on microsomal Ca^{2+} release.** *A*, cortical microsomes were loaded by two sequential additions of 25 nmol of Ca^{2+} prior to addition of palmitoyl CoA (30 μM). Ca^{2+} was also added at the end of each experiment to confirm the functional integrity of the microsomes with respect to Ca^{2+} uptake. In *panels B* and *C*, C8-GlcCer (10 μM) was added before Ca^{2+}, and in *panel D*, after Ca^{2+}. Ryanodine (350 μM) was added to microsomes before palmitoyl CoA in *panel C*. *Asterisks* indicate examples of Ca^{2+} sparks (see Table II). Data are representative traces showing absorbance change ($A_{710}-A_{790}$) of antipyrylazo III *versus* time, with an increase in absorbance demonstrating an increase in free Ca^{2+} in the cuvette, and a decrease in absorbance demonstrating a decrease in free Ca^{2+} due to microsomal Ca^{2+} uptake.

activity. LC-GlcCer enhanced Ca^{2+} release to a similar extent to that of C8-GlcCer, using either palmitoyl CoA or GalSph as RyaR agonists (Table I and Fig. 3). C8-GalCer, over a range of concentrations (Fig. 3), and long-acyl chain GalCer (Table I), were completely ineffective in modulating agonist-induced Ca^{2+} release, as were a variety of other sphingolipids (Table I), demonstrating a highly specific mode of sensitization of the RyaR by GlcCer.

Although GlcCer did not induce Ca^{2+} release by itself (Fig. 2*D*), a significant increase in spontaneous quantal Ca^{2+} release (calcium sparks) (41, 42) was observed in the presence of C8-GlcCer. In untreated microsomes, ~2 sparks per hour were observed over the time-course of a typical experiment, which increased to ~6 sparks per hour in the presence of C8-GlcCer, both of which could be completely blocked by ryanodine (Table II). The amount of Ca^{2+} released per spark was higher in the presence of C8- and LC-GlcCer than in controls, although it was not statistically significant (Table II). Likewise, LC-GlcCer caused a significant increase in spark frequency, but no increase was observed with other GSLs. These data strengthen the idea that GlcCer sensitizes the RyaR.

We next examined the ability of GlcCer to mobilize Ca^{2+} via

TABLE I
Effect of SLs on microsomal Ca^{2+} release

Cortical microsomes were incubated with or without the indicated SLs (10 μM) prior to induction of Ca^{2+} release using either GalSph (100 μM) or palmitoyl CoA (30 μM), as in Fig. 2B. Results are means ± S.D. for four independent experiments.

Sphingolipid	Agonist	
	GalSph	Palmitoyl CoA
	Ca^{2+} release (percent of total)	
None	18 ± 4	19 ± 4
C8-GlcCer	52 ± 3	61 ± 5
C8-GalCer	21 ± 3	16 ± 4
C8-LacCer	23 ± 3	16 ± 2
C8-Ceramide	23 ± 4	16 ± 1
C8-sphingomyelin	22 ± 3	11 ± 4
LC-GlcCer	61 ± 8	55 ± 3
LC-GalCer	17 ± 2	19 ± 4
LC-LacCer	23 ± 4	16 ± 4

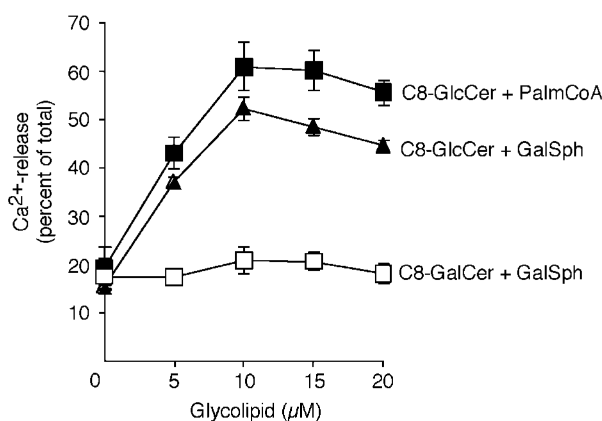

FIG. 3. **Concentration dependence of C8-GlcCer-enhanced agonist-induced Ca^{2+} release.** Cortical microsomes were incubated with varying concentrations of C8-GlcCer or C8-GalCer prior to induction of Ca^{2+} release using either GalSph (100 μM) or palmitoyl CoA (30 μM). Results are means ± S.D. for 3–4 independent experiments.

