Lyso-glycosphingolipids mobilize calcium from brain microsomes via multiple mechanisms

Emyr LLOYD-EVANS[1], Dori PELLED, Christian RIEBELING and Anthony H. FUTERMAN[2]

Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

Recently, we demonstrated that the GSL (glycosphingolipid), GlcCer (glucosylceramide), modulates Ca^{2+} release from intracellular stores and from microsomes by sensitizing the RyaR (ryanodine receptor), a major Ca^{2+}-release channel of the endoplasmic reticulum, whereas the lyso derivative of GlcCer, namely GlcSph (glucosylsphingosine), induced Ca^{2+} release via a mechanism independent of the RyaR [Lloyd-Evans, Pelled, Riebeling, Bodennec, de-Morgan, Waller, Schiffmann and Futerman (2003) J. Biol. Chem. **278**, 23594–23599]. We now systematically examine the mechanism by which GlcSph and other lyso-GSLs modulate Ca^{2+} mobilization from rat brain cortical and cerebellar microsomes. GlcSph, lactosylsphingosine and galactosylsphingosine all mobilized Ca^{2+}, but at significantly higher concentrations than those required for GlcCer-mediated sensitization of the RyaR. GlcSph-induced Ca^{2+} mobilization was partially blocked by heparin, an inhibitor of the Ins(1,4,5)P_3 receptor, and also partially blocked by thapsigargin or ADP, inhibitors of SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase), but completely blocked when both acted together. In contrast, neither lactosylsphingosine nor galactosylsphingosine had any effect on Ca^{2+} release via either the Ins(1,4,5)P_3 receptor or SERCA, but acted as agonists of the RyaR. Finally, and surprisingly, all three lyso-GSLs reversed inhibition of SERCA by thapsigargin. We conclude that different lyso-GSLs modulate Ca^{2+} mobilization via different mechanisms, and discuss the relevance of these findings to the GSL storage diseases in which lyso-GSLs accumulate.

Key words: galactosylsphingosine, glucosylsphingosine, Ins(1,4,5)P_3, lactosylsphingosine, ryanodine, thapsigargin.

INTRODUCTION

GSLs (glycosphingolipids) are important structural and regulatory components of biological membranes, in particular neuronal membranes [1]. Their lyso (i.e. *N*-deacylated) derivatives (lyso-GSLs) are found in much lesser amounts in normal tissues, and have also been implicated in a variety of regulatory roles, e.g. the regulation of Ca^{2+} homoeostasis [2–4], modulation of enzyme activities [5,6] and modulation of protein kinases [7,8]. Most research studies on lyso-GSLs have focused on determining their functions in the GSL storage diseases, where both GSLs and the lyso-GSL derivatives accumulate. Indeed, lyso-GSLs have been implicated as pathogenic molecules in these diseases, particularly in those associated with neuronal pathology [9]. For instance, in Krabbe's [10] and Gaucher's disease [11], GalSph (galactosylsphingosine, psychosine) and GlcSph (glucosylsphingosine) accumulate respectively to relatively high levels, and have been assigned pathogenic roles [12].

Recently, we demonstrated that the GSL GlcCer (glucosylceramide) modulates Ca^{2+} release from the endoplasmic reticulum [13] by sensitizing the RyaR (ryanodine receptor), although it does not act as an agonist of the RyaR [14]. As a consequence, neurons that accumulate GlcCer are more sensitive to agents that induce neuronal cell dysfunction or death via the RyaR [15]. Importantly, the amount of exogenously added GlcCer required to sensitize the RyaR was close to that which accumulated in microsomes derived from the brain of a neuronopathic patient with Gaucher's disease [14]. In contrast, the mechanism by which GlcSph modulated Ca^{2+} release did not involve the RyaR, as ryanodine did not block GlcSph-mediated Ca^{2+} release [14]. In the present study, we examine the mechanism by which GlcSph, GalSph and LacSph (lactosylsphingosine) release Ca^{2+} from microsomes derived from rat brain cortex, a region enriched in the RyaR, and from rat brain cerebellum, a region enriched in the other major Ca^{2+}-release channel, the InsP_3R [Ins(1,4,5)P_3 receptor] [16]. We demonstrate that all three lyso-GSLs modulate Ca^{2+} release from microsomes by mechanisms different from one another and also different from the one found for GlcCer.

