Original Article

C16:0 Sulfatide Inhibits Insulin Secretion in Rat β-Cells by Reducing the Sensitivity of K_{ATP} Channels to ATP Inhibition

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Sulfatide (3′-sulfo-β-galactosyl ceramide) is a glycosphingolipid present in mammalians in various fatty acid isoforms of which the saturated 16 carbon-atom length (C16:0) is more abundant in pancreatic islets than in neural tissue, where long-chain sulfatide isoforms dominate. We previously reported that sulfatide isolated from pig brain inhibits glucose-induced insulin secretion by activation of ATP-sensitive K^+ channels (K_{ATP} channels). Here, we show that C16:0 sulfatide is the active isoform. It inhibits glucose-stimulated insulin secretion by reducing the sensitivity of the K_{ATP} channels to ATP. (The half-maximal inhibitory concentration is 10.3 and 36.7 μmol/l in the absence and presence of C16:0 sulfatide, respectively.) C16:0 sulfatide increased whole-cell K_{ATP} currents at intermediate glucose levels and reduced the ability of glucose to induce membrane depolarization, reduced electrical activity, and increased the cytoplasmic free Ca^{2+} concentration. Recordings of cell capacitance revealed that C16:0 sulfatide increased Ca^{2+}-induced exocytosis by 215%. This correlated with a stimulation of insulin secretion by C16:0 sulfatide in intact rat islets exposed to diazoxide and high K^+. C24:0 sulfatide or the sulfatide precursor, β-galactosyl ceramide, did not affect any of the measured parameters. C16:0 sulfatide did not modulate glucagon secretion from intact rat islets. In βTC3 cells, sulfatide was expressed (mean [±SD] 0.30 ± 0.04 pmol/μg protein), and C16:0 sulfatide was found to be the dominant isoform. No expression of sulfatide was detected in αTC1-9 cells. We conclude that a major mechanism by which the predominant sulfatide isoform in β-cells, C16:0 sulfatide, inhibits glucose-induced insulin secretion is by reducing the K_{ATP} channel sensitivity to the ATP block. *Diabetes* 55:2826–2834, 2006

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$[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; ELISA, enzyme-linked immunosorbent assay; GalCer, galactosyl ceramide; HPTLC, high-performance thin-layer chromatography; K_{ATP} channel, ATP-sensitive K^+ channel; KRBH, Krebs-Ringer bicarbonate HEPES buffer; PIP, phosphatidylinositol phosphate; SulfLacCer, sulfated lactosyl ceramide; TLC, thin-layer chromatography.

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Sulfatide is a glycosphingolipid present in mammalian organisms (1). The fatty acids of the ceramide moiety vary in chain length and saturation and may also be hydroxylated (2). In neural tissue, ~90% of the sulfatide molecules are composed of long-chain isoforms, often unsaturated and hydroxylated (3). In pancreas, the dominating forms are saturated with chain length of 16 (C16:0) and 24 (C24:0) carbon atoms with only low levels of unsaturated and hydroxylated forms (3). This means that in pancreas, the C16:0 isoform is relatively more common than in neural tissues (3). The fatty acid composition influences the physical and chemical properties of the sulfatide molecule and possibly also its biological functions between different organs and cells (1).

Several biological properties have been addressed to sulfatide during the last decade (4–7). In the pancreatic β-cell, sulfatide is involved in the folding of proinsulin in a chaperone-like fashion, preserves insulin crystals, and facilitates monomerization (8). The insulin crystal preservation has been assigned to the C16:0 sulfatide form (9). Patients with type 2 diabetes have low serum concentrations of sulfatide, and some animal models of type 2 diabetes have low pancreatic expression of C16:0 sulfatide (9,10). Administration of C16:0 sulfatide increases insulin secretion and improves first-phase insulin response in Zucker fatty rats (11).

In a previous study, using sulfatide purified from pig brain and without any further selection of fatty acid isoforms, we showed that sulfatide stimulated ATP-sensitive K^+ channel (K_{ATP} channel) activity leading to inhibition of glucose-induced insulin secretion (12). In the β-cell, K_{ATP} channels play a pivotal role in coupling cell metabolism to electrical activity and Ca^{2+} influx and stimulation of insulin secretion (13). Interestingly, despite the inhibitory activity of sulfatide on insulin secretion, the glycosphingolipid stimulated Ca^{2+}-dependent exocytosis of the insulin-containing secretory granules (12). This effect was, however, only observed under experimental conditions that bypassed the activity of the K_{ATP} channels (12). In the present study, we have explored the ability of specific isoforms (C16:0 and C24:0) of sulfatide to modulate both proximal and distal steps in β-cell stimulus-secretion coupling. We demonstrate that C16:0 sulfatide is the active isoform and inhibits insulin secretion by reducing K_{ATP}-channel sensitivity to ATP inhibition. This results in increased K_{ATP} channel activity at intermediate glucose

concentrations, reduced glucose-induced electrical activity, and elevation of the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Furthermore, we show that the ability of C16:0 sulfatide action is specific to the β-cell and that the glycosphingolipid does not modulate glucagon secretion from the pancreatic α-cell.

RESEARCH DESIGN AND METHODS

Preparation of islets and cell culture. Male Lewis rats (250–300 g; Møllegaard, Ll. Skensved, Denmark) were anesthetized with pentobarbital (100 mg/kg i.p.) and killed by cervical dislocation. The experimental procedures were approved by the local ethical committees in Copenhagen, Denmark. After removal of the pancreas, islets were isolated by collagenase digestion (Boehringer Mannheim, Mannheim, Germany) and dispersed into single cells using dispase. Single β-cells were identified by their size (only cells with a cell capacitance >6 pF were used [$n = 144$]; average 7.6 ± 0.2 pF), which is not different from the size of fluorescence-activated cell sorted rat β-cells ($n = 33$; 7.4 ± 0.4 pF). Single β-cells were also identified by stimulation of electrical activity or increase in $[Ca^{2+}]_i$ at high glucose concentration. The cells were plated on plastic dishes (Nunc, Roskilde, Denmark) and, for microfluorometry of the $[Ca^{2+}]_i$, on 22-mm glass coverslips. The cells were maintained for 2 days in RPMI-1640 medium (Invitrogen, Carlsberg, CA) supplemented with 10% (vol/vol) heat-inactivated FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere.

αTC1-9 glucagonoma cells were cultured in Dulbecco's modified Eagle's medium tissue culture medium (Invitrogen) in 5% CO_2 supplemented with 4,500 mg/l glucose, 10% (vol/vol) heat-inactivated fetal bovine serum, 1 mmol/l natriumpyruvate, nonessential amino acids (1:100), 1.5 mmol/l HEPES, L-glutamine (1:50), 0.5% BSA, 100 IU/ml penicillin, and 100 mg/ml streptomycin. The culture was passaged every 3rd day by gentle trypsinization, and cells were seeded at a density of 4×10^4 cells/cm^2 in 162-cm^2 Corning bottles with 27 ml complete medium. βTC3 insulinoma cells were cultured essentially as described above for αTC1-9 cells, except that the tissue culture medium was supplemented with 15% heat-inactivated horse serum and 3% (vol/vol) heat-inactivated fetal bovine serum. The cell culture was passaged once a week, and the cells were seeded at a density of 4×10^4 cells/cm^2 in 162-cm^2 Corning bottles with 15 ml complete medium.