TABLE II
Effect of GSLs on spontaneous RyaR-mediated Ca^{2+} release

Cortical microsomes were incubated with GSLs (10 μM) or with ethanol or ethanol/dodecane (final concentration of 1% (v/v)), prior to Ca^{2+} loading, as in Figs. 2, *B* and *C*; ryanodine (350μM) or DTT (10 mM) were added prior to C8-GlcCer. The number and size of the Ca^{2+} sparks was calculated from between 3 (ryanodine + C8-GlcCer) to 10 (C8-GlcCer) individual traces. Statistical differences were analyzed by ANOVA comparing C8-GSLs with the ethanol control, and LC-GSLs with the ethanol/dodecane control. *, $p < 0.01$.

	Number of Ca^{2+} sparks per min	Ca^{2+} release per spark
		nmol
No addition	0.028 ± 0.007	2.34 ± 0.23
Ethanol	0.024 ± 0.011	1.90 ± 0.59
Ethanol/dodecane	0.052 ± 0.020	1.36 ± 0.06
C8-GlcCer	0.104 ± 0.019*	3.17 ± 0.40
Ryanodine + C8-GlcCer	0.006 ± 0.006	1.42[a]
DTT + C8-GlcCer	0.015 ± 0.015	1.28[a]
LC-GlcCer	0.115 ± 0.033*	3.13 ± 0.39
C8-GalCer	0.054 ± 0.023	2.02 ± 0.63
LC-GalCer	0.034 ± 0.013	1.52 ± 0.21
C8-LacCer	0.033 ± 0.031	1.94 ± 0.36
LC-LacCer	0.023 ± 0.008	1.84 ± 0.38

[a] Only one spark was observed.

other mechanisms. C8-GlcCer did not affect $InsP_3$-induced Ca^{2+} release from cerebellar microsomes, a rich source of the $InsP_3R$ (43), which could be blocked by the $InsP_3R$ antagonist, heparin (44) (Fig. 4*A*). Neither C8-GlcCer, C8-GalCer (Fig. 4*B*), nor 10 μM LC-GlcCer (0.45 ± 0.08 nmol/sec/mg of protein) or 10 μM LC-GalCer (0.39 ± 0.05 nmol/sec/mg of protein) had an

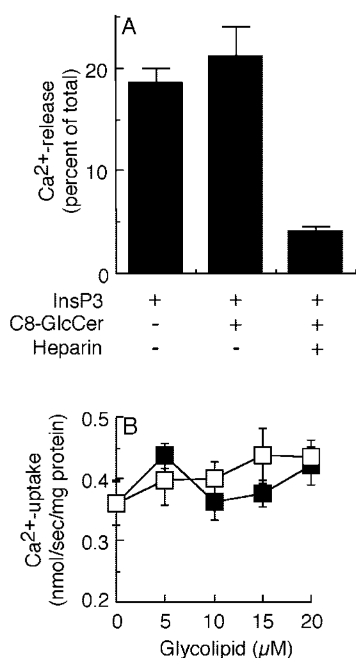

FIG. 4. **Effect of C8-GlcCer on $InsP_3Rs$ and on SERCA.** *A*, cerebellar microsomes were incubated with C8-GlcCer (10 μM) prior to induction of Ca^{2+} release using $InsP_3$ (25 μM), with or without preincubation with heparin (150 μg/ml). Results are means ± S.D. for five independent experiments. *B*, cortical microsomes were incubated with C8-GlcCer (*closed squares*) or C8-GalCer (*open squares*) prior to Ca^{2+} addition, and the rate of microsomal Ca^{2+} uptake analyzed spectrophotometrically. Results are means ± S.D. for four independent experiments.