EXPERIMENTAL

Materials

GalSph, GlcSph, antipyrylazo III, thapsigargin, A23187, heparin, InsP_3 [Ins(1,4,5)P_3], palmitoyl CoA, creatine kinase, phosphocreatine, ATP, ADP and Ruthenium red were from Sigma. LacSph was from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Ryanodine was from Alomone Labs (Jerusalem, Israel). $^{45}Ca^{2+}$ (30 mCi/mg) was from Amersham Biosciences (Little Chalfont, Bucks., U.K.).

Rat brain microsomes

Wistar rats, obtained from the Weizmann Institute Breeding Center (Weizmann Institute of Science, Rehovot, Israel), were killed, their brains removed, separated into cerebral cortex and cerebellum, rapidly frozen in liquid nitrogen and stored at − 80 °C. Microsomes (from 10 to 12 g of tissue) were prepared essentially as described in [17] with some modifications [14]. Tissue was suspended in 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 PMSF, 0.8 μg/ml leupeptin and aprotinin (1.4 trypsin inhibitor

Abbreviations used: GalSph, galactosylsphingosine; GlcCer, glucosylceramide; GlcSph, glucosylsphingosine; GSL, glycosphingolipid; InsP_3, Ins(1,4,5)P_3; InsP_3R, InsP_3 receptor; LacSph, lactosylsphingosine; RyaR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase.

[1] Present address: Department of Biochemistry, Glycobiology Institute, University of Oxford, U.K.

[2] To whom correspondence should be addressed (e-mail tony.futerman@weizmann.ac.il).

unit) (buffer A), and homogenized at 4 °C using eight up- and downstrokes of a rotating Potter–Elvehjem-type homogenizer. After centrifugation (at 700 ***g*** for 10 min), the resulting pellet (P1) was gently resuspended in one-fourth of the original volume of buffer A, centrifuged (at 700 ***g*** for 10 min) and the two supernatants were pooled (S1). Mitochondria were removed by centrifugation (at 8000 ***g*** for 45 min) of S1 and the resulting supernatant (S2) was centrifuged (at 115 000 ***g*** for 90 min) to obtain a microsomal pellet (P3), which was resuspended in 0.4–0.8 ml of buffer A. Protein was determined [18] and the microsomes were subsequently flash-frozen in liquid nitrogen. Microsomes were stored at − 80 °C and used up to 6 months after their preparation, when there was no change in their activity with respect to Ca^{2+} release and uptake.

Spectrophotometric assay of Ca^{2+} uptake and release

Ca^{2+} uptake and release were measured by a spectrophotometric assay using the Ca^{2+}-sensitive dye, antipyrylazo III [17,19,20], with some modifications. Rat brain microsomes from either the cortex or cerebellum (330 μg in 8–15 μl, buffer A) were added to 0.95 ml of 8 mM Na-Mops (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 Mg-ATP, 40 μg/ml creatine kinase and 5 mM phosphocreatine (pH 7.0) were added. Ca^{2+} uptake and release were measured in a Cary spectrophotometer (Varian, Melbourne, Australia) at 37 °C by determining $A_{790}-A_{710}$ at 2 s intervals. The effect of lyso-GSLs was tested by their addition after Ca^{2+} loading. Lyso-GSLs were dissolved in ethanol and the final ethanol concentration did not exceed 1 % (v/v) in the cuvette.

The amount of Ca^{2+} released from microsomes was expressed as a percentage of the total Ca^{2+} in the microsomes, 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 the 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 the addition of Ca^{2+}, agonist or lipid.

Kinetic assay of SERCA (sarcoplasmic/endoplasmic reticulum ATPase)

Ca^{2+} uptake by SERCA was determined radiometrically using a rapid filtration technique [21]. Rat cortical microsomes (350 μg of protein) were incubated at 37 °C in 1.5 ml of buffer B [40 mM imidazole (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 5 mM NaN_3, 5 mM potassium oxalate, 0.5 mM EGTA, 1 μM Ruthenium red [21] (which blocks spontaneous Ca^{2+} release via the RyaR [22]), 10 μCi $^{45}Ca^{2+}$ and $CaCl_2$] to yield a final concentration of 1 μM free Ca^{2+} (determined using an algorithm [23] and software available at http://www.stanford.edu/~cpatton/maxc.html). Lyso-GSLs (100 μM) were added to buffer B for 2 min at 37 °C, with or without 50 μM thapsigargin. Ca^{2+} uptake was initiated by 5 mM ATP, and was terminated after 1, 3 and 5 s by adding 3 ml of ice-cold washing solution (20 mM Hepes, pH 7.4/150 mM KCl/1.4 mM $MgCl_2$/2 mM KH_2PO_4) [24], followed by filtration through a HAWP 0.45 μm Millipore filter. The initial rate (v_o) of Ca^{2+} uptake was calculated by linear regression analysis.