Insulin and glucagon secretion. Insulin and glucagon release from intact islets cultured for 24 h was performed as described previously (12,14) using 10 size-matched islets per well in 300 μl Krebs-Ringer bicarbonate HEPES buffer (KRBH) consisting of (in mmol/l) 115 NaCl, 4.7 KCl, 2.6 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 20 $NaHCO_3$, 0.5% BSA (fraction V), and 10 HEPES (pH 7.4 with NaOH) and supplemented with glucose and test substances as indicated in the text. For experiments in depolarized islets, a modified KRBH was used containing (in mmol/l) 89.7 NaCl, 30 KCl, 2.6 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 20 $NaHCO_3$, 0.5% BSA (fraction V), 10 HEPES (pH 7.4 with NaOH), and 0.25 diazoxide and supplemented with glucose as indicated in the test. The glycosphingolipids were either present during the entire preincubation period (24 h) or were added to the culture medium for the last hour, as indicated in the text. Insulin and glucagon were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (Linco Research, St. Charles, MO). For determination of insulin content, islets were subjected to a lysis buffer containing 75% ethanol, 23.5% water, and 1.5% concentrated HCl. Insulin concentration in the islet lysates were measured with ELISA.

βTC3 cells were seeded on polyornithine-coated 24-well plates (25,000 cells per well) and cultured overnight in RPMI-1640 medium supplemented with 15% heat-inactivated horse serum and 3% (vol/vol) heat-inactivated fetal bovine serum. Cells were washed twice with 0.5 ml RPMI-1640 medium and then preincubated for 30 min at 37°C. After a second wash, cells were incubated at 37°C for 30 min with RPMI-1640 medium supplemented with 16.8 mmol/l glucose and different glycosphingolipids as indicated in the text.

Electrophysiology. Patch pipettes were pulled from borosilicate glass capillaries and, before use, were coated with Sylgard near their tips and fire polished thereafter. The resistance of the pipettes was 2.5–4 MΩ when filled with the pipette filling solutions. Before establishment of the seal with the pipette in bath, the zero-current potential was adjusted. All patch-clamp experiments were performed using an endothelial progenitor cell-9 amplifier and Pulse software (version 8.31; HEKA Elektronik, Lamprecht, Pfalz, Germany). Whole-cell K^+ current was estimated by applying 10-mV hyper- and depolarizing voltage pulses (duration 200 ms; pulse interval 2 s) from a holding potential of −70 mV using the perforated whole-cell configuration of the patch-clamp technique. The effect of C16:0 and C24:0 sulfatide on β-cell membrane potential was measured using the perforated-patch whole-cell configuration. Single-channel activity was recorded from inside-out patches. Exocytosis was measured in single β-cells as increases in cell capacitance. The interval between two successive points was 0.4 s, and the measurements of cell capacitance were initiated ~5 s after establishment of the standard whole-cell configuration of the patch-clamp technique. Exocytosis was elicited by infusion of Ca^{2+}-EGTA buffer through the recording electrode. All effects of C16:0 sulfatide, C24:0 sulfatide, and galactosyl ceramide (GalCer) on whole-cell K_{ATP} currents, membrane potential, and exocytosis were determined following a 30-min preincubation. For inside-out measurements, the test substances were added directly to the bath solution facing the inside of the membrane.

Recordings of exocytosis, whole-cell K_{ATP} currents, and the membrane potential were performed at 33°C with the cells immersed in an extracellular solution consisting of (in mmol/l) 138 NaCl, 4.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES (pH 7.4 with NaOH), and D-glucose as indicated in the text. In inside-out patch-clamp experiments, the extracellular solution contained (in mmol/l) 125 KCl, 30 KOH, 10 EGTA, 1 $MgCl_2$, and 5 HEPES (pH 7.15 with KOH) and 0–1 mmol/l Mg-ATP. The volume of the recorded chamber was 0.4 ml with a flow rate of 1.5–2.0 ml/min.

For measurements of membrane potential and K_{ATP} channel activity, the pipette solution was composed of (in mmol/l) 76 K_2SO_4, 10 NaCl, 10 KCl, 1 $MgCl_2$, and 5 HEPES (pH 7.35 with KOH). Electrical contact was established by adding 0.24 mg/ml amphotericin B to the pipette solution. Exocytosis was determined by infusion of the following Ca^{2+}-EGTA buffer through the recording pipette (in mmol/l): 125 κ-glutamate, 10 KCl, 10 NaCl, 1 $MgCl_2$, 5 HEPES, 3 Mg-ATP, 10 EGTA, and 5 $CaCl_2$. The resulting Ca^{2+} concentration was 220 nmol/l according to the binding constants previously described (15).

$[Ca^{2+}]_i$ measurements. Single rat β-cells were loaded with 0.4 μmol/l fura-2/acetoxymethyl ester (Moleculer Probes, Eugene, OR) for 20 min at 37°C in extracellular solution with 5 mmol/l glucose. The coverslips were then transferred to a custom-made perifusion chamber and mounted on the stage of an Axiovert 100 inverted microscope equipped with a Plan-Neofluar 100×/1.30 objective (Carl Zeiss, Oberkochen, Germany) and an Ionoptix (Milton, MA) fluorescence imaging system. The volume of the recorded chamber was 0.4 ml with a flow rate of 1.5–2.0 ml/min of extracellular solution with 2.8–16.8 mmol/l glucose. The temperature in the recording chamber was 33°C.

Preparation of glycolipids. The glycosphingolipid preparations used in this study were sulfatide (3′-sulfo-galactocyl ceramide) and GalCer purified from pig brain (16,17) and without further selection due to fatty acid composition. Structural identification, using fast-atom bombardment mass spectrometry showed that this sulfatide preparation contained sulfatide with different fatty acid length and different degrees of saturation, dominated by C:24 species but C16:0 and C18:0 were also present (3). The sulfatide isoforms C16:0 and C24:0 sulfatide were synthesized from the pig brain sulfatide mixture by using alkaline hydrolysis to produce lysosulfatide followed by reacylation with the specific fatty acid (9).

Stock solutions of each glycosphingolipid were made in chloroform/methanol/water (C/M/W; 60:30:4.5 by volume) in a concentration of 1 μmol/ml. After 1 h at room temperature, the tubes were ceiled and stored at 4°C. Before use, the glycosphingolipids were transferred to glass tubes and evaporated at room temperature overnight. Dried glycolipids were redissolved in PBS (pH 7.1) and sonicated for 20 s using a Branson sonicator (Danbury, CT). The final concentrations of the various sulfatide molecules were verified by scintillation counting of the ^{3}H-labeled molecule added to the stock solutions. Even though C24:0 sulfatide is more hydrophobic than C16:0 sulfatide, they were dissolved in PBS to similar degree (>90%).

Standards of glycolipids. Sulfatide used as standard was purified from pig brain and sulfated lactosyl ceramide (SulfLacCer) from post mortem kidney tissue from a patient with metachromatic leukodystrophy, as previously reported (18). Seminolipid (1-alkyl-2-acyl-glycerol form) was a gift from Dr. Ineo Ishizuka, Department of Biochemistry, Teikyo University School of Medicine, Tokyo, Japan.