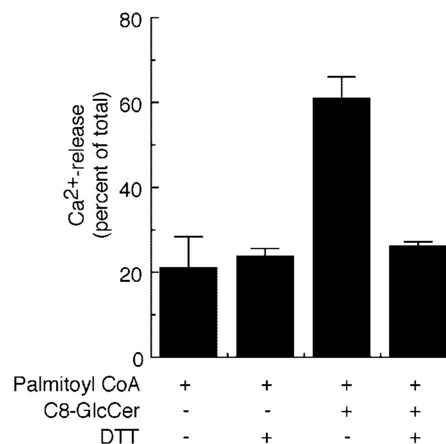

FIG. 5. **Effect of DTT on C8-GlcCer-enhanced agonist-induced Ca^{2+} release.** Cortical microsomes were incubated with or without DTT (10 mM) prior to addition of C8-GlcCer (10 μM), Ca^{2+} (2×25 nmol) and palmitoyl CoA (30 μM). Results are means ± S.D. for three independent experiments.

effect on the rate of Ca^{2+} influx into microsomes via the Ca^{2+}-ATPase, SERCA. Thus, we conclude that GlcCer specifically modulates Ca^{2+} mobilization via the RyaR and not via the $InsP_3R$ or SERCA. Since neither pretreatment with C8-GlcCer, LC-GlcCer, or LC-GalCer had any effect on the B_{max} or K_D of [^{3}H]ryanodine binding to the RyaR (B_{max} of [^{3}H]ryanodine binding (fmol/mg of protein) for control (ethanol or ethanol/dodecane-treated) microsomes = 341 ± 9, B_{max} for C8-GlcCer = 350 ± 46, B_{max} for LC-GlcCer = 291 ± 15, and B_{max} for LC-GalCer = 335 ± 16; K_D (nM) for control = 1.7 ± 0.1, K_D for C8-GlcCer = 1.8 ± 0.7, K_D for LC-GlcCer = 1.4 ± 0.1, K_D for LC-GalCer = 1.7 ± 0.2), we further conclude that GlcCer does not affect the affinity, and hence the efficacy of ryanodine binding to the RyaR.

Recent studies have demonstrated that RyaR activity can be enhanced by its redox state (45–47). Preincubation with the reducing agent, DTT, completely abolished the ability of C8-GlcCer (Fig. 5) to enhance agonist-induced Ca^{2+} release, and blocked calcium sparks (Table II), suggesting that GlcCer may modulate the redox state of the RyaR via its redox sensor (48, 49).

We next examined the extent of Ca^{2+} release from brain tissue obtained from a Gaucher disease type 2 patient to determine the physiological significance of these findings. Previous studies have suggested that GlcCer accumulates in Gaucher brain tissue (50, 51), but the extent of accumulation was highly variable, ranging from 5–80-fold compared with normal brains. Using a new method for separation of GSLs and lyso-GSLs (34), and a new method to quantify these lipids in which the free NH_2 group of lyso-GSLs is derivatized with [^{3}H]acetic anhydride, [2] an ~13-fold higher level of GlcCer was detected in microsomes prepared from the temporal lobe of a type 2 Gaucher patient (*i.e.* a neuronopathic patient) compared with a control brain,[3] and GlcSph was also detected in the Gaucher brain, although at levels ~5-fold less than GlcCer, with no detectable GlcSph in control brain microsomes (Table III). Intriguingly, palmitoyl CoA-induced Ca^{2+} release from Gaucher brain microsomes was significantly higher than from human control brain microsomes, and could be reduced by DTT to control levels (Fig. 6). Thus, these data demonstrate a physiological and pathophysiological link between GlcCer accumulation in Gaucher brains and enhanced levels of Ca^{2+} release via the RyaR.