RESULTS AND DISCUSSION

In a recent study [14], we demonstrated that the maximal level of sensitization of the RyaR by GlcCer was obtained using a concentration of 10 μM, for both short- and long-acyl-chain GlcCer.

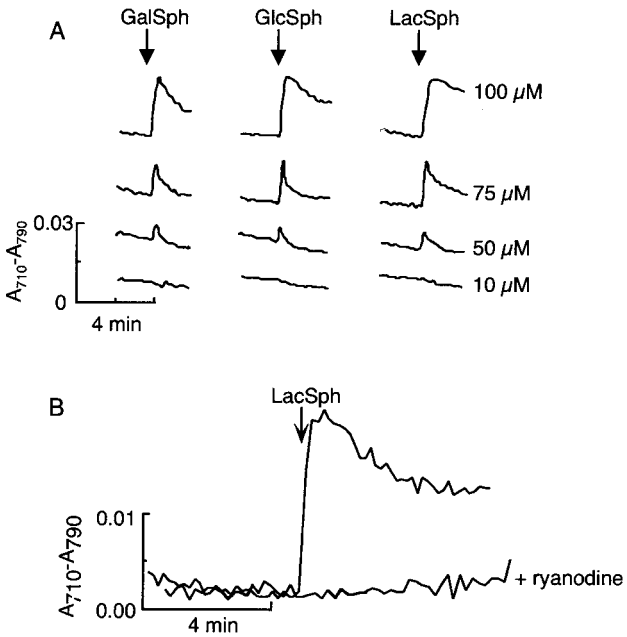

Figure 1 Effects of lyso-GSLs on Ca^{2+} release

Cortical microsomes were loaded by two sequential additions of 25 nmol of Ca^{2+} (not shown in the Figure), and then incubated with (**A**) increasing concentrations of lyso-GSLs or (**B**) with or without ryanodine (350 μM) before addition of 100 μM LacSph. Data are representative traces showing absorbance change ($A_{710}-A_{790}$) of antipyrylazo III versus time.

We now examine the effects of the lyso derivative of GlcCer, GlcSph and two other lyso-GSLs on Ca^{2+} mobilization. In contrast with GlcCer, 10 μM GlcSph, GalSph or LacSph had no effect on Ca^{2+} release from cortical microsomes (Figure 1A). A small amount of Ca^{2+} was released using 50 μM of lyso-GSLs, and approx. 20 % of microsomal Ca^{2+} was released at 100 μM (Figure 1A), an amount close to that released by agonists of the RyaR such as palmitoyl CoA [14]. LacSph-mediated Ca^{2+} release was completely blocked by preincubation with 350 μM ryanodine (Figure 1B), demonstrating that LacSph is an agonist of the RyaR, as is GalSph [14]. The ability of ryanodine to abolish completely LacSph- (Figure 1B) and GalSph-mediated Ca^{2+} release [14] indicates that these lyso-GSLs do not affect the integrity of the microsomal membranes. GlcSph-mediated Ca^{2+} release was not affected by ryanodine [14], demonstrating that GlcSph mobilizes Ca^{2+} by a mechanism independent of the RyaR.

To determine the mechanism by which GlcSph stimulates Ca^{2+} release, we next analysed the ability of heparin, an InsP_3R antagonist [25], to modulate GlcSph-induced Ca^{2+} release from cerebellar microsomes, which contain higher levels of the InsP_3R when compared with cortical microsomes [26]. Significantly more Ca^{2+} was released from cerebellar when compared with cortical microsomes by 100 μM GlcSph [approx. 35 % (Figure 2) compared with 20 % (see Figure 3) respectively]. GlcSph-induced Ca^{2+} release was blocked to a significant extent (approx. 60 %) by heparin (Figure 2), as was InsP_3-induced Ca^{2+} release, suggesting that at least part of the GlcSph-induced Ca^{2+} release is mediated via the InsP_3R. In contrast, heparin had no effect on either GalSph- or LacSph-induced Ca^{2+} release (Figure 2), consistent with the suggestion that GalSph and LacSph are specific RyaR agonists.