Isolation and quantification of sulfatide and related glycolipids from αTC1-9 and βTC3 cells. Lipids were extracted from cell homogenates after protein determination (BCA protein assay reagent method; Pierce, Rockford, IL) by adding methanol and chloroform to give a final C/M/W ratio of 4:8:3 (by volume) (19). Silica gel-60 chromatography (Merck, Darmstadt, Germany) was used for further purification as detailed elsewhere (3). Sulfatide, SulfLacCer, seminolipid, and GalCer were eluted with C/M/W (65:25:4 by volume). This "sulfatide fraction" was saponified in methanol, 1 mol/l KOH (1:1 vol/vol) (9), before quantification of sulfatide and SulfLacCer by thin-layer chromatography (TLC)-ELISA and orcinol staining of GalCer. Quantification of seminolipid was performed by TLC-ELISA on the same fraction before saponification.

Sulfatide, SulfLacCer, and seminolipid were identified and quantified by a previously described TLC-ELISA method (20) using the Sulf I antibody (21) and purified standards of these antigens. Briefly, purified glycolipid standard and aliquots of the "sulfatide fraction" were applied as 5-mm lanes to TLC plates (10 × 20 plastic backed, Merck) and chromatographed in C/M/W 65:25:4

(by volume for sulfatide/SulfLacCer) or twice in 75:25:3 (by volume for seminolipid, using disodium-tetraborate-decahydrate–treated plates) (22). Plates were sequentially incubated with the monoclonal Sulf I antibody, alkaline-phosphatase–conjugated anti-mouse antibody (The Jackson Laboratories, West Grove, PA) and 5′-bromo-4′chloro-3′indolylphosphate. All steps were performed at room temperature. The intensity of the developed color was determined by densitometric scanning at 620 nm. GalCer was quantified by application of sample and reference GalCer on high-performance TLC (HPTLC) plates (Merck), chromatographed twice in 75:25:3 (using disodium-tetraborate-decahydrate–treated plates), and detected by orcinol staining.

Ceramide characterization of sulfatide isolated from βTC3 cells by electrospray ionization mass spectrometry. The saponified sulfatide fraction cell homogenates was further purified by preparative HPTLC (Merck) (11). Samples were dissolved in C/M/W (30:60:20 by volume) at a concentration of 10 μmol/l before ceramide characterization by electrospray ionization mass spectrometry (quadrupol time of flight, equipped with a z-spray nanospray ion source; Micromass, Manchester, U.K.). A capillary voltage of 900 V and a source block temperature of 80°C were used. Samples were manually loaded into nanoflow probe tips (type F thin wall; Micromass). For mass spectrometry–mass spectrometry, the gas cell was pressurized with argon gas and fragmentation of the precursor ion was affected at a collision energy of 60 eV.

Sulfatide synthesis in βTC3 cells. Cells were cultured as described above. When confluent, the culture media was changed, and ^{14}C-serine (3 μCi/ml, n = 3) and ^{35}S-sulfate (20 μCi/ml, n = 3) were added to fresh culture medium containing heat-inactivated FCS. Lipid extraction, separation, and saponification of the "sulfatide fraction" were performed as described above. Aliquots of the saponified "sulfatide fractions," corresponding to 50–130 μg protein, were applied as 8-mm bands to alumina-backed HPTLC plates (Merck) and chromatographed in C/M/W (65:25:4 by volume). The plate was air-dried, sprayed with Enhance spray (NEN Life Science Products, Zaventem, Belgium), and exposed to X-ray film for 7 days. The individual bands after chromatography were identified by similar migration as structurally identified glycosphingolipid standard(s). Liquid scintillation counting (Tri-Carb 1500; Packard) was performed on scrape-out fractions of the individual lipid bands after chromatography.

Sulfotransferase assay. The enzyme preparation was performed using a modification of the procedure described by Lingwood et al. (23). The procedure was performed at 4°C. Cells (~25×10^6 βTC3 cells or 10×10^6 αTC1-9 cells) were homogenized in 300 μl 0.32 mol/l sucrose to give a final concentration of 10–20 mg protein/ml. The homogenates were centrifuged at 35,000g for 30 min. The supernatant was removed and pellet resuspended by homogenization in 300 μl 10 mmol/l Tris HCl-buffer (pH 7.4)/25% glycerol/0.5% triton X-100 (wt/vol). Thereafter, the homogenate was left at 4°C for 1 h with occasional shaking and then centrifuged at 35,000g for 60 min. The supernatant, constituting the enzyme source, was transferred to a new tube, and an aliquot was withdrawn for protein determination (BCA protein assay reagent method; Pierce). The enzyme preparation was immediately used in the enzyme assay but is stable at −80°C up to 2 months.

The glycosphingolipid acceptor substrate *N*-palmitoyl-galactosyl sphingosine (C16:0-galactosyl ceramide) was synthesized as described (9). The conditions of the sulfotransferase assay were described elsewhere (24). GalCer (20 nmol) and Triton X-100 (1 mg) dissolved in chloroform methanol 2:1 (vol/vol), were evaporated to dryness under N_2, and finally under vacuum for 1 h to remove the last traces of organic solvent. Triton X-100 (1 mg) alone was used as blank. The substrate/blank was dissolved in 200 μl reaction buffer (0.1 mol/l imidazole buffer, pH 7.2, containing 1% Triton X-100, 20 mmol/l $MgCl_2$, 4 mmol/l dithiothreitol, 10 mmol/l ATP, 2 μmol/l PAPS, and ~5×10^6 cpm [^{35}S]-PAPS), sonicated for 5 min, and preincubated at 37°C for 5–30 min. To this mixture, 50 μl of the enzyme preparation (0.5–1 mg protein/ml) was added and incubated for 4 h at 37°C. The reaction was stopped by adding 500 μl ice-cold 0.05 mol/l NaAc, pH 4.4. Formed sulfated lipid products were isolated by chromatography on Licrosorb RP-18 (Merck) microcolumns (100 mg) as described previously (25).

To verify that sulfatide had been formed, the sulfatide-containing fraction was subjected to chromatography on a silica gel-60 column (0.1 mg), equilibrated in $CHCl_3$. The column was washed by adding $CHCl_3$ and C:M 9:1, after which the sulfated product was eluted by C/M/W (65:25:4 by volume). An aliquot of this fraction was applied to alumina-backed HPTLC plates (Merck), chromatographed in 75:25:2:1 C/M/W/HAc and developed by autoradiography (3). The activity of sulfotransferase is expressed as picomoles per milligram of protein per hour.

Data analysis. The rate of exocytosis is presented as an increase in cell capacitance occurring during the first 60 s after establishment of the whole-cell configuration, not including any rapid changes occurring during the initial 10 s corresponding to the time required for equilibration of the pipette solution with cytosol. All voltage signals were filtered at 500 Hz and sampled at a rate of 1 kHz. The results are given as means ± SE for the indicated number of experiments. Statistical significances were evaluated using Student's t test for pairs of data, Dunnett's test for multiple comparisons with a control, and Tukey's test when multiple comparisons between groups were required.