In contrast to GlcCer, GlcSph and GalSph directly stimulated Ca^{2+} release from rat cortical microsomes, albeit at concentrations of ~5–10-fold higher than GlcCer. Unexpectedly, and in contrast to the ability of ryanodine to block GlcCer-enhanced, agonist-induced Ca^{2+} release (Fig. 2*C*), ryanodine was unable to block GlcSph-mediated Ca^{2+} release (Fig. 7*A*), and GlcSph-induced Ca^{2+} release was not enhanced by GlcCer (not shown), suggesting that GlcSph mediates Ca^{2+} release via a mechanism independent of the RyaR. However, ryanodine did inhibit GalSph-mediated Ca^{2+} release (Fig. 7*B*), demonstrating that GalSph is a RyaR agonist. Neither GlcSph- nor GalSph-induced Ca^{2+} release could be blocked (not shown) by preincubation with GDPβS (100 μM), an inhibitor of G-protein activation, or by pertussis toxin (1 μg/ml), an inhibitor of the heterotrimeric G proteins, G_i and G_o, implying that GPCRs are not involved in mediating the action of GalSph and GlcSph on Ca^{2+} release; this is further supported by the inability of sphingosine-1-phosphate, a GPCR agonist, and of sphinganine-1-phosphate and sphingosylphosphorylcholine (all at 100 μM), to induce Ca^{2+} release from rat brain microsomes (not shown).

DISCUSSION

The major finding of the current study is that GlcCer mobilizes Ca^{2+} from microsomes via a mechanism involving modulation of the activity of a major Ca^{2+} channel of the ER, the RyaR. This finding could be of relevance for understanding the pathophysiology of neuronal forms of Gaucher disease, in which GlcCer accumulates. This contention is strongly supported by our observation that GlcCer accumulates in microsomes prepared from a type 2 Gaucher disease brain, in which

[3] An extensive and systematic analysis of GlcCer and GlcSph levels in human brain tissue, both from control and type 2 and 3 Gaucher patients, is currently underway. Based on previously published data (29, 50, 51, 65), we do not anticipate a large variation in GlcCer levels in control human brains.

TABLE III
GlcCer and GlcSph levels in human brain microsomes

Lipids were extracted from the same human brain microsomes as used in Fig. 6, and GlcCer and GlcSph levels determined by acetylation with [^{3}H]acetic anhydride. Results are means of 2–4 independent analyses for each sample.

	Control brain	Gaucher type 2 brain
	nmol/mg of protein	
GlcCer	2.09	27.95
GlcSph	ND[a]	4.88

[a] ND, not detected, *i.e.* <0.01 nmol/mg of protein (see Footnote 2).

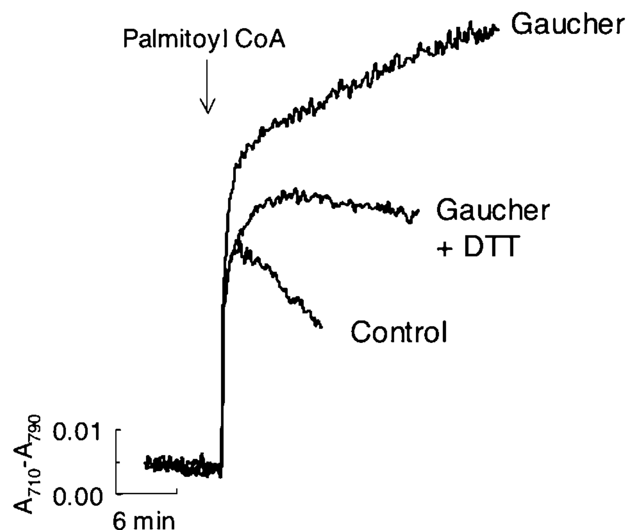

FIG. 6. **Ca^{2+} release from human brain microsomes.** Human temporal lobe microsomes were incubated with or without DTT (10 mM), and then loaded by two sequential additions of 25 nmol of Ca^{2+} (not shown) prior to addition of palmitoyl CoA (30 μM). Data are representative traces showing absorbance change (A_{710}−A_{790}) of antipyrylazo III *versus* time.