Since only part of the GlcSph-induced Ca^{2+} release could be blocked by heparin (Figure 2), and not at all by ryanodine, we sought an additional mechanism to explain the residual levels of GlcSph-mediated Ca^{2+} mobilization. Preincubation with either

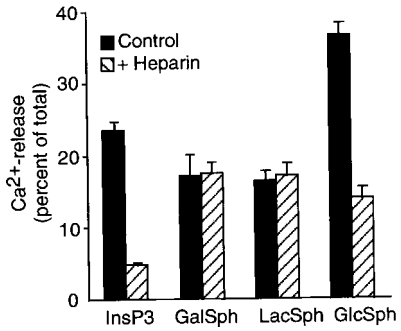

Figure 2 Lyso-GSL-induced Ca^{2+} release via the InsP_3R in cerebellar microsomes

Cerebellar microsomes were loaded by two sequential additions of 25 nmol Ca^{2+} and then incubated with or without heparin (150 μg/ml) before addition of 25 μM InsP_3 or 100 μM lyso-GSLs. Results are means $\pm$ S.D. from 3 to 4 independent experiments.

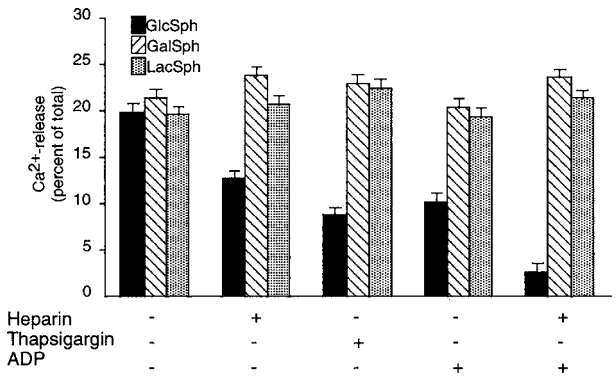

Figure 3 Lyso-GSL-induced Ca^{2+} mobilization via SERCA and the InsP_3R in cortical microsomes

Cortical microsomes were incubated with or without ADP (1 mM) for 10 min and loaded with Ca^{2+}. Note that in the experiment in which ADP was added, ATP, creatine kinase and phosphocreatine were not added to the cuvette. Microsomes were then incubated either with 150 μg/ml heparin or 150 μM thapsigargin for 10 min, and then incubated with 100 μM lyso-GSLs. In microsomes incubated with thapsigargin, an additional dose of 25 nmol of Ca^{2+} was added to confirm inhibition of SERCA-mediated Ca^{2+} uptake. Results are means $\pm$ S.D. from three independent experiments.

thapsigargin (a specific SERCA inhibitor [27]) or ADP (which inhibits the ATPase activity of SERCA [28]) blocked GlcSph-induced Ca^{2+} release by approx. 55% in cortical microsomes (Figure 3). Incubation with both ADP and heparin almost completely blocked Ca^{2+} release (Figure 3), suggesting that GlcSph induces Ca^{2+} release via both the InsP_3R and from thapsigargin-sensitive Ca^{2+} stores, and confirms that 100 μM GlcSph does not affect microsomal integrity. Surprisingly, not only did thapsigargin block GlcSph-induced Ca^{2+} release (Figure 3), but addition of GlcSph either before (results not shown) or after (Figure 4) the addition of thapsigargin restored Ca^{2+} uptake into microsomes. Moreover, GalSph and LacSph also restored Ca^{2+} uptake to levels comparable with those in the absence of thapsigargin (Table 1), whereas neither thapsigargin nor ADP had any effect on Ca^{2+} release by these two lyso-GSLs (Figure 3). The ability of the three lyso-GSLs to reverse thapsigargin inhibition of Ca^{2+} uptake via SERCA was supported by analysis of v_o of Ca^{2+} influx into cortical microsomes using a kinetic assay. In untreated microsomes, the v_o of Ca^{2+} uptake was 2.8 and 1.1 nmol of $Ca^{2+} \cdot s^{-1} \cdot (mg\ of\ protein)^{-1}$ in the presence of 50 μM thapsigargin (Table 2). The v_o of Ca^{2+} uptake was restored to