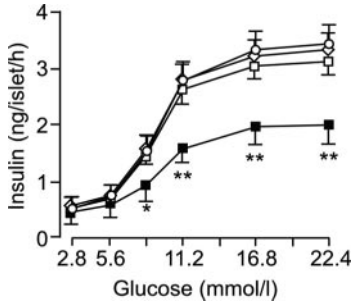

FIG. 1. Effects of C16:0 sulfatide, C24:0 sulfatide, and GalCer on insulin secretion in isolated rat islets. Insulin release was measured for 1 h from groups of 10 sized-matched islets at increasing glucose concentrations (2.8 to 22.4 mmol/l) alone (○) or following incubation for 24 h with 20 nmol/ml of C16:0 sulfatide (■), C24:0 sulfatide (□), or GalCer (◇). Data are means ± SE of three individual experiments, each performed in quadruplicate. *$P < 0.05$; **$P < 0.01$.

RESULTS

C16:0 sulfatide inhibits glucose-induced insulin secretion. Figure 1 shows stimulation of insulin secretion from intact rat islets in response to increasing glucose concentrations in the extracellular medium. Preincubation of islets with C16:0 sulfatide (20 nmol/ml for 24 h) did not affect insulin release at ≤5.6 mmol/l glucose but significantly reduced the insulin response to higher glucose concentrations (≥8.4 mmol/l) compared with untreated islets ($P < 0.05$). Neither C24:0 sulfatide nor GalCer did affect insulin release at any of the tested glucose concentrations (Fig. 1). Table 1 shows that preincubation of rat islets for 24 h with C16:0 sulfatide, C24:0 sulfatide, or GalCer (all at 20 nmol/ml) did not change insulin content.

Short-term incubation (1 h) of rat islets with C16:0 sulfatide (20 nmol/ml) inhibited insulin secretion at 16.8 mmol/l glucose from 3.67 ± 0.12 ng insulin · islet^{-1} · h^{-1} under control conditions to 2.76 ± 0.09 ng insulin · islet^{-1} · h^{-1} in C16:0 sulfatide–treated islets ($P < 0.05$; data not shown). As expected, under these experimental conditions, C24:0 sulfatide and GalCer were ineffective (data not shown). Furthermore, sulfatide isolated from pig brain inhibited insulin secretion following both short- (1 h) and long-term (24 h) incubation to a similar extent as that observed with C16:0 sulfatide (data not shown; see Fig. 1*A* in ref. 12).

Effects of C16:0 sulfatide on membrane potential. Figure 2*A* shows a membrane potential recording from a single rat β-cell in the presence of 2.8 mmol/l glucose. The

TABLE 1
Insulin content in rat islets cultured for 24 h in RPMI-1640 medium alone (control) or supplemented with 20 nmol/ml of sulfatide, C16:0 sulfatide, C24:0 sulfatide, or GalCer

Treatment	Insulin content (ng/islet)
Control	56.7 ± 5.4
Sulfatide	52.1 ± 4.3
C16:0 sulfatide	54.4 ± 6.1
C24:0 sulfatide	58.9 ± 6.1
GalCer	57.5 ± 4.4

Data are means ± SE of four different experiments.

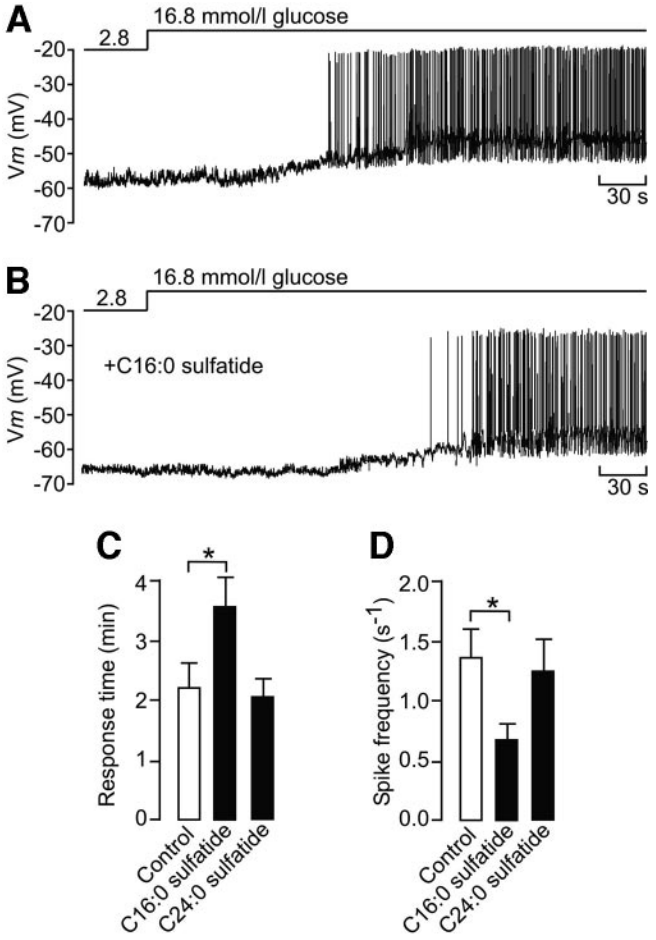

FIG. 2. Effect of C16:0 sulfatide on membrane potential and electrical activity. The recordings of membrane potential (V*m*) were obtained from individual rat β-cells using the perforated patch whole-cell configuration. The cells were exposed to 2.8 mmol/l glucose before stimulation with 16.8 mmol/l of the sugar as indicated by the horizontal lines. Membrane potential and electrical activity was recorded from untreated cells (*A*) or from cells incubated for 30 min with 20 nmol/ml C16:0 sulfatide (*B*). The recordings are representative of five different cells under both experimental conditions. Histograms summarizing response time from glucose stimulation to initiation of electrical activity (*C*) and frequency of the action potentials in untreated cells (Control) and in cells incubated for 30 min with 20 nmol/ml of either C16:0 sulfatide or C24:0 sulfatide (*D*). The analysis of the action-potential frequency was performed for the first 3 min after initiation of electrical activity in response to glucose stimulation (16.8 mmol/l). *$P < 0.05$.

β-cell was electrically silent, and the membrane potential was close to −60 mV. Increasing the glucose concentration in the extracellular medium to 16.8 mmol/l depolarized the β-cell, and electrical activity was observed after 2 min. Figure 2*B* shows the membrane potential in a single rat β-cell preincubated for 30 min with 20 nmol/ml C16:0 sulfatide before application of 16.8 mmol/l glucose. On average, the resting membrane potential at 2.8 mmol/l glucose was not different between the two experimental conditions (control, −56 ± 7 mV and C16:0 sulfatide treated, −64 ± 6 mV; $n = 5$). However, the response time from glucose stimulation to initiation of electrical activity was 62% longer in the C16:0 sulfatide–treated cells ($P < 0.05$; $n = 5$) (Fig. 2*C*). Furthermore, the spike frequency was reduced in cells exposed to C16:0 sulfatide ($P < 0.05$; $n = 5$) (Fig. 2*D*). No effect of C24:0 sulfatide was observed on any of the above parameters (Fig. 2*C* and *D*).