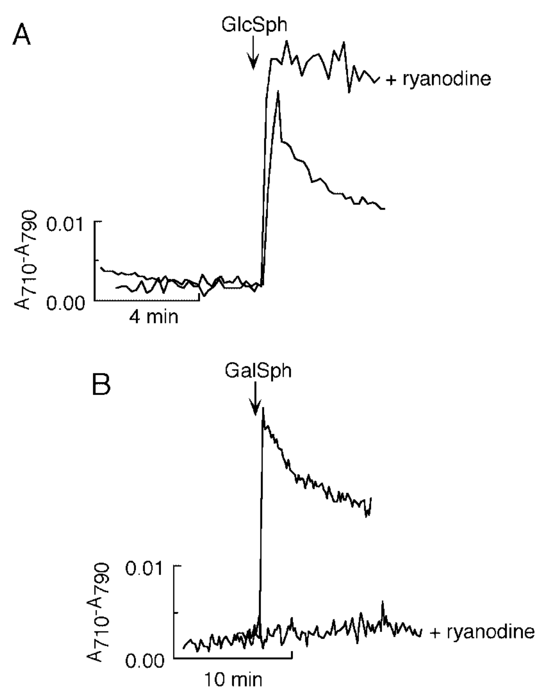

FIG. 7. **Effects of lyso-GSLs on Ca^{2+} release.** Cortical microsomes were loaded by two sequential additions of 25 nmol of Ca^{2+} (not shown in the figure), and then incubated with or without ryanodine (350 μM) prior to addition of 100 μM GlcSph (*A*) or GalSph (*B*). Data are representative traces showing absorbance change (A_{710}−A_{790}) of antipyrylazo III *versus* time.

Ca^{2+} release is also enhanced. Remarkably, the molar concentration of endogenous GlcCer in the human brain microsomes was similar to that added exogenously to rat brain microsomes in order to enhance agonist-induced Ca^{2+} release[4] via the RyaR.

The impetus for the current study was our earlier observation that upon its accumulation in cultured hippocampal neurons, GlcCer caused changes in neuronal functionality, inasmuch as a large increase in Ca^{2+} release from intracellular stores was observed in response to glutamate or caffeine stimulation. Moreover, neurons were more sensitive to glutamate-induced neuronal toxicity and to toxicity induced via various other cytotoxic agents, which could be blocked by preincubation with antagonistic concentrations of ryanodine (22, 25). Our current finding that GlcCer modulates agonist-induced Ca^{2+} release from brain microsomes via the RyaR is consistent with these earlier observations, and the lack of effect of GalCer and other related GSLs and SLs might even imply that GlcCer plays a physiological role in regulation of the RyaR. This is further supported by the increase in the frequency of spontaneous quantal Ca^{2+} release (sparks) upon incubation of microsomes with GlcCer. Ca^{2+} sparks, sudden localized increases in intracellular Ca^{2+} (52), have been observed in muscle and in brain (41, 53–55), and have been suggested to be of key importance in Ca^{2+} signaling in the nervous system (56). The specificity of C8-GlcCer and LC-GlcCer to increase spark frequency strongly supports a central role for GlcCer in the regulation of Ca^{2+} homeostasis via its sensitization of the RyaR. However, it is difficult to directly extrapolate the findings reported herein concerning sparks in brain microsomes to live neurons. Although we do not know, as yet, the precise molecular mechanism by which GlcCer sensitizes the RyaR, the ability of DTT to abolish GlcCer modulation of both Ca^{2+} sparks and of agonist-stimulated Ca^{2+} release suggests that GlcCer may modulate the redox state of the RyaR via its redox sensor. A number of ion channels have been predicted to have an oxidoreductase domain (48). In the case of the RyaR, various cysteines within the channel have been proposed to regulate oxidative and nitrosative responses (46, 57), and GlcCer may interact with the putative redox sensor (45).

