Synthesis and Characterization of *N*-Parinaroyl Ganglioside G_{M1}: Effect of Choleragen Binding on Fluorescence Anisotropy in Model Membranes†

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ABSTRACT: *N-cis*-Parinaroyl ganglioside G_{M1} and *N-trans*-parinaroyl ganglioside G_{M1} were synthesized and characterized by HPLC, TLC, component analysis, absorbance spectroscopy, and proton NMR spectroscopy. Steady-state fluorescence anisotropy of the purified compounds, incorporated into phosphatidylcholine liposomes, was measured in the presence and absence of choleragen (cholera toxin) and choleragenoid (cholera toxin B subunit). In gel-phase liposomes, anisotropy measurements indicated that the motion of the parinaroyl ganglioside was not affected by addition of choleragen or choleragenoid. In fluid-phase liposomes, however, addition of toxin resulted in increased anisotropy (decreased rotational motion) of the fluorescent gangliosides. This decreased motion was not observed with other parinaroyl lipid probes, such as phosphatidylcholine, glucosylceramide, or free fatty acids, indicating that the effect was due to specific ganglioside/toxin interactions. Varying the amount of ganglioside or the amount of toxin suggested that the effect of toxin on probe motion was saturable at approximately 1 choleragen (or choleragenoid) molecule/5 ganglioside molecules. These results are consistent with previous hypotheses regarding the ganglioside/choleragen interaction and indicate that parinaroyl ganglioside probes will be useful in elucidation of the molecular details of this interaction.

Glycosphingolipids are ubiquitous components of mammalian cell surface membranes. Included in this class of lipids are gangliosides, which contain one or more sialic acid residues in the carbohydrate head group. The distribution of gangliosides in the plasma membrane is asymmetric, as they are predominantly and likely exclusively located in the extracellular leaflet of the membrane (Singer, 1974; Fishman & Brady, 1976; Miller-Podraza et al., 1982). A large literature implicates ganglioside involvement in a wide variety of biological processes including cell differentiation and growth control, oncogenic transformation, cell/cell interactions, and the binding of toxins, viruses, lectins, and agents of the immune system to the plasma membrane [for review, see Hakomori (1981); Thompson and Tillack (1985), and Curatolo (1987)]. However,the molecular basis for the involvement of gangliosides in many of these processes is still unknown.

Ganglioside G_{M1}[1] serving as the cell surface receptor for choleragen on eukaryotic cells is an example of a ganglioside function that has been well studied. Choleragen consists of two subunits, A and B. The B subunit has five identical polypeptides. Previous experiments suggested that each of the five polypeptides of the B subunit binds to one molecule of G_{M1} (Dwyer & Bloomfield, 1982; Fishman & Atikkan, 1980). The A subunit has two dissimilar polypeptides linked by a disulfide bond. The A_1 polypeptide contains ADP-ribosylase activity (Moss et al., 1979; Moss & Vaughan, 1980), but the A subunit does not bind to cell surfaces on its own [for review, see Fishman (1982) and Holmgren (1981)]. Choleragen specifically binds G_{M1} with a dissociation constant of 10^{-9}–10^{-10} M (Cuatrecasas, 1973). The mechanism by which the interaction between choleragen and G_{M1} triggers A-peptide insertion into the membrane remains mysterious.

To investigate the molecular behavior and function of gangliosides, ganglioside derivatives that can be used as spectroscopic probes and that have properties similar to natural gangliosides are useful. Parinaric acid is a naturally occurring fluorescent fatty acid that can be incorporated into membrane lipids and that is very similar in structure to the saturated and unsaturated fatty acids found commonly in biomembranes (Sklar et al., 1979; Welti & Silbert, 1982). PnA and its derivatives have been widely used as probes of membrane lipid structure (Rintoul et al., 1979; Hyslop et al., 1984; Pugh et al., 1982). Previously, we have synthesized ceramides, glucosylceramides, lactosylceramides, and sulfatides containing PnA and have used them to investigate the thermodynamic properties of glycosphingolipids in model membranes (Rintoul et al., 1986). These experiments demonstrated that such fluorescent probes were useful tools with which to study lipid behavior in the membrane. Schwarzmann and Sandhoff (1986) have previously reported the synthesis of *N*-parinaroyl G_{M1}, but no further characterization of its structure and biological function has been reported.