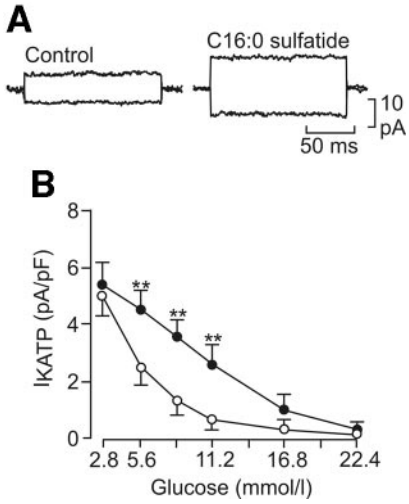

FIG. 3. Effect of C16:0 sulfatide on K_{ATP} channel activity. *A*: Whole-cell K_{ATP} currents were recorded in response to 10 mV of de- and repolarizing voltage pulses from a holding potential of −70 mV using the perforated-patch whole-cell configuration. The current traces were obtained from either untreated individual rat β-cells (Control) or cells preincubated for 30 min with C16:0 sulfatide (20 nmol/ml). *B*: Whole-cell K_{ATP} currents recorded at different glucose concentrations (2.8 to 22.4 mmol/l) from either untreated β-cells (○) or from cells incubated for 30 min with 20 nmol/ml C16:0 sulfatide (●). **$P < 0.01$.

C16:0 sulfatide increases whole-cell K_{ATP} channel current. Figure 3*A* shows that incubation of single rat β-cells in 5.6 mmol/l glucose with C16:0 sulfatide (20 nmol/ml for 30 min) increased the activity of the K_{ATP} channels (estimated by 200 ms of 10 mV hyper- and depolarizing pulses) by 92% using the perforated patch whole-cell configuration. The stimulatory effect of C16:0 sulfatide amounted, on average, to 98 ± 8% ($P < 0.05$; $n = 5$). No effect on K_{ATP} channel activity was observed with either C24:0 sulfatide or GalCer (20 nmol/ml for 30 min; data not shown). The currents were inhibited when adding 0.1 mmol/l tolbutamide to the bath, indicating that these are indeed K_{ATP} channel currents (data not shown).

Figure 3*B* shows the glucose dependence of the stimulatory action of C16:0 sulfatide on K_{ATP} channel activity. As expected, glucose caused a concentration-dependent decrease in K_{ATP} channel activity with nearly complete inhibition of channel activity at ≥11.2 mmol/l glucose (Fig. 3*B*). C16:0 sulfatide (20 nmol/ml for 30 min) stimulated K_{ATP} channel activity at intermediate glucose concentrations (5.6–11.2 mmol/l), whereas the glycoshingolipid did not significantly affect K_{ATP} channel activity at low (2.8 mmol/l) or high (16.8 and 22.4 mmol/l) glucose concentrations (Fig. 3*B*). These results suggest that C16:0 sulfatide did not cause an acute recruitment or translocation of K_{ATP} channels to the plasma membrane. C16:0 sulfatide also did not enhance K_{ATP} currents when the K_{ATP} channels were completely inhibited by high glucose concentrations, suggesting that C16:0 sulfatide did not activate any K^+ conductance other than K_{ATP} channels.

C16:0 sulfatide decreases ATP sensitivity of rat β-cell K_{ATP} channels. Figure 4*A* shows K_{ATP} channel activity recorded from an inside-out patch containing at least six channels. In nucleotide-free solution, intense channel activity was observed. Channel activity was partially inhibited by 10 μmol/l ATP. Interestingly, inclusion of 20 nmol/ml C16:0 sulfatide in the perfusion medium in the continuous presence of 10 μmol/l ATP gradually reversed the inhibitory action of ATP on channel activity.

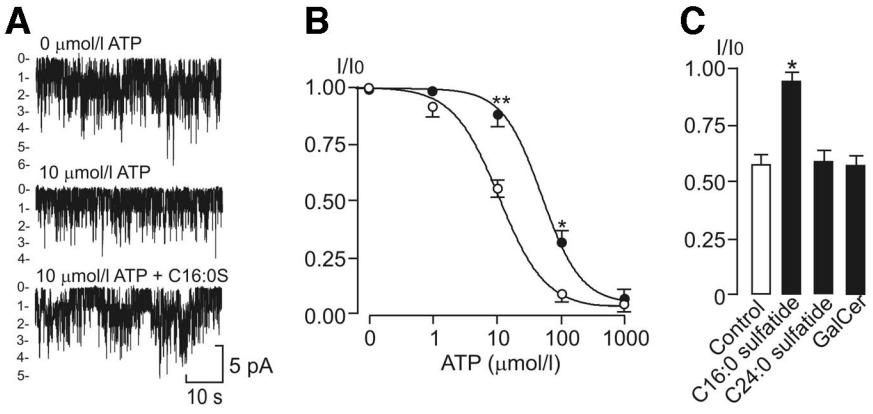

FIG. 4. C16:0 sulfatide reverses the inhibitory action of ATP on K_{ATP} channel activity. Channel activity recorded from an inside-out patch isolated from a rat β-cell at −70 mV. Recordings were started 17 ± 5 s after patch excision and terminated with 7.8 ± 0.7 min. *A*: Representative channel activity recorded in the absence (*top panel*) or presence (*middle panel*) of 10 μmol/l ATP (1 mmol/l free Mg^{2+} was present throughout the experiments) or 3 min after application of 20 nmol/ml C16:0 sulfatide (C16:0S) in the perfusion solution in the continuous presence of 10 μmol/l ATP (*bottom panel*). *B*: ATP dose-response relationship for K_{ATP} channel activity in the absence (○) or following application of 20 nmol/ml C16:0 sulfatide (●) for 3 min. Channel activity is expressed relative to control solution before and after exposure to ATP (I/Io). Channel activity was calculated from steady state to end of nucleotide application. The lines are the best fit of the data to the Hill equation. *C*: Normalized K_{ATP} channel activity relative to control solution before and after exposure to 10 μmol/l ATP (I/Io) alone (Control) or following application of 20 nmol/ml C16:0 sulfatide, C24:0 sulfatide, or GalCer for 3 min. Data are means ± SE of 4–6 experiments. *$P < 0.05$; **$P < 0.01$.

The stimulatory action of C16:0 sulfatide on K_{ATP} channel activity commenced 87 ± 15 s ($n = 5$) after the addition of the glycosphingolipid. Figure 4*B* shows the normalized relationship between different ATP concentrations and channel activity. The relationship could be fitted by the Hill equation with a K_i of 10.3 μmol/l and a cooperativity factor of 0.9 ± 0.2. Figure 4*B* also shows that C16:0 sulfatide treatment caused the ATP sensitivity curve to shift to the right (K_i of 36.7 μmol/l and a cooperativity factor of 1.0 ± 0.2) by 3.6-fold, indicating that C16:0 sulfatide can effectively decrease the ATP sensitivity of rat β-cell K_{ATP} channels. Figure 4*C* shows that the reversibility of the inhibitory action of ATP (10 μmol/l) on K_{ATP}-channel activity was specific for C16:0 sulfatide and was not observed by perfusion of β-cells with 20 nmol/ml C24:0 sulfatide or GalCer.

C16:0 sulfatide inhibits glucose-induced increase in $[Ca^{2+}]_i$. Stimulation of rat β-cells with 16.8 mmol/l glucose elicited a pronounced increase in $[Ca^{2+}]_i$ after an initial delay of 2.4 ± 0.6 min ($n = 11$; Fig. 5*A*). On average, glucose (16.8 mmol/l) increased $[Ca^{2+}]_i$ by 446 ± 62 nmol/l from a resting level of 134 ± 19 nmol/l ($n = 11$). Incubation of β-cells with C16:0 sulfatide (20 nmol/ml for 30 min) did not affect the basal $[Ca^{2+}]_i$ (121 ± 18 nmol/l; $n = 13$) but reduced the ability of glucose to elicit an increase in $[Ca^{2+}]_i$ by 52% (232 ± 42 nmol/l; $P < 0.05$; $n = 13$). Furthermore, the initial delay in the increase in $[Ca^{2+}]_i$ was accentuated by 44 ± 12% ($P < 0.05$; $n = 13$).