If GlcCer acts as a physiological or pathophysiological modulator of the RyaR, which is located in the ER, GlcCer must presumably be present in this organelle, even though it is mainly synthesized distal to the ER, in the Golgi apparatus (58). However, our understanding of the intracellular distribution of GSLs may need to be re-evaluated in light of recent findings demonstrating that GlcCer affects a number of activities associated with the ER and other intracellular organelles (59, 60). Indeed, a recent study demonstrated that sphingolipid-specific glycosyltransferases are found in a mitochondrial-associated ER subcompartment of rat liver (61) which could not be ascribed to contaminating Golgi apparatus membranes. Interestingly, the ceramide glucosyltransferase showed specificity for ceramide bearing phytosphingosine as sphingoid base, suggesting that different pools of GlcCer may be synthesized at different subcellular locations. In support of this are our current findings that microsomes obtained from human Gaucher brain tissue contains significant levels of GlcCer, although no

[4] GlcCer was present at levels of ~30 nmol/mg of protein in Gaucher type 2 microsomes (Table III). 330 μg of protein were used per Ca^{2+} release experiment in a final volume of 1 ml, thus giving a molar concentration of endogenous GlcCer of ~9 μM in the reaction mixture, similar to the concentration used to enhance Ca^{2+} release when added exogenously (Fig. 3).

assessment of the purity of these microsomes was attempted due to limited availability of the human brain tissue.

GlcCer is the only GSL used in this study that sensitizes the RyaR, whereas GalSph and GlcSph both acted as agonists to stimulate Ca^{2+} release from microsomes. Recently, GalSph was shown to induce Ca^{2+} release from cultured cells (62) via a cell surface GPCR, T-cell death-associated gene 8 (TDAG8). However, TDAG8 is expressed at very low levels in brain and has a narrow tissue distribution (63). The lack of effect of GDPβS and pertussis toxin on GalSph and GlcSph-induced Ca^{2+} release from brain microsomes suggests that they do not act via GPCRs in microsomes, although the possibility that GalSph also binds to a cell surface orphan GPCR in neurons, as has been suggested in HL-60 cells (11), cannot be excluded. The lack of effect in microsomes of sphingosine-1-phosphate and sphingosylphosphorylcholine, which bind to cell surface GPCRs (6), together with the antagonistic effect of ryanodine on GalSph-induced Ca^{2+} release, support the notion that GalSph (9–13) acts as an agonist of the RyaR, whereas GlcSph mediates Ca^{2+} release via a mechanism independent of the RyaR.

Whether any or all of the effects of GalSph and GlcSph on Ca^{2+} mobilization are of physiological relevance in normal cells is a matter of debate since lyso-GSL concentrations in cells are normally in the subnanomolar range (see Ref. 34).[5] However, lyso-GSLs accumulate at much higher levels in GSL storage diseases, such as Gaucher and Krabbe's disease, particularly in the brain, and the lyso-GSLs, rather than the GSLs, have been implicated in the mechanisms underlying disease pathology, especially neuropathology (34, 64). Irrespective of the physiological relevance of lyso-GSLs in mobilizing Ca^{2+} from microsomes, the specificity of GlcCer compared with both other GSLs and lyso-GSLs on Ca^{2+} mobilization reported both in this study in microsomes and in our previous study in cultured neurons (22), and our recent study on activation of CTP:phosphocholine cytidylyltransferase by GlcCer (60), suggest that GlcCer is an important intracellular messenger that plays keys roles in both the regulation of phospholipid synthesis and in intracellular Ca^{2+} homeostasis.

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[5] GlcSph was present at levels of ~5 nmol/mg of protein in Gaucher type 2 microsomes (Table III) but was below the detection limit in a control brain. Thus, the molar concentration of endogenous GlcSph in the human microsomes used for Ca^{2+} release studies was ~1.5 μM (estimated as in Footnote 4), a concentration significantly lower than that required to induce Ca^{2+} release from microsomes when added exogenously.