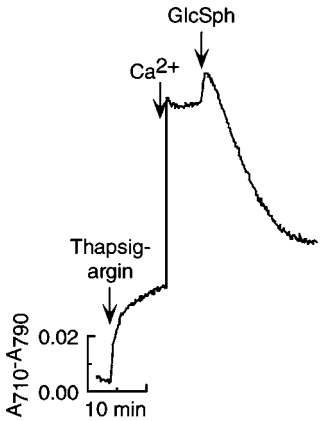

Figure 4 Reversal of thapsigargin inhibition of SERCA by GlcSph

Cortical microsomes were loaded with Ca^{2+} (not shown in the Figure), incubated with 150 μM thapsigargin, loaded with a further dose of 25 nmol Ca^{2+} to confirm inhibition of SERCA-mediated Ca^{2+} uptake, followed by addition of 100 μM GlcSph. A representative trace of absorbance change ($A_{710}-A_{790}$) versus time is shown.

Table 1 Reversal of thapsigargin inhibition of SERCA by lyso-GSLs

Cortical microsomes were loaded with Ca^{2+}, incubated with or without thapsigargin (150 μM), loaded with a further dose of 25 nmol of Ca^{2+} to confirm inhibition of SERCA-mediated Ca^{2+} uptake, followed by addition of 30 μM palmitoyl CoA or 100 μM lyso-GSL. Results are means $\pm$ S.D. from 3 to 4 independent experiments.

	Rate of Ca^{2+} uptake [nmol $\cdot$ s^{-1} $\cdot$ (mg of protein)$^{-1}$]		
Agonist or lyso-GSL	Before agonist or lyso-GSL	After agonist or lyso-GSL*	After thapsigargin followed by agonist or lyso-GSL
Palmitoyl CoA	0.41 $\pm$ 0.07	0.12 $\pm$ 0.03	0.0 $\pm$ 0.0
GlcSph	0.36 $\pm$ 0.05	0.08 $\pm$ 0.02	0.13 $\pm$ 0.03
GalSph	0.35 $\pm$ 0.01	0.10 $\pm$ 0.02	0.08 $\pm$ 0.01
LacSph	0.38 $\pm$ 0.07	0.11 $\pm$ 0.01	0.11 $\pm$ 0.00

* The rate of Ca^{2+} uptake after the addition of palmitoyl CoA or lyso-GSL is lower than that in their absence, since both induce Ca^{2+} release (via different mechanisms), and the values measured are therefore a combination of Ca^{2+} uptake and release.

Table 2 Effect of lyso-GSLs on reversing thapsigargin-mediated SERCA inhibition

Cortical microsomes were preincubated with or without thapsigargin (50 μM) followed by incubation with 100 μM lyso-GSLs, and the v_0 of Ca^{2+} uptake was measured using $^{45}Ca^{2+}$. Results are means $\pm$ S.D. from 3 to 4 independent experiments.

Lipid	Initial rate (v_0) of SERCA activity [nmol of Ca^{2+} uptake $\cdot$ s^{-1} $\cdot$ (mg of protein)$^{-1}$]
Control	2.8 $\pm$ 0.2
GlcSph	2.9 $\pm$ 0.3
GalSph	1.9 $\pm$ 0.1
LacSph	1.9 $\pm$ 0.3
Thapsigargin	1.1 $\pm$ 0.1*
GlcSph + thapsigargin	2.3 $\pm$ 0.3
GalSph + thapsigargin	2.3 $\pm$ 0.4
LacSph + thapsigargin	2.0 $\pm$ 0.3

* $P < 0.01$ (by ANOVA followed by Tukey–Kramer all pairs analysis).