Figure 5*B* shows that the percentage of rat β-cells responding with an elevation of $[Ca^{2+}]_i$ following a glucose challenge increased from 34% (at 8.4 mmol/l) to 88% (at 16.8 mmol/l) glucose. Preincubation with C16:0 sulfatide (20 nmol/ml) for 30 min before glucose stimulation reduced the number of cells responding with an increase in $[Ca^{2+}]_i$ by 45–79% (Fig. 5*B*). Only cells responding with a pronounced increase in $[Ca^{2+}]_i$ to subsequent tolbutamide application were included in the analysis.

C16:0 sulfatide increases Ca^{2+}- and glucose-dependent exocytosis in rat β-cells. Increases in cell capacitance, which is indicative for exocytosis, are shown in Fig. 6*A*. Increases in cell capacitance were elucidated by infusion of single rat β-cells with a Ca^{2+}-EGTA buffer with free Ca^{2+} concentration of 220 nmol/l. Under control conditions, a small capacitance increase was observed, which reached a steady-state level within 2–4 min. In contrast, cells incubation with C16:0 sulfatide (20 nmol/ml for 30 min) displayed a strong stimulation of exocytosis. On average (Fig. 6*B*), C16:0 sulfatide stimulated exocytosis by 215 ± 19% ($P < 0.05$; $n = 6$). No acceleration of the exocytotic response was observed in the presence of C24:0 sulfatide or GalCer (Fig. 6*B*).

In Fig. 6*C*, we investigated the effects of C16:0 sulfatide on insulin release from intact islets depolarized with 30 mmol/l K^+ and in the presence of diazoxide (250 μmol/l).

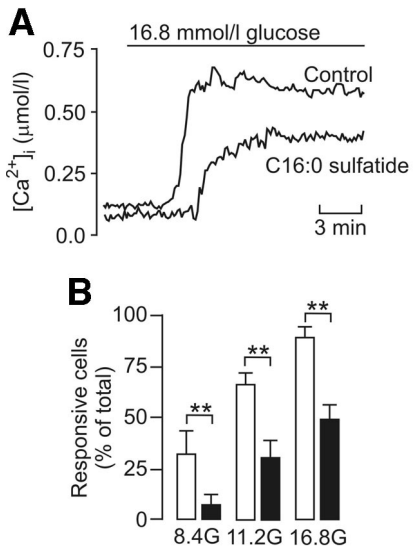

FIG. 5. Effect of C16:0 sulfatide on glucose-stimulated increase on $[Ca^{2+}]_i$. *A*: Cytoplasmic Ca^{2+} concentration measured in KRBH with 2.8 mmol/l glucose and following stimulation with 16.8 mmol/l glucose (indicated by the horizontal line) in an individual rat β-cell under control conditions or in another cell incubated for 30 min with 20 nmol/ml C16:0 sulfatide. The recordings are representative of 11 (Control) and 13 different cells (C16:0 sulfatide treated). *B*: Histogram depicting percentage cells responding to indicated glucose stimulation with an increase in $[Ca^{2+}]_i$ under control conditions (□) and following a 30-min preincubation with 20 nmol/ml C16:0 sulfatide (■). Data are means ± SE of 9–17 experiments. **$P < 0.01$.

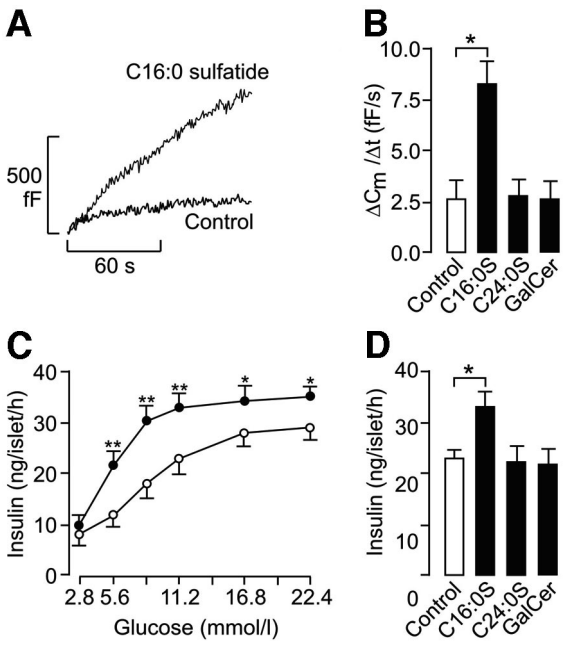

FIG. 6. Effect of C16:0 sulfatide on Ca^{2+}- and glucose-dependent exocytosis. *A*: Increases in cell capacitance elicited by intracellular infusion of a pipette solution with free Ca^{2+} concentration of 220 nmol/l under control conditions and in a rat β-cell preincubated for 30 min with 20 nmol/ml C16:0 sulfatide. The traces depict changes in cell capacitance measured over the first 2 min after establishment of the whole-cell configuration. Throughout the recordings, the cells were clamped at −70 mV to avoid activation of the voltage-dependent Ca^{2+} channels that would otherwise interfere with the measurements. *B*: Rates of exocytosis detected 10–70 s after break in into individual β-cells under control conditions (Control) or in cells treated for 30 min with either C16:0 sulfatide (C16:0S), C24:0 sulfatide (C24:0S), or GalCer at a concentration of 20 nmol/ml. *C*: Effects of C16:0 sulfatide on insulin secretion in clamped intact rat islets. Batches of 10 islets were incubated for 1 h in KRBH containing 30 mmol/l K^+, 250 μmol/l diazoxide, and increasing glucose concentrations (2.8 to 22.4 mmol/l). Insulin release was determined from control islets (○) and islets incubated for 24 h with 20 nmol/ml C16:0 sulfatide (●). *D*: Insulin secretion from clamped rat islets in the presence of 11.2 mmol/l glucose under control conditions or following incubation for 24 h with 20 nmol/ml of either C16:0 sulfatide (C16:0S), C24:0 sulfatide (C24:0S), or GalCer. Insulin release was measured as described in *C*. Data are means ± SE for 10 batches of islets. *$P < 0.05$; **$P < 0.01$.

Under these experimental conditions, the membrane potential is clamped and action-potential firing is suppressed. In accordance with previous data (26,27), glucose enhanced insulin secretion in a dose-dependent manner (half-maximal effective concentration of 8.6 mmol/l). This effect must have occurred independent of the K_{ATP}-channel closure because this pathway is bypassed under these conditions. Figure 6*C* also shows the corresponding insulin release data from islets incubated with C16:0 sulfatide (20 nmol/ml for 24 h). Under these conditions, insulin secretion was not affected at 2.8 mmol/l glucose but enhanced by 22–69% at the higher glucose concentrations tested (Fig. 6*C*). Figure 6*D* shows that the stimulatory action was specific for C16:0 sulfatide and was not observed in islets incubated with C24:0 sulfatide or GalCer (20 nmol/ml for 24 h).