In the present investigation, *N*-parinaroyl ganglioside G_{M1} has been synthesized and characterized. The specific binding of choleragen to Pn G_{M1} has been examined by using steady-state fluorescence anisotropy. We show that this fluorescent G_{M1} binds choleragen specifically and with the same stoichiometry as natural G_{M1}.

Experimental Procedures

Reagents. Ganglioside G_{M1} used in the synthesis of Pn G_{M1} was isolated from bovine brain as described by Svennerholm (1973) and purified by DEAE-Sephadex A-25 column chro-

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[1] Abbreviations: GSL, glycosphingolipid; tPnA, *all-trans*-9,11,13,15-octadecatetraenoic acid; cPnA, *cis,trans,trans,cis*-9,11,13,15-octadecatetraenoic acid; G_{M1}, Gal(β1–3)GalNAc(β1–4)[NeuAc(α2–3)]Gal(β1–4)GlcCer; lyso G_{M1}, Gal(β1–3)GalNAc(β1–4)[NeuAc(α2–3)]Gal(β1–4)GlcSph; Pn G_{M1}, *N*-parinaroyl G_{M1}; DEC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; DEPC, 1,2-dielaidoylphosphatidylcholine; Pn PC, 1-oleoyl-2-parinaroylphosphatidylcholine; $(CH_3)_2SO$-d_6, dimethyl-d_6 sulfoxide; CD_3OD, methyl-d_3 alcohol-*d*; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid.

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matography (Momoi et al., 1976). Ganglioside G_{M1} used as a TLC standard and as a component of liposomes was obtained from Sigma Chemical Co. (St. Louis, MO). DEC and DEAE-Sephadex A-25 were also purchased from Sigma. 1,2-Dielaidoylphosphatidylcholine was from Avanti Polar Lipids, Inc. (Pelham, AL). PnA was obtained from Molecular Probes, Inc. (Eugene, OR). 1-Oleoyl-2-parinaroylphosphatidylcholine was the generous gift of Dr. R. Welti, Division of Biology, Kansas State University. TLC plates were purchased from Analtech, Inc. (Newark, DE). Sep-Pak C_{18} cartridges were from Anspec Co., Inc. (Ann Arbor, MI) and Unisil silicic acid was from Clarkson Chemical Co., Inc. (Williamsport, PA). Methyl-d_3 alcohol-d and dimethyl-d_6 sulfoxide were from Aldrich Chemical Co., Inc. (Milwaukee, WI). All solvents were of reagent or HPLC grade from Allied Fisher Scientific Co. (Fair Lawn, NJ).

Synthesis of N-Parinaroyl G_{M1}: (A) Preparation of Deacetyl Lyso G_{M1} (Compound II). Ganglioside G_{M1} was deacylated by the method of Neuenhofer et al. (1985). Purified G_{M1} (50 mg) was mixed with 6 mL of 1 M methanolic potassium hydroxide that had been dried over Na_2SO_4. After flushing with nitrogen, the reaction mixture was incubated at 102 °C for 25 h. The mixture was neutralized with acetic acid, and the solvent was evaporated under a nitrogen stream. The residue then was dissolved in water and dialyzed against water for at least 3 h, followed by lyophilization. Reaction progress was monitored by TLC. Deacetyl lyso G_{M1} could be detected on TLC plates by two specific spray reagents, resorcinol (specific for sialic acid) and ninhydrin (specific for free amino groups). The residue was dissolved in solvent A ($CHCl_3$/CH_3OH/H_2O, 30/60/8 v/v/v), and deacetyl lyso G_{M1} was purified by TLC in $CHCl_3$/CH_3OH/12 mM $MgCl_2$/NH_4OH (40/40/7.5/3 v/v/v/v). Alternatively, deacetyl lyso G_{M1} was purified by DEAE-Sephadex A-25 column chromatography. For column chromatography, a 1 × 20 cm colume was equilibrated with solvent A. The sample, dissolved in solvent A, was eluted with the same solvent, and 1-mL fractions were collected. Deacetyl G_{M1} was eluted first, followed by deacetyl lyso G_{M1}. Fatty acid and unreacted G_{M1} remained on the column; they could be eluted with 0.7 M sodium acetate in methanol.