2.0–2.3 nmol of $Ca^{2+} \cdot s^{-1} \cdot (mg\ of\ protein)^{-1}$ when thapsigargin-treated microsomes were incubated with the lyso-GSLs, suggesting that these lyso-lipids reverse the inhibition of SERCA

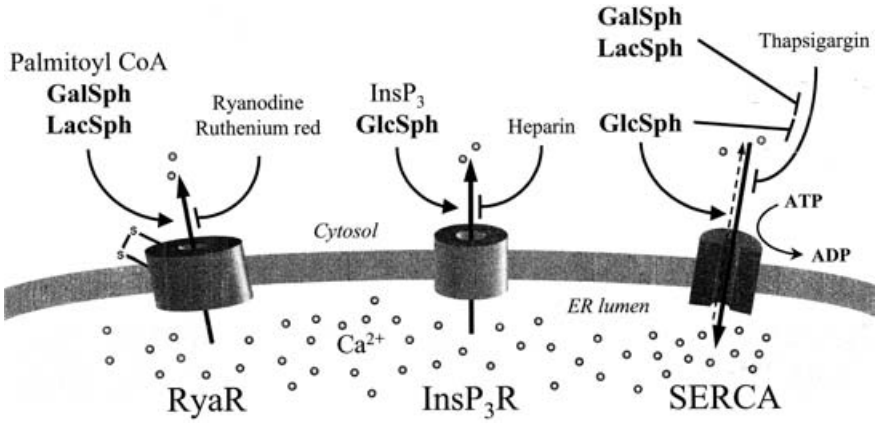

Figure 5 Summary of effects of lyso-GSLs on Ca^{2+} mobilization

GalSph and LacSph are agonists of the RyaR, as their action, similarly to that of the known RyaR agonist, palmitoyl CoA, can be blocked by high concentrations of ryanodine or by Ruthenium Red. GlcSph is an agonist of the InsP_3R, as its activity can be partially blocked by heparin. GlcSph as well as GalSph and LacSph reverse thapsigargin-mediated inhibition of SERCA, but GlcSph also mediates Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores.

by thapsigargin. This surprising result suggests that thapsigargin may not be an irreversible inhibitor of SERCA as assumed previously [27].

Together, these results show that lyso-GSLs mediate Ca^{2+} mobilization from microsomes via multiple mechanisms, with a high degree of specificity (Figure 5). However, none of the lyso-GSLs mediate their actions via G-protein-coupled receptors since their effects could not be blocked by GDPβS or pertussis toxin ([14]; C. Riebeling and A. H. Futerman, unpublished work). Thus GlcSph mobilizes Ca^{2+} as an InsP_3R agonist from thapsigargin-sensitive Ca^{2+} stores (Figure 5), but does not sensitize the RyaR like GlcCer [14], whereas both GalSph and LacSph are RyaR agonists (Figure 5). This might suggest that lyso-GSLs with terminal galactose moieties act in a manner differently from GSLs with terminal glucose moieties. Similarly, GlcCer, but not galactosylceramide, activates CTP:phosphocholine cytidylyltransferase [29]. Also, GlcCer, but not galactosylceramide or lactosylceramide, sensitizes the RyaR [14].

It is a matter of debate whether the effects of lyso-GSLs on Ca^{2+} mobilization are relevant for understanding the molecular mechanisms of neuronal dysfunction in the GSL storage diseases in which lyso-GSLs accumulate. The lyso-GSL concentrations required to elicit an effect are significantly higher than those of the GSLs, at least for GlcSph versus GlcCer, and are also significantly higher than the levels found in tissues from GSL patients and animal models. We have recently measured GlcSph levels in the cortex [30] of a glucocerebrosidase-deficient mouse [31] and in brain microsomes [14] of a patient suffering from a neuronopathic form of Gaucher's disease, in which GlcSph levels were 2 and 5 nmol/mg of protein respectively. Calculation of the molar concentration of GlcSph in human brain microsomes gives a value of approx. 1.5 μM [14], levels at which GlcSph has no effect on Ca^{2+} release (Figure 1), implying that GlcSph would need to accumulate at levels 10–50-fold higher than that found in tissues from patients with Gaucher's disease. We therefore conclude that, despite the specificity of lyso-GSL-induced Ca^{2+} release, these effects are unlikely to account for the pathophysiological mechanisms of neuronal dysfunction in GSL storage diseases, such as Gaucher's disease, in which GlcCer accumulates at higher levels than GlcSph [14,30,32], and affects Ca^{2+} mobilization at lower levels. Similarly, the effects of lyso-GSLs on other enzyme activities and on protein kinase C may be of pharmacological but not of physiological relevance.

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