C16:0 sulfatide does not affect glucagon secretion. Figure 7*A* shows that incubation of intact rat islets with C16:0 sulfatide (20 nmol/l for 24 h) did not affect glucagon secretion at 2.8 or 16.8 mmol/l glucose, whereas glucose stimulation itself inhibited glucagon release by 46% ($P < 0.05$; $n = 10$). In these experiments, C16:0 sulfatide inhibited insulin secretion by 42% at 16.8 mmol/l glucose (data not shown).

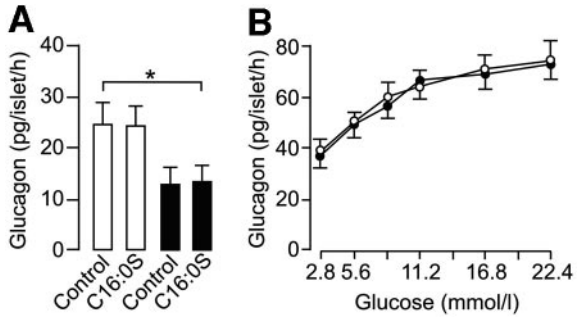

FIG. 7. Effect of C16:0 sulfatide on glucagon release from rat islets. *A*: Histogram summarizing glucagon release from batches of 10 intact rat islets in the presence of 2.8 (□) or 16.8 (■) mmol/l glucose under control conditions or in islets incubated for 24 h with 20 nmol/ml C16:0 sulfatide (C16:0S). *B*: Effect of C16:0 sulfatide on glucagon secretion in clamped intact rat islets. Batches of 10 islets were incubated for 1 h in KRBH containing 30 mmol/l K^+, 250 μmol/l diazoxide, and increasing glucose concentrations (2.8 to 22.4 mmol/l). Glucagon release was determined from control islets (○) and islets incubated for 24 h with 20 nmol/ml C16:0 sulfatide (C16:0S; ●). Data are means ± SE for 10 batches of islets. *$P < 0.05$.

In accordance with our previous data (28), the results in Fig. 7*B* show that glucose produced a concentration-dependent stimulation of glucagon release from rat islets clamped with 30 mmol/l K^+ and 250 mmol/l diazoxide. Under these experimental conditions, C16:0 sulfatide incubation (20 nmol/ml for 24 h) did not affect the secretory response at any of the tested glucose concentrations (Fig. 7*B*). No effect of C16:0 sulfatide on glucagon secretion (2.8 mmol/l glucose) or Ca^{2+}-stimulated exocytosis was observed in αTC1–9 glucagonoma cells (data not shown).

Analysis of sulfatide expression in βTC3 cells. For the study of sulfatide expression, we used βTC3 insulinoma cells. Incubation of βTC3 cells with C16:0 sulfatide (20 nmol/ml for 1 h) reduced glucose (11.2 mmol/l)-induced insulin secretion by 46 ± 9% ($P < 0.05$; $n = 5$), whereas C24:0 sulfatide or GalCer were without effects. These data suggest that βTC3 cells represent a good cell model for studying sulfatide expression.

After extraction of glycolipids from cultures of βTC3 cells and analysis using TLC and the specific monoclonal antibody Sulf I, the content of sulfatide was determined to 0.30 ± 0.04 pmol/μg protein ($n = 7$), approximately one-tenth of the sulfatide amount found in isolated rat islets (3). The upper sulfatide band on the TLC-ELISA plate (Fig. 8) represents sulfatide containing long-chain fatty acids and the lower band(s), short-chain and hydroxylated fatty acids. However, since the TLC-ELISA only represents a guide as to the sulfatide isoforms present in the samples, mass spectrometry was performed on pooled βTC3 cell samples for ceramide characterization. The βTC3 cells showed a dominance of the C16:0 fatty acid isoform (>50%), but also the C22:0, C24:1, and C24:0 isoforms could be detected. SulfLacCer and seminolipid were present only in trace amounts (<0.01 pmol/μg protein) in βTC3 cells. GalCer was not detected. Figure 8 also shows that only trace amounts of sulfatide could be detected in αTC1-9 cells (<0.01 pmol/μg protein; $n = 6$). None of the sulfatide-related glycolipids, SulfLacCer, seminolipid, or GalCer, could be detected in this cell line.

Sulfatide was shown to be synthesized by βTC3 cells, as demonstrated by the incorporation of ^{35}S-sulfate into the sulfatide molecule (Fig. 9). Other sphingolipids, measured by ^{14}C-serine incorporation, found to be produced by these cells had a similar migration as standard ceramide, GalCer,

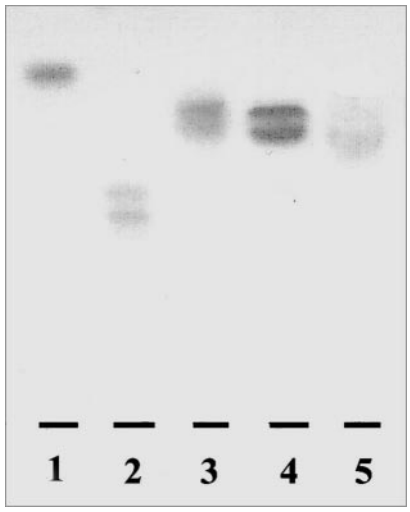

FIG. 8. TLC of the Sulf I antigens seminolipid, SulfLacCer, and sulfatide together with the "sulfatide fraction" isolated from cultured βTC3 and αTC1-9 cells. Seminolipid (25 pmol; *lane 1*), SulfLacCer (20 pmol; *lane 2*), pig brain sulfatide (40 pmol; *lane 3*), the "sulfatide fraction" isolated from βTC3 cells (100 μg cell protein applied; *lane 4*), and the "sulfatide fraction" isolated from αTC1-9 cells (500 μg cell protein applied; *lane 5*).

and sphingomyelin (Fig. 9). The βTC3 cells were analyzed for cerebroside sulfotransferase activity, an enzyme that adds the sulfate group to the galactose moiety–producing sulfatide from GalCer, and the specific activity was 26 ± 2 pmol · mg protein^{-1} · h^{-1} ($n = 5$). The cerebroside sulfotransferase activity in the αTC1-9 cells was below detection limit ($n = 2$), using 10×10^6 cells, which corresponds to ~150 μg protein.

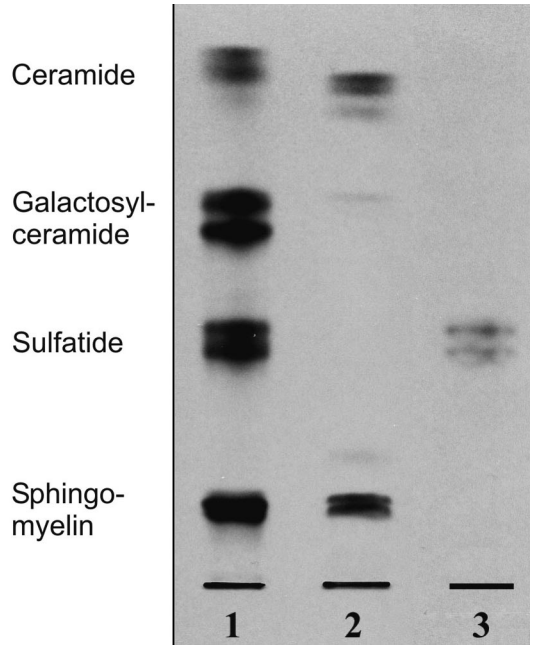

FIG. 9. Autoradiogram of ^{35}S-sulfate– or ^{14}C-serine–labeled lipids in the "sulfatide fraction" from cultured βTC3 cells. One experiment is shown. Lipids corresponding to 50 μg (^{14}C-serine–labeled cells) or 130 μg (^{35}S-sulfate–labeled cells) cell protein were applied. The double spots reflect variation in fatty acids; the higher hydrophobicity, the faster migration on the plate. Reference lipids (^{3}H-sphingomyelin, ^{3}H-sulfatide, ^{3}H-galactosyl ceramide, and ^{3}H-ceramide; *lane 1*), ^{14}C-serine–labeled lipids in the "sulfatide fraction" from cultured βTC3 cells (*lane 2*), ^{35}S-sulfate–labeled sulfatide from cultured βTC3 cells (*lane 3*).