(B) Preparation of Deacetyl Parinaroyl G_{M1} (Compound III). Deacetyl lyso G_{M1} (2 μmol) was mixed with either cPnA or tPnA (4 μmol), DEC (40 μmol), and 0.5 mL of CH_2Cl_2/CH_3OH (95/5 v/v) in a screw-capped vial. After flushing with nitrogen, the mixture was sealed and stirred overnight at room temperature. The solvent was evaporated, and the reaction products, suspended in chloroform, were applied to a Unisil column (0.3 g). The column was eluted with $CHCl_3$, $CHCl_3$/CH_3OH (6/4 v/v), $CHCl_3$/CH_3OH (1/1 v/v), and CH_3OH sequentially. The elution process was monitored by TLC. The first two eluates contained unreacted and degraded PnA and the catalyst. Deacetyl Pn G_{M1} was found in the third eluate. Unreacted deacetyl lyso G_{M1} was in the fourth eluate and could be recycled.

(C) Preparation of N-Parinaroyl G_{M1}. Deacetyl Pn G_{M1} was mixed with 1 mL of ethyl ether and 1 mL of saturated $NaHCO_3$. Acetic anhydride (10 μL) was added, with stirring, every 5 min until the pH reached 5.5–6.0. After evaporation of the ether, the products were desalted by passage through a Sep-Pak C_{18} cartridge (Kubo & Hoshi, 1985). Finally, Pn G_{M1} was purified by HPLC according to the method of Kundu and Scott (1982) and analyzed by TLC. Pn G_{M1} could be detected by its fluorescence under UV light and with resorcinol spray reagent, but not with ninhydrin. Starting with 30 μmol of G_{M1}, the yield was approximately 200 nmol of Pn G_{M1} from this procedure.

Thin-Layer Chromatography. Analytical TLC of Pn G_{M1} and reaction intermediates was performed on HPTLC-GHL plates. All TLC was performed at room temperature. Solvent systems used were $CHCl_3$/CH_3OH/12 mM $MgCl_2$/NH_4OH, 60/35/7.5/3 (v/v), $CHCl_3$/CH_3OH/H_2O, 55/45/10 (v/v), containing 0.02% $CaCl_2$, and $CH_3CH_2CH_2OH$/H_2O, 7/3 (v/v). Detection methods included spraying with 50% H_2SO_4, followed by heating at 180–200 °C overnight, for detection of any organic compounds; spraying with 0.2% ninhydrin in butanol saturated with water, followed by heating at 100 °C, for detection of compounds containing free amino groups; and spraying with resorcinol (2% resorcinol in water/concentrated HCl/0.1 M $CuSO_4$/H_2O, 10/80/0.25/9.75 v/v), followed by heating at 110 °C for a few minutes, for detection of compounds containing sialic acid.

Preparation of Liposomes. Lipids were dissolved in 10 μL of absolute ethanol after they were dried under vacuum for 30 min to remove all organic solvents. Then the lipids in ethanol were slowly injected into 2.5 mL of buffer (50 mM Hepes, 100 mM KCl, pH 7.2) while vortexing. Unilamellar liposomes containing 95–100% of the lipids were obtained by this method (Rintoul et al., 1986; Batzri & Korn, 1973) and used for measurements of steady-state fluorescence anisotropy.

Fluorescence Anisotropy Measurement. Liposomes containing a total of 400 nmol of lipids (varying amounts of DEPC and nonfluorescent G_{M1} and 1 nmol of Pn G_{M1}) were prepared in 2.5 mL of buffer as described above. Fluorescence of the samples was measured with a Spex Fluorolog spectrofluorometer, which was equipped with a thermostated cuvete chamber and quartz polarizers. Excitation wavelengths was 320 nm (bandpass 5 nm) for tPn G_{M1} and 325 nm (bandpass 5 nm) for cPn G_{M1}. Fluorescence emission was monitored at 420 nm (bandpass 20 nm) for both of the probes. The anisotropy (r) is defined as

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$$

where I_{VV} and I_{VH} refer respectively to fluorescence intensity parallel and perpendicular to the vertically polarized excitation beam and I_{HV} and I_{HH} refer respectively to fluorescence intensity parallel and perpendicular to the perpendicularly polarized excitation beam. G, which is equal to I_{HH}/I_{HV}, is a factor to correct for residual polarization due to the monochromator diffraction gratings (Chen & Bowman, 1965).

Absorbance Spectra of Parinaric Acid and N-Parinaroyl G_{M1}. The absorbance spectra were obtained on an SLM-Aminco DW-2C UV–vis spectrophotometer, scanning from 270 to 360 nm.

Proton NMR Spectroscopy. Samples were repeatedly dried from 99.5% CD_3OD to remove exchangeable protons and then dissolved in 0.5 mL of 99.9% $(CH