Glycosphingolipid-facilitated Membrane Insertion and Internalization of Cobra Cardiotoxin

THE SULFATIDE-CARDIOTOXIN COMPLEX STRUCTURE IN A MEMBRANE-LIKE ENVIRONMENT SUGGESTS A LIPID-DEPENDENT CELL-PENETRATING MECHANISM FOR MEMBRANE BINDING POLYPEPTIDES

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Cobra cardiotoxins, a family of basic polypeptides having lipid- and heparin-binding capacities similar to the cell-penetrating peptides, induce severe tissue necrosis and systolic heart arrest in snakebite victims. Whereas cardiotoxins are specifically retained on the cell surface via heparan sulfate-mediated processes, their lipid binding ability appears to be responsible, at least in part, for cardiotoxin-induced membrane leakage and cell death. Although the exact role of lipids involved in toxin-mediated cytotoxicity remains largely unknown, monoclonal anti-sulfatide antibody O4 has recently been shown to inhibit the action of CTX A3, the major cardiotoxic from Taiwan cobra venom, on cardiomyocytes by preventing cardiotoxin-induced membrane leakage and CTX A3 internalization into mitochondria. Here, we show that anti-sulfatide acts by blocking the binding of CTX A3 to the sulfatides in the plasma membrane to prevent sulfatide-dependent CTX A3 membrane pore formation and internalization. We also describe the crystal structure of a CTX A3-sulfatide complex in a membrane-like environment at 2.3 Å resolution. The unexpected orientation of the sulfatide fatty chains in the structure allows prediction of the mode of toxin insertion into the plasma membrane. CTX A3 recognizes both the headgroup and the ceramide interfacial region of sulfatide to induce a lipid conformational change that may play a key role in CTX A3 oligomerization and cellular internalization. This proposed lipid-mediated toxin translocation mechanism may also shed light on the cellular uptake mechanism of the amphiphilic cell-penetrating peptides known to involve multiple internalization pathways.

Glycosphingolipids in eukaryotic cells have important biological functions in membrane trafficking and cell signaling (1, 2). The number of disease-related proteins found to interact specifically with glycosphingolipids is also increasing (3–5). For instance, recent studies have shown that natural killer cells can be activated by glycosylceramides from the cell surface of Gram-negative bacteria that do not contain lipopolysaccharide (6, 7). There is also evidence indicating that, in response to tumor necrosis factor-α, trafficking of ganglioside GD3 from the plasma membrane to mitochondria can trigger several cell death signaling responses (3, 8, 9). Although the molecular mechanisms accounting for the observed glycosphingolipid transport and glycolipid-triggered cell responses remain elusive, recent progress in determining the three-dimensional structure of protein-glycosphingolipid complexes has shed some light on the process (10–13). These glycosphingolipid-binding proteins function by either extracting lipids from the membrane and forming a water-soluble protein/lipid complex, as in the cases of glycosphingolipid transfer protein, saposin B, and lipid-presenting CD1a (10–12), or by localizing to a glycosphingolipid domain of the plasma membrane and eventually being internalized via the endocytic pathway, as seen for cholera and Shiga toxins (13, 14).

Cobra produces small, three-fingered β-sheet cardiotoxins (CTXs) with heparin- and lipid-binding capability (18–21) that cause systolic heart arrest by forming cytolytic pores in the cell membrane (22). Extracellular heparan sulfate may be involved in the observed specificity of CTX for cardiomyocytes by mediating a cell surface retention mechanism (22–24). CTXs are also recognized as cytotoxins, with the ability to induce a general lytic effect on human erythrocytes (21), necrotic cell death in fetal rat cardiomyocytes (25), and apoptosis in cortical neurons and human leukemia cells (25, 26). The major CTX from Taiwan cobra, CTX A3, targets the mitochondrial membrane after internalization and acts synergistically with a CTX-induced cytosolic calcium increase to disrupt the mitochondrial network (27). Interestingly, by applying the monoclonal antibody O4, which is specific for the 3′-sulfated galactose moiety of sulfatides (28), CTX internalization and CTX-induced cytotoxicity of cardiomyocytes can be inhibited efficiently (25). These results suggest that sulfatides in the plasma membrane may be a specific target of CTX action.

§ The abbreviations used are: CTX, cardiotoxin; SGC, sulfatide or 3′-sulfated β1,4-galactosylsphingosine; C12E6, hexadecylamine glycol monodecylether; HBSS, Hanks’ balanced salt solution; DRM, detergent-resistant membrane; FITC, fluorescein isothiocyanate; BODIPY, dipyrromethene boron difluoride; LDL, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SpM, sphingomyelin; START, sterol-degenerate acyl regulatory protein-related lipid transfer; GM1, Neu5Acα2-3Galβ1-3Galβ14GlcCer; GM3, Neu5Acα2-3Galβ14GlcCer; GD3, Neu5Acα2-8Neu5Acα2-3Galβ14GlcCer.

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The atomic coordinates and structure factors (code 2BH4) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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Although the trivial name "sulfatides" is used as a generic term for a class of amphiphiles containing sulfate esters and carbohydrates (28), the sulfatide used in this study is 3′-sulfated β1-α-galactosylceramide (SGC) and, as sphingolipids, is composed of an 18-carbon sphingosine base that is N-amine-linked to a fatty acid ranging in length from C14 to C24. In light of current interest in the role of glycosphingolipids in toxin internalization and lipid domain formation, it is important to examine whether direct interaction between CTX and SGC indeed triggers CTX-related toxicity. In addition, if antibody application can be useful for cobra snakebite victims, it is desirable to learn how an SGC-specific antibody exerts its inhibitory effect on CTX action. Here, we used a combined biochemical and confocal microscopic approach to show that anti-SGC blocks the binding of CTX A3 to SGC in the plasma membrane of cardiomyocytes. Electrophysiological and confocal microscopic investigations on H9C2 cardiomyoblast cells showed that CTX A3 molecules interact specifically with SGC at both the headgroup and the ceramide interfacial region to cause CTX A3-mediated membrane pore formation and internalization. This was further confirmed by the structure of a CTX A3-SCG complex determined in the presence of C10E8 (hexethylene glycol monodetergent) detergent, which mimics the membrane environment. In sharp contrast to the effect of other glycosphingolipid binding proteins in forming water-soluble protein-lipid complexes, the CTX A3-SCG structure provides a prototype for understanding how membrane-soluble protein-lipid complexes can be formed. The results also illustrate an important role of glycosphingolipid conformation at the ceramide interfacial region to cause CTX A3-mediated membrane pore formation and internalization.

**EXPERIMENTAL PROCEDURES**

**CTX A3 and Antibody Preparation**—CTX A3 was purified from crude venom of *Naja atra* (purchased from Snake Education Farms, Taiwan) by a chromatographic method previously described (29). Polyclonal antibodies against CTX were purified from the antivenin of *Bungarus multicinctus* and *N. atra* (Center for Disease Control, Taiwan) by immunoprecipitation (27). The specificity of isolated CTX antibodies was verified by immunoblotting. Monoclonal anti-SGC IgM O4 (Chemicon) and anti-SGC IgG Sulf1 (provided by Dr. Pam Fredman, Sahlgrenska Academy at Göteborg University, Göteborg, Sweden) were used throughout the experiments.

**Cell Cultures**—Cardiomyocytes were isolated from day 18 fetal Wistar rats. Hearts were immersed in ice-cold Hanks’ balanced salt solution (HBSS) immediately after their removal. After thorough washing, the minced ventricles were placed in sterile HBSS containing 0.25% trypsin at 37 °C for 30 min. To obtain purified cardiomyocytes, the dissociated cells were plated for 1 h, and the unattached cardiomyocytes were collected, counted, and seeded in culture medium (72% Dulbecco’s modified Eagle’s medium, 18% Medium 199, 10% fetal bovine serum, 100 units ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin) on poly-l-lysine-coated plates or glass coverslips. 

H9C2 cardiomyoblast cells (ATCC number CRL-1446) were maintained in Dulbecco’s modified Eagle’s medium with 10% (v/v) fetal calf serum (Hyclone), 100 units ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin. For image recording, H9C2 cells were cultured on coverslips at 10⁴ cells cm⁻². For internal CTX quantification, H9C2 cells were cultured in 24-well plates at 10⁶ cells/plate.

**TLC Overlay**—Lipid standards (Avanti Polar Lipids) or lipid extracts of plasma membrane fraction were loaded on silica 60 HPTLC plates (Merck). For separating phospholipids, SGC, and cholesterol, the solvent system was chloroform/methanol/acetic acid (26:5:3, v/v/v). For ganglioside mixtures and GM1 separation, the solvent system was chloroform, methanol, 0.02% CaCl₂, and NaCl (60:35:8, v/v/v). To visualize lipids, the plates were first developed by iodine vapor. Then they were discolored in vacuo, dipped in 0.4% polysobutylmethacrylate, and dried for further overlaying with 5 μm CTX A3. CTX A3 bound to TLC plates was detected by immunostaining with anti-CTX (31).

**Plasma Membrane Isolation**—Cardiomyocytes or H9C2 Cells were washed three times with ice-cold phosphate-buffered saline and then scraped off of the plate and collected by centrifugation at 4 °C. The pellet was homogenized in 0.1 ml of STM buffer (0.25 M sucrose, 5 mM Tris-HCl, 1 mM MgCl₂, pH 7.4) with 1 mM phenylmethylsulfonyl fluoride on ice. The homogenate was centrifuged at 220 × g for 5 min using an Eppendorf 5804R (Hamburg, Germany) centrifuge. The supernatant fraction was saved, and the pellet was rehomogenized in 0.1 ml of STM buffer with 1 mM phenylmethylsulfonyl fluoride. The suspension was again centrifuged at 220 × g at 4 °C for 5 min. The first and second supernatant fractions were combined and centrifuged at 100,000 × g at 4 °C for 2 h using a 70P-72 ultracentrifuge with an RPS 56T rotor (Hitachi, Japan). The resulting pellet was collected for use.

**Isolation of Detergent-resistant Membrane (DRM) Fraction**—Cardiomyocyte plasma membrane was treated with 1% (v/v) ice-cold Triton X-100 and shaken gently at 4 °C for 30 min. DRM fraction of plasma membrane was floated to the top of a sucrose density step gradient of 0.1 ml of 40% (w/v, 1.37 M) sucrose, 0.6 ml of 30% (w/v, 1.1 M) sucrose, and 0.4 ml of 5% (w/v, 0.15 M) sucrose in MBS buffer (25 mM MES, 150 mM NaCl, pH 6.5, with 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride) with centrifugation at 240,000 × g at 4 °C for 18 h (32). Amounts of individual lipids and proteins in each sucrose gradient fraction were assayed by TLC (33) and immunoblotting, respectively, and then quantitated by densitometry.

**Extraction of Plasma Membrane Lipids**—The plasma membrane preparation was incubated and shaken with methanol/chloroform/phosphate-buffered saline (1:1:2, v/v/v) for 1 h at room temperature and then centrifuged at 3,000 × g at 4 °C for 15 min using an Eppendorf 5804R centrifuge. The resultant organic layer was collected and dried for use.

**Confocal Microscopy**—Unless otherwise specified, fluorescent dyes were purchased from Molecular Probes, Inc. (Eugene, OR). Fluorescein isothiocyanate (FITC)-CTX A3 was prepared as described previously (25, 27), and dipyromethene boron difluoride (BODIPY)-SGC was provided by Dr. Richard E. Pagano (Mayo Clinic College of Medicine, Rochester, MN). To monitor the internalization of CTX A3, 5 μM FITC-CTX A3 was applied to cardiomyocytes or H9C2 cells in the HBSS. Images were recorded using a Zeiss LSM 510 confocal microscope using a full-width half-maximum of 0.7 μm to set pinhole size. The amount of FITC-CTX A3 internalized was quantified by integrating the cytosolic FITC-CTX A3 intensity signal. CTX-induced intracellular calcium increase in the presence or absence of anti-SGC was monitored using a full-width half-maximum of 5 μm to set the pinhole size at the desired wavelengths of 515 and 590 for calcium indicator dyes Fluo-3AM and FuraRed-AM, respectively (25). For BODIPY-SCG incorporation, H9C2 cells were incubated with 0.5 μg μl⁻¹ BODIPY-SCG with 1% bovine serum albumin at 10 °C for 15 min and then washed twice with HBSS plus 1% bovine serum albumin and once with HBSS. After CTX A3 was applied, cells were kept at 37 °C for 5 min and then washed six times with 5% bovine serum albumin in HBSS for back-exchange (34). MitoTracker Red was used as a marker for mitochondria in SGC internalization experiments.
Membrane Insertion and Internalization of Cobra Cardiotoxin

Mass Spectrometry—Mass analysis was performed with a triple quadrupole instrument (VG micromass model Quattro II). Samples were infused at a flow rate of 20 nl min \(^{-1}\) and detected in negative ion mode for characterization of the SGC standard or the SGC lipid extract from preparative TLC (35, 36).

Cell Surface Modification and Specific Lipid Incorporation—To mask surface SGC, cardiomyocytes or H9C2 cells were preincubated with the desired concentrations of monoclonal anti-SGC IgM (O4), anti-SGC IgG (Sulf1), or anti-GM3 IgM (GM6, Seikagaku, Japan) at 37 °C for 30 min. To degrade sulfated glycoconjugates, H9C2 cells were preincubated with the desired concentrations of sulfatase (EC 3.1.6.1; Sigma) at 37 °C for 30 min. Antibody staining with anti-SGC Sulf1 and Alexa Fluor 546-conjugated secondary antibody was used to check the effectiveness of desulfation. To reduce heparan sulfate, H9C2 cells were preincubated with the desired concentrations of heparinase I (EC 4.2.2.7; Sigma) or III (EC 4.2.2.8; Sigma) at 37 °C for 60 h. The efficiency of heparinase I and III treatment was evaluated by immunostaining with monoclonal anti-heparan sulfate (3G10 and 10E4; Seikagaku) (37, 38) and rhodamine-conjugated secondary antibodies. For specific lipid incorporation, H9C2 cells were incubated with 200 μM SGC (Avanti Polar Lipids) or SGE at 37 °C for 30 min and then washed twice with HBBS plus 1% bovine serum albumin and once with HBSS. SGE, the synthetic SGC analog without the ceramide structure, was synthesized and provided by Dr. Dennis Whitfield (Institute of Biological Science, National Research Council Canada). Efficiency of lipid incorporation was assessed by TLC of the plasma membrane fraction, which was isolated by a modified method (39).

Cell Viability Assay—Cardiomyocytes or H9C2 cells were cultured in 96-well plates at 10\(^6\) cells/plate. Cell survival after treatment with 5 μM CTX A3 for 1 h was assessed by lactate dehydrogenase (LDH) leakage (Sigma) or mitochondrial activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (25). The LDH leakage from cells was defined as the percentage of LDH activity in the culture medium to total LDH activity in the culture medium and the cell lysates. The cell viability was expressed as the percentage of MTT reduction, assuming the absorbance of control cells as 100%. Electrophysiological Studies—H9C2 cells were cultured on coverslips at 10\(^6\) cells/cm\(^2\). The pipette used for patch membranes contained a solution of 140 mM NaCl, 5 mM KCl, 1.3 mM CaCl\(_2\), 10 mM glucose, 10 mM HEPES, pH 7.4. The external solution for host cell membranes contained 140 mM NaCl, 5 mM KCl, 1.3 mM CaCl\(_2\), 3 mM MgCl\(_2\), 10 mM glucose, 10 mM HEPES, 5 mM tetraethylammonium chloride, pH 7.4, and 5 μM ouabain. CTX A3-induced extra conductance of H9C2 cells was recorded at room temperature by an Axopatch 200B amplifier under voltage clamp conditions and digitized by an analog-to-digital interface (Digidata 1322A; Molecular Devices). The patch electrodes had a resistance of 2–4 MΩs for whole-cell recording and 1.5–2.5 MΩs for outside-out patch. K\(^+\), Na\(^+\), or Ca\(^{2+}\) in the external and internal solutions was replaced with Li\(^+\) or NH\(_4\)\(^+\) in an ion selectivity assay (data not shown). We did not test anion selectivity in either whole-cell recording or outside-out patch, because both sulfate and phosphate bind effectively to the anionic binding pocket of CTX A3 (40).

Crystallization and Data Collection—The CTX A3-SGC-C\(_{10}\)E\(_6\) complex was crystallized by the hanging drop vapor diffusion technique at room temperature. The hanging drops were formed by mixing 1 μl of the complex solution (10 mg ml\(^{-1}\) CTX A3, 13 mg ml\(^{-1}\) SGC (Sigma) in 0.5% (w/v) C\(_{10}\)E\(_6\), and 10 mM Tris-HCl, pH 8.5) with 5 μl of reservoir solution containing 1.8 M sodium maitanone, 7.5% (v/v) tert-butanol, 7.5% (v/v) pentaerythritol ethoxylate (15:4 EO/OH), 0.08% (w/v) C\(_{10}\)E\(_6\), 50 mM imidazole, and 50 mM Tris-HCl, pH 7.0. Crystals were flash-frozen in liquid nitrogen followed by cryodata collection on the ADSC Quantum 210 detector at Beamline 17B2 of National Synchrotron Radiation Research Center in Taiwan. Diffraction data from a single crystal was processed to 2.3 Å using the HKL2000 package (41) in space group P6\(_{3}\)22 (unit cell dimensions: a = b = 63.32 Å; c = 120.87 Å) (Table 1).

Structure Determination and Refinement—The structure was solved by the molecular replacement method using the crystal structure of the CTX A3-SDS complex (Protein Data Bank code 1H0J) as the search model. Using AMoRe (42), the initial solution containing two CTX A3 molecules in the asymmetric unit had an R-factor of 40.1%. Model building was performed using the program XtalView (43), and the structure was further refined with a simulated annealing procedure using CNS (44). At a later stage of refinement, one SGC and 11 complete or partial C\(_{10}\)E\(_6\) detergent molecules were assigned in the model. Starting coordinates and CNS parameter and topology files for SGC and C\(_{10}\)E\(_6\) were obtained from the Dundee PRODRG2 Server (45) and verified manually. The final model had an R-factor/Rmerge of 21.9%/22.2% for all reflections above 2σ between 26.5 and 2.3 Å resolution. The quality of the model was assessed with the program PROCHECK (46). Refinement statistics are summarized in Table 1. The program PyMOL (available on the World Wide Web at pymol.sourceforge.net) was used to prepare Fig. 10, and DINO (available on the World Wide Web at www.dino3d.org) was used to prepare Fig. 11.

RESULTS

Anti-SGC Antibody O4 Blocks CTX A3 Binding to SGC in the Plasma Membrane of Cardiomyocytes—We have previously reported that CTX A3 can bind negatively charged phosphatidylserine and phosphatidylglycerol to form pores in large unilamellar vesicles (22, 47). In Fig. 1, we used TLC overlay assays to examine the binding between various lipids and CTX A3. CTX A3 bound effectively to negatively charged phosphatidylserine (PS), cardiolipin (CL), and SGC and bound weakly to sphingomyelin (SpM) (Fig. 1, left). It also interacted with phosphatidylinositol (Pl), phosphatidylinositol 3-phosphate (PIP), and phosphatidylinositol 4,5-diphosphate (PIP2) (Fig. 1, middle). CTX A3 did not bind phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cholesterol (C) (Fig. 1, left). It also failed to interact with gangliosides (GG), the sialic acid-containing glycosphingolipids, and purified GM1 (Fig. 1, right). These results suggest that most negatively charged lipids with small headgroups can interact with CTX

| TABLE 1 Data collection and refinement statistics |
|---------------------------------|----------------|
| **Parameters** | **Values** |
| Wavelength (Å) | 1.127 |
| Resolution range (Å) | 26.5–2.3 (2.4–2.3) |
| Space group | P6\(_{3}\)22 |
| Redundancy | 6.1 |
| Completeness (%) | 99.8 (100.0) |
| Average I/σ(I) | 34.6 (5.1) |
| Rmerge (a) (%) | 5.0 (33.9) |

(a) Values in parentheses indicate those for the highest resolution shell.

(b) Rmerge (I) = \(\sum_{i} \sum_{h} I_{hi} - \sum_{i} \sum_{h} I_{hi} \) / \(\sum_{i} \sum_{h} I_{hi} \), where I represents the mean intensity of the i observations of reflection h.
A3. In addition, CTX A3 exhibited preferential binding toward the sphingolipid moiety, because both SpM and phosphatidylcholine contain phosphocholine as the polar headgroup, but SpM had higher binding affinity to CTX A3. Among the tested lipids that showed significant binding to CTX A3, only SGC is known to be located in the outer leaflet of the plasma membrane (28).

In the plasma membrane, most sphingolipids, including SGC, segregate with cholesterol to form microdomains termed “lipid rafts” (2), and much of the data on rafts have been derived from biochemical studies of DRMs. DRMs are low density membrane fragments insoluble in cold, nonionic detergents such as Triton X-100 and can be readily purified by ultracentrifugation on sucrose density gradient (32). To determine whether CTX A3 interacts directly with SGC in the membrane, we obtained the DRM fraction of rat cardiomyocytes previously incubated with CTX A3, because the plasma membrane of fetal rat cardiomyocytes contains \( \sim 0.07 \pm 0.03 \text{nmol of SGC/mg of protein} \) (25). As shown in Fig. 2A, although the location of CTX A3 in the DRM fraction did not correspond exactly to the location of caveolin-1 and the ganglioside GM1, two established raft markers, there was significant overlap between the two populations. Nevertheless, SGC was found in the same fractions that contain CTX A3, supporting the conjecture that CTX A3 and SGC co-localize in the outer leaflet of the plasma membrane.

Additional support for the co-localization of SGC and CTX A3 was also found by confocal microscopy of the binding of fluorescein-labeled CTX A3 (FITC-CTX A3) to cardiomyocytes (Fig. 3). Both FITC-CTX A3 and anti-SGC immunofluorescence signals were seen in a discrete, punctate pattern that is indicative of plasma membrane lipid microdomains. The fact that CTX A3 binding sites only partially (~20%) co-localized with GM1 (Fig. 3, top) is consistent with the partial overlap of GM1 and SGC in the DRM fraction (Fig. 2, A and C). However, we have
consistently observed over 85% co-localization of FITC-CTX A3 with anti-SGC in the plasma membrane of cardiomyocytes (Fig. 3, bottom). While determining the optimal experimental conditions for confocal microscopy, we noticed that application of anti-SGC to localize SGC significantly affects the amount of observable FITC-CTX A3, consistent with the biochemical evidence for competitive binding between anti-SGC and CTX A3 for SGC (Fig. 2C).

**CTX A3-induced Membrane Leakage Is Partially Due to SGC-dependent CTX Pore Formation**—To examine the origin of the observed CTX-induced membrane leakage, we performed electrophysiological studies to address the possibility that CTX A3 induces membrane pores. Many previous model membrane studies suggest that CTX A3 forms pores in vesicles containing negatively charged lipids (22). We found it difficult to perform patch clamping experiments on fetal rat cardiomyocytes due to their beating behavior and thus switched to another cultured cardiomyoblast cell line, H9C2.

Before beginning experiments with H9C2 cells, it was important to determine the availability of SGC in H9C2 cells because synthesis of SGC in cardiomyoblasts has not been reported. Fig. 4A shows that ~0.1 ± 0.02 nmol of SGC/mg of protein was present in the plasma membrane of H9C2 cells as judged from orcinol-positive spots on TLC plates. SGC from porcine brains was used as a standard in these experiments. Results from mass spectrometric analyses (Fig. 4B) suggested that the majority of the fatty acyl components of SGC from the plasma membrane of H9C2 cells were 24 carbons in length (nonhydroxylated 24:1 and 24:0 and hydroxylated 24:1 and 24:0), similar to those from the brain tissues (28).

In a manner similar to that reported previously for cardiomyocytes (25), pretreatment of H9C2 cells with anti-SGC O4 blocked CTX A3-induced membrane leakage, as monitored by intracellular calcium increase (Fig. 5A) and cytotoxicity (Fig. 5, B and C). The increased intracellular calcium concentration was due to both extracellular calcium influx and release from intracellular calcium stores (25). We also showed the effect of cell surface modification on CTX-induced cytotoxicity on H9C2 cells as monitored by LDH leakage and the MTT reduction assay. We tested this in three ways: by masking SGC on the cell membrane using antibody O4 (Fig. 5C), by modifying preexisting sulfated glycoconjugates with sulfatase (Fig. 5C), or by removing heparan sulfate in the extracellular matrix using heparinase I or III treatments (Fig. 5D). Both anti-SGC and sulfatase treatment reduced cell death caused by CTX A3, but heparinase I or III treatment failed to inhibit CTX A3-induced cytotoxicity. The results strongly suggest that SGC is indeed a potential target for CTX A3 action, but not other anionic glycoconjugates located at the outer leaflet of the plasma membrane of H9C2 cells, such as ganglioside GM3 or heparan sulfate.

The whole-cell recording showed that CTX A3 induced pore formation in the plasma membrane of H9C2 cells (Fig. 6A, top row). An additional nonspecific pathway with slightly outward rectifying ion conductivity was detected upon treating cells with CTX A3. CTX A3-induced conductance was sensitive to pretreatment of the cells with sulfatase,
anti-SGC IgG (Sulf1) (Fig. 6A, middle row), or anti-SGC IgM (O4; data not shown). Removal of any preexisting sulfate moiety on the cell surface by sulfatase treatment or masking the 3'-sulfated galactose head-group of SGC by binding with anti-SGC completely abolished the inward CTX A3-induced conductance, consistent with the MTT reduction assay results (Fig. 5C). However, their respective effects on the outward conductance were incomplete but differed slightly, suggesting that part of the CTX A3-induced conductance was SGC-independent. The interfacial amide linkage of SGC is important for the effect of CTX A3, because exogenously incorporated SGC could largely restore CTX A3-induced conductance in sulfatase-pretreated H9C2 cells, but SGE, an SGC analog with an ether linkage (Fig. 6B), was ineffective in restoring conductance (Fig. 6A, bottom row). Our results indicate that CTX A3-induced conductance contains an SGC-dependent pathway and that the pathway requires both the headgroup and the ceramide interfacial region of SGC for its action.

Quantitative analysis of the voltage-dependent profile of H9C2 cells as affected by CTX A3 also revealed the presence of SGC-independent pathway(s). The I-V curve indicated a residual outwardly rectifying CTX A3-induced conductance in H9C2 cells after complete blockage by antibody Sulf1 treatment of any SGC suitable for CTX A3 action (Fig. 6C, left) or after removal by sulfatase treatment of any sulfate moiety on the cell surface (data not shown). When we subtracted this residual conductance from the CTX A3-induced conductance, the resulting conductance from the CTX A3-induced conductance, the resulting residual outwardly rectifying profile was linear (Fig. 6D, right), in sharp contrast to the outwardly rectifying SGC-independent component. The dose-response curves of the SGC-dependent CTX A3-induced pathway corresponded roughly to the square of the CTX A3 concentrations (Fig. 6D, right, with best-fitted b values of 1.87 ± 0.10, 2.16 ± 0.11, 2.27 ± 0.10, and 2.50 ± 0.11; see legend to Fig. 6 for details). These results suggest that a bimolecular interaction, such as homodimerization of CTX A3, might be the rate-limiting step for the observed effect. In contrast, the SGC-independent pathway demonstrated a linear increase in conductance with respect to CTX A3 concentrations (Fig. 6D, left).

The SGC-dependent CTX A3-induced single channel activity was further investigated using an outside-out patch of the H9C2 cell membrane held at negative potential (Fig. 7A). Upon the addition of CTX A3 from 50 to 100 nM, a stepwise conducting event could be observed. Quantification of the observed conductance, as shown in Fig. 7B, suggested that the single channel conductance was about 18.5 ± 10.2 picosiemens with an apparent open lifetime of 12.9 ± 5.8 ms. The CTX A3-induced single channel conductance also required the presence of SGC, since there was no detectable conductance from membrane patches pretreated with anti-SGC O4 (Fig. 7C).

Interestingly, in contrast to the whole-cell recording, a desensitizing effect following repeated CTX A3 treatment became apparent in the outside-out patch clamp experiment (Fig. 7C, lower trace). In the continuous presence of CTX A3, as shown in the recording trace of Fig. 7B, detectable desensitization was observed within 10 s. Because the observed conductance depended on the presence of SGC, it follows that SGC was either masked by the added CTX A3 or became internalized during the experiment after the repeated addition of CTX A3. Alternatively, the CTX-induced conductance became silent after initial pore formation in the cell membrane.

SCG-facilitated CTX Internalization Occurs after CTX Pore Formation in the H9C2 Membrane—We have shown previously that CTX A3 targets mitochondria after internalization by H9C2 cells and disrupts the mitochondrial network (27) and that anti-SGC treatment of rat cardiomyocytes can block CTX A3 internalization (25). Fig. 8A shows internalization of FITC-CTX A3 by H9C2 cells. Anti-SGC O4 inhibited uptake of FITC-CTX A3 in H9C2 cells, indicating the involvement of the 3'-sulfated galactose headgroup of SGC. Removal of the preexisting sulfate moiety on the cell surface by sulfatase also inhibited CTX A3 internalization (Fig. 8, A and B), suggesting that the negatively charged sulfate moiety is involved in the CTX-SGC interaction, leading to CTX A3 internalization. In addition, exogenously incorporated SGC with an amide linkage, but not SGE with an ether linkage, into the plasma membrane significantly enhanced CTX A3 internalization (Fig. 8, A and B). We conclude that CTX A3 internalization requires both the headgroup
Membrane Insertion and Internalization of Cobra Cardiotoxin

FIGURE 6. Whole-cell recording of SGC-dependent and SGC-independent CTX A3-induced extra conductance in H9C2 cells. A, voltage-dependent conductance as a function of time. The role of sulfoglycolipids was demonstrated by removing, masking, or raising the level of sulfu components on the cell surface. Upward deflections show outward currents; downward deflections show inward currents. Top row, cells treated with 1.5 μM CTX A3; middle row, cells pretreated with 50 mIU ml⁻¹ sulfatase or 5 μg ml⁻¹ anti-SGC Sulf1 at 37 °C for 30 min were then exposed to 1.5 μM CTX A3; bottom row, cells pretreated with 50 μM ml⁻¹ sulfatase were incorporated with 200 μM SGC or SGE at 37 °C for 30 min and then perfused with 1.5 μM CTX A3. Clamping from −90 to +100 mV with V_m = 0 mV was performed in 10-mV increments for 200 ms with a 300-ms interval between each pulse; n = 3–5. B, schematic presentation of the acidic glycosphingolipid structure of SGC and SGE. C, current-voltage (I-V) curves after control subtraction. Closed squares, cells without CTX A3 treatment; open circles, Sulf1-masked cells treated with CTX A3; closed diamonds, current of cells treated with CTX A3 alone minus the current of cells masked by Sulf1; open diamonds, current of sulfatase-modified cells incorporated with SGC and treated with CTX A3 minus the current of cells modified by sulfatase. D, dose-response curves of CTX A3-induced extra conductance by steady-state current at ±70 mV and fitted to an exponential function, $y = ax^n$. The closed and open symbols are the same as in C; n = 5–7.

and the ceramide interfacial region of SGC, as we observed for CTX A3-facilitated membrane pore formation.

Further support for the conclusion that CTX A3 internalization and targeting to mitochondria requires SGC located in the plasma membrane of H9C2 cells was obtained by masking the 3'-sulfated galactose headgroup of SGC using anti-SGC Sulf1 (data not shown). Although Sulf1 differs slightly from O4 with regard to binding specificity toward SGC (28), both of the anti-SGC antibodies blocked CTX A3 internalization effectively. As expected for most membrane-delimited pro-

FIGURE 7. CTX A3-induced single channel events as detected by outside-out patch clamp experiments in H9C2 cells. A, representative traces of CTX A3-induced transient rising conductance ($I_m = -70$ mV, $n = 3–5$). B, histograms of the single channel conductance and its open lifetime. Single channel conductance was calculated with an open channel current histogram with a bin width of 1 picosiemens and fitted to the sum of four Gaussian functions (left in lower row). A continuous trace in the presence of 50 nM CTX A3 (upper row) was stepped as four sets of single channel conductance for open lifetime calculation. The events of channel opening (at time t) and shutting (at $t + \Delta t$) were calculated and fitted to the sum of four Gaussian functions (right in lower row). C, desensitization of SGC-dependent conductance after repeated treatment with CTX A3 (lower trace) as compared with the anti-SGC O4 (5 μg ml⁻¹)-pretreated sample (upper trace). CTX A3 (0.2 μM) was applied for 50 ms at the designated time (*) for the conductance recording ($n = 3$).

FIGURE 8. Effect of SGC on CTX A3 internalization by H9C2 cells. A, the cell surface was modified by treatment with 5 μg ml⁻¹ anti-SGC O4, 50 μIU ml⁻¹ sulfatase, 200 μM SGC, or 200 μg ml⁻¹ SGE, and then cells were incubated with 5 μM FITC-CTX A3 at 37 °C for 5 min. FITC-CTX A3 localization in living cells was visualized by confocal microscopy. Bar, 10 μm. B, the time course of CTX A3 internalization by H9C2 cells pretreated with the indicated reagents. (mean ± S.E.; $n = 12–15$). C, temperature-dependent CTX A3 internalization as detected by time course studies of H9C2 cells exposed to 5 μM FITC-CTX A3 at 4 or 37 °C (mean ± S.E.; $n = 21$).
The SGC lipid headgroup is buried within the pocket formed by the D1 dimer, in contrast to the exposed lipid headgroup at the protein surface for glycosphingolipid transfer protein (10) and two other SGC-binding proteins, CD1a (52) and saposin B (11). However, similar to the lactosylceramide that binds to glycosphingolipid transfer protein or the SGC self-antigen in complex with CD1a, the specificity of the CTX A3–SGC interaction is achieved through recognition and anchoring of both the headgroup and the ceramide interfacial region. The galactose moiety of the bound SGC forms extensive hydrogen bonds with the side chain nitrogens of Lys(12) and Lys(18) and the backbone oxygens of Arg(36) and Cys(38) of CTX A3 monomer A. The side chain of Lys(44) is the only residue in CTX A3 monomer B that lies above the galactose moiety and interacts with the amide region of the ceramide moiety (Fig. 10B). Like a slide on the stage of a microscope, the 3′-sulfated galactose headgroup lies on the surface of monomer A composed of Tyr(22) and Arg(36)–Ile(39). Lys(44) from monomer B acts like a stage clip and fastens the headgroup tightly to the surface. In addition, the sulfate moiety of SGC forms a hydrogen bond with the side chain nitrogen of Lys(30) from monomer A, just as in the heparin-derived disaccharide that binds to CTX A3 (40).

Both cerebroside and SGC are in a preferred “bent shovelful” bilayer-planar conformation in the membrane (Fig. 11A) due to the bifurcated intramolecular hydrogen bonds between the amide N-H and the glycosidic oxygen and the fatty acid α-hydroxyl group (29, 53). However, the bound SGC adopts an extended conformation in the crystal, and surprisingly its hydrophobic tails are not attached to the presumed membrane-binding surface comprising the three loops of CTX A3 (Figs. 10A and 11C). The insertion of the Lys(44) side chain from CTX A3 monomer B seems crucial for straightening the bend of SGC after the SGC headgroup has already been anchored in the binding pocket of monomer A (Figs. 10B and 11C). The Lys(44) insertion abolishes the SGC intramolecular hydrogen bonds, provides special restraints within this area, and makes new hydrogen bonds between the CTX A3 protein and the amide region of the bound SGC. Unlike the complete burial of lipid tails in glycosphingolipid transfer proteins (10) or saposin B (11) to allow the formation of water-soluble lipid-protein complexes, the extended bound SGC exposes its hydrophobic tails from the headgroup binding pocket. As shown in Fig. 10C, the sphingosine alkyl chain of SGC protrudes into the detergent-rich solvent area, and the fatty acid alkyl chain inserts into a symmetry-related D1 dimer. This buried hydrophobic tail adopts a curved, U-shaped conformation and makes van der Waals contacts with the L3 region containing Asp(40)–Lys(44) of monomer A as well as regions including Pro(15)–Lys(18) and Ile(39)–Asp(40) of monomer B.

Barring the involvement of side chain reorientations near the tip of the loops in mediating the dimerization of CTX A3, the overall structure of CTX A3 remains unchanged after CTX-membrane interaction due to the presence of four disulfide bonds. Although in the absence of SGC, the D1 dimer can form near the membrane surface via a hydrophobic clustering of Met(24) and Met(26) (Fig. 11C) (22), the SGC can function as a glue to effect tighter binding between monomers in the CTX A3 dimeric complex. For instance, in the presence of SGC, the dimer gains a pair of intermolecular hydrogen bonds from the side chain nitrogen of Lys(12) and the backbone oxygen of Lys(44) (Fig. 10B), which is not observed in the CTX A3–SGC complex. The total surface area of the D1 dimer in the CTX A3–SGC complex buried through dimerization increases from 1,035 to 1,133 Å² compared with the D1 dimer in the CTX A3–SGD complex.

**DISCUSSION**

We have established that CTX A3 binds SGC, based on TLC overlay assays, co-localization in a purified DRM fraction and in confocal
micrographs, and the x-ray crystal structure. Our work addresses the mechanism of CTX A3-induced cytotoxicity in rat cardiomyocytes and H9C2 cells and indicates the importance of SGC with regard to CTX A3-induced membrane pore formation and CTX A3 internalization. The observed efficacy of anti-SGC antibodies in preventing CTX A3 cytotoxicity also suggests the application to cobra snakebite victims. The observed SGC-mediated CTX A3 pore formation and internalization in this study is distinct from that of all other known glycosphingolipid-binding toxins. Recent studies have shown that the scorpion toxin maurocalcine crosses the plasma membrane, reaches its target channel located in the endoplasmic reticulum, and consequently causes calcium release from intracellular stores (54). It has been suggested that maurocalcine behaves similarly to the well-known cell-penetrating peptides (55), such as the human immunodeficiency virus Tat peptide (56), although recent efforts to understand the translocation mechanism of cell-penetrating peptides have revealed greater complexity involving multiple pathways (57–59). Interestingly, similar to CTX A3, polypeptides with cell penetrating capability usually are amphiphilic and contain positively charged clusters that are capable of targeting the cell surface by binding to heparan sulfate in the extracellular matrix and/or anionic lipids in the membrane bilayer (60–62). Because of the growing importance of cell-penetrating peptides in delivering functionally important molecules into cells, future investigations on the detailed molecular processes involved in the lipid-mediated protein translocation, as we observed for CTX A3 and SGC, should shed new light on the cell-penetrating mechanism of cell-penetrating peptides.

Protein-SGC Interactions at the Membrane Interface: Extraction Versus Insertion—The three-dimensional structure of the CTX A3-SGC complex in a membrane mimetic environment provides a prototype for...
Membrane Insertion and Internalization of Cobra Cardiotoxin

understanding how a lipid could direct insertion of peripheral binding polypeptides into membrane bilayers. It is instructive to compare two groups of SGC binding proteins: saposin B, which extracts SGC from lipid membranes (11) to allow the action of hydrolytic enzymes on the lipids, and CTX A3, which inserts into lipid membranes and forms pores with the assistance of SGC. In the former case, a protein conformational change must occur to facilitate the insertion of the lipid hydrophobic tails into the inner cavity of the saposin B dimer. This mechanism is further supported by the direct shuttle model for the lipid transfer proteins containing the START (steroidogenic acute regulatory protein-related lipid transfer) domain (63), which suggests that during interaction with a membrane, a partial opening of the domain structure is necessary for lipid uptake and release, because the inner hydrophobic cavity or tunnel is usually too small to accommodate a lipid molecule.

However, membrane insertion of CTX A3 probably requires both protein and lipid conformational changes. The lipid-facilitated protein insertion mechanism suggests a rearrangement of the lipid to move the protein from a peripheral to an insertional binding mode (Fig. 11). Previous studies indicated that CTX A3 monomer peripherally binds to the phospholipid membrane surface (22, 24, 47) (Fig. 11A), and therefore a peripherally bound CTX A3 monomer can use its sulfated carbohydrate-binding site to directly interact with a SGC molecule in "bent shovel" conformation in the outer leaflet of the plasma membrane (Fig. 11B). When the CTX-SGC interaction couples with CTX A3 dimerization near the membrane surface, a conformational change in SGC occurs at its ceramide interfacial region, leading the bound SGC to adopt an extended conformation (Fig. 11C). However, instead of burying the hydrophobic tails of the lipid within the CTX A3 dimer, as for saposin B (11) and START proteins (63), or keeping the lipid in a normal orientation perpendicular to the membrane surface, as for AB_{13} ganglioside-binding toxins (13), the lipid tails reorient 90° by anchoring the SGC headgroup to the sulfated carbohydrate-binding site of CTX A3. We propose that the observed lipid conformational changes induce additional CTX A3 oligomerization by allowing SGC to interact with other CTX A3 dimers via its hydrophobic tails, as suggested by the crystal packing of the CTX A3 tetramer (Figs. 10C and 11D). The proposed mechanism could also facilitate the CTX A3 D1 and D2 dimer formation required for CTX A3 pore formation, as we suggested previously based on the whole-cell recording studies for the SGC-dependent CTX A3-induced conductance (Fig. 6D) and the CTX A3-SDS complex structure (22).

CTX A3 Aggregates as an Explanation for Pore Desensitization and Further CTX A3 Internalization—In patched membranes, the single channel conductance of CTX A3 pores apparently desensitized in 10 s even with continuous perfusion of CTX A3. Previous model membrane studies with various peptides capable of inducing membrane leakage have generated several models to explain the desensitization effect. It has been suggested that lipid flip-flop or peptide translocation across the membrane may be responsible for the incomplete vesicle leakage induced by lytic polypeptides (62, 64). In light of the fact that many β-sheet polypeptides form neurodegenerative disease-related amyloid aggregates near the membrane surface with or without membrane pore formation (65, 66), it is also possible that CTX A3 aggregates having differing stabilities may also form near the membrane surface to account for the observed pore desensitization.

The distinct difference between the lifetimes of CTX A3 pore formation and desensitization we observed in this study and the lifetime of CTX A3 internalization to target mitochondria (27) provides hints to distinguish between the aforementioned mechanisms. In the patch clamp experiment on H9C2 cell membranes (Fig. 7B), we estimated an average open lifetime of 12.9 ± 9.8 ms for CTX A3-induced single-channel conductance, which is consistent with the lifetime of ~8 ms for CTX A3 pore formation in SGC-containing 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylcholine vesicles (data not shown). Therefore, CTX A3 pore formation observed in cell membranes might share some similarities with that observed in model membranes, although the SGC concentration available to the outer leaflet of the plasma membrane is much less than that used in model membrane studies. It is likely that SGC segregates as microdomains in both systems, as also evidenced by the punctate staining pattern of SGC in the confocal microscopic study of H9C2 cell plasma membrane (Fig. 3). In fact, CTX A3 was found co-localized with these SGC domains.

Significant CTX A3 internalization can only be observed after 5 minutes (27), whereas the estimated lifetime for CTX A3 pore formation is ~12.9 ms. It seems unlikely that the internalization process results directly from SGC-induced CTX A3 pore formation without intermediate states. Both lipid flip-flop and CTX A3 insertion into the membrane appear to be too fast to be directly responsible for the observed

FIGURE 11. SGC-facilitated insertion of the CTX A3 dimer as structural intermediates during membrane pore formation. The lipid bilayer is drawn as zones (orange-to-light gray gradients; the hydrophilic regions of the bilayer; light gray zones; the hydrophobic regions). A, a peripherally bound CTX A3 monomer (green ribbon) and SGC molecule (stick model) located in the bilayer outer leaflet. Two different views of the conformation of SGC are presented throughout the figure. B, SGC headgroup interacting with a CTX A3 monomer at its sulfated carbohydrate-binding site near the membrane surface. C, structural intermediate during the process of membrane insertion based on the CTX-SGC complex structure. D, CTX A3 tetramer formed by two D1 dimers (green-cyan and yellow-blue) detected in crystal packing. The binding to SGC connects two D1 dimers and promotes the formation of the D2 dimer (green-yellow and cyan-blue) required for membrane pore formation by the CTX A3 oligomer. The surrounding detergent C_{12}E_{6} molecules are shown as black sticks. The bound SGC molecules are in red.
Membrane Insertion and Internalization of Cobra Cardiotoxin

CTX A3 internalization, although these processes are somehow involved in the interaction with SGC. Because protein internalization in cell membranes often involves segregation of membrane domains with distinct lipid and protein composition to form endocytic vesicles (67, 68), metastable SGC-induced aggregation of CTX A3 may also be involved in the process. The insertion of CTX A3 into sphingolipid domains is expected to perturb the equilibrium and the bending energy of noncaveolar lipid rafts during endocytosis and could possibly stimulate the membrane-delimited endocytotic process, since the balance between caveolin and glycosphingolipids appears to regulate the endocytic potential of caveolae (34). Alternatively, some other components in the membrane may have to associate with the CTX A3-SGC complex to complete CTX A3 internalization. In this regard, future analysis of CTX aggregates under different crystallization conditions could provide useful information for understanding the mechanism of CTX A3 internalization.

Heparan Sulfate Versus SGC as a Target for CTX Action—Many toxins likely hijack glycosaminoglycans in the extracellular matrix for specific tissue targeting (69). However, surface modification of either the high sulfated or low sulfated domain of heparan sulfate in the extracellular matrix of H9C2 cells by using heparinase I or III did not significantly affect CTX-induced cytotoxicity (Fig. 5D). The binding sites on CTX A3 for the SGC headgroup and for heparin partially overlap, and Lys<sup>35</sup> is responsible for interacting with the sulfate moiety in both SGC (Fig. 10A) and heparin (40). Therefore, negatively charged glycosaminoglycans may serve as a high capacity region for concentrating basic toxins that can be further transferred with high specificity to a sulfated glycosphingolipid domain in the plasma membrane outer leaflet (70). Although CTX A3 failed to bind gangliosides, such as GM1, that localize to lipid rafts, the observed interaction between CTX A3 and SGC was tied to the formation of different CTX A3 aggregates at the membrane surface and may also account for the apparent differences in biological activities of membrane insertion, pore formation, and cell internalization.

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REFERENCES

1. Holhuijs, J. C., and Levine, T. P. (2005) Nat. Rev. Mol. Cell Biol. 6, 209–220
2. Munro, S. (2003) Cell 115, 377–388
3. Morales, A., Coelle, A., Mari, M., Garcia-Ruiz, C., and Fernandez-Checka, J. C. (2004) Glycoconjug J. 20, 579–588
4. Tettamanti, G. (2004) Glycoconjug. J. 20, 301–317
5. C.-H. Wang, S.-C. Lee, and W. Wu, unpublished results.

5 C.-H. Wang, S.-C. Lee, and W. Wu, unpublished results.
Membrane Insertion and Internalization of Cobra Cardiotoxin