DISCUSSION

Our results demonstrate that C16:0 sulfatide inhibits glucose-induced insulin secretion. This effect of C16:0 sulfatide is specific, as C24:0 sulfatide or GalCer had no effect on insulin release. We have previously shown that sulfatide isolated from pig brain augments β-cell K_{ATP} channel activity in the presence of a low concentration of intracellular ATP (0.3 mmol/l) (12). In that report, it was not investigated which isoform of sulfatide is responsible for the increase in the K_{ATP} current and if it involves K_{ATP} channel surface translocation or changes in its sensitivity to ATP inhibition. In the present study, we demonstrate that C16:0 sulfatide is responsible for the stimulation of K_{ATP} channel activity. C16:0 sulfatide only enhanced K_{ATP} currents in the presence of intermediate glucose concentrations. However, C16:0 sulfatide did not enhance currents at low glucose concentration, suggesting that C16:0 sulfatide did not cause the enhancement in K_{ATP} current by acutely recruiting or translocating K_{ATP} channels to the plasma membrane or simply increasing the open probability. C16:0 sulfatide treatment also did not enhance currents when the K_{ATP} channels were completely inhibited by high glucose, suggesting that C16:0 sulfatide did not activate conductances other than the K_{ATP} conductance. We therefore concluded that C16:0 sulfatide enhancement of K_{ATP} channels are due to a reduction in ATP sensitivity of the K_{ATP} channels. This is supported by our findings that C16:0 sulfatide reduced the sensitivity of the K_{ATP} channels to ATP more than threefold in inside-out patches.

Recent work has uncovered two distinct classes of lipids as potent activators of K_{ATP} channels: long-chain CoAs (29–31) and phosphatidylinositol phosphates (PIPs) (32–34). Both types of lipids possess three negatively charged phosphate groups and one or two long hydrophobic acyl chains. Both long-chain CoAs and PIPs have been shown to increase the open probability and to reduce the ATP sensitivity of K_{ATP} channels. The molecular mechanisms by which the two types of lipid stimulate K_{ATP} channels involve interactions with multiple common residues on Kir6.2, the pore-forming subunit of the K_{ATP} channel (35–37). Given the apparent similarity of sulfatide with long-chain-CoAs and PIPs, it is tempting to speculate that sulfatide may activate the K_{ATP} channel by interacting with the same residues on Kir6.2. Additional studies are required to address this question.

The increase in K_{ATP} conductance by C16:0 sulfatide may serve to set the membrane potential below the threshold of L-type Ca^{2+}-channel activation and generation of electrical activity, hence dampening β-cell excitability. This is consistent with the observation that C16:0 sulfatide reduced the number of cells responding with an increase in $[Ca^{2+}]_i$ at different glucose concentrations. C16:0 sulfatide treatment also decreased the frequency of action-potential firing and the associated increase in $[Ca^{2+}]_I$, as well as increased the time from glucose stimulation of β-cells to initiation of electrical activity and increase in $[Ca^{2+}]_i$.

The lack of response of the C24:0 isoform of sulfatide on insulin secretion might be due to the fact that the C16:0 isoform is more hydrophilic and dissolves more easily in aqueous solutions than the C24:0 isoform. However, in this study, similar concentrations of C16:0 and C24:0 sulfatide, as measured by scintillation counting of [^{3}H]-labeled isoforms, were added to the cells. Another factor that could influence the effect of sulfatide in our experiments is the

size of the micelles that are produced in the incubation solution. The micelle size differs with the fatty acid length of the glycosphingolipid and might affect the exogenous supply of these molecules. However, the sulfatide mixture from pig brain, with mainly long-chain fatty acids but also short-chain isoforms, showed similar results as the C16:0 sulfatide, and supports that the C16:0 sulfatide isoform mediates the effect on β-cells.

The present study demonstrates that C16:0 sulfatide does not only modulate the proximal steps in the β-cell stimulus-secretion coupling but also stimulates Ca^{2+}-dependent exocytosis, as well as insulin secretion from clamped islets. It has previously been demonstrated that long-chain-CoAs acutely stimulate exocytosis in β-cells by a mechanism involving protein acylation (38). As discussed above, given the overall similarity between sulfatide and long-chain-CoAs, it is tempting to speculate that C16:0 sulfatide might stimulate exocytosis by a similar mechanism. Indeed, preliminary data suggest that cerulenin, an inhibitor of protein acylation, blocked the stimulatory action of C16:0 sulfatide on Ca^{2+}-induced exocytosis. This suggests that the stimulatory effect of C16:0 sulfatide is mediated through an association with a protein, most likely a component of the exocytotic machinery.

In previous studies, we have demonstrated that β-cells isolated from islets (mouse, rat, and human) contain sulfatide (39) and also that sulfatide was produced by these cells (3). The βTC3 cells used in this study were found to contain and produce sulfatide in agreement with the isolated β-cells. In contrast to freshly isolated islet non–β-cells (mainly consisting of α-cells), the αTC1-9 cells contained only trace amounts of sulfatide. This discrepancy might be due to hampered production of sulfatide caused by the immortalization of the cells. Another plausible explanation is that sulfatide in non–β-cells of intact islets reflect an uptake from an exogenous source, e.g., β-cell secretion. Interestingly, we were not able to demonstrate any effects of C16:0 sulfatide on glucagon secretion in intact islets or Ca^{2+}-dependent exocytosis from αTC1-9 cells. The reason for the selective action of C16:0 sulfatide on β-cell function is unknown, especially since rat α-cells express K_{ATP} channels (40) and that their stimulus-secretion coupling resembles that of the β-cell (28).

In conclusion, our study demonstrates that C16:0 sulfatide plays an important role in determining the activity of K_{ATP} channels in β-cells by reducing their sensitivity to ATP inhibition. This leads to decreased responsiveness to glucose and reduced insulin secretion. Our findings raise the possibility that variations in membrane C16:0 sulfatide under normal and pathological conditions (41) may affect insulin secretion by altering K_{ATP} channel activity. The ability of C16:0 sulfatide to activate K_{ATP} channels might have important implications for inducing β-cell rest. A beneficial effect of K_{ATP} channel activators, like diazoxide, on insulin secretion has been shown in type 2 diabetes (42,43), as well as in pre–type 1 diabetes (44,45). Induction of β-cell rest may also underlie the improvement of first-phase and overall insulin secretion during a glucose tolerance test in Zucker rats, an animal model of type 2 diabetes treated with C16:0 sulfatide (11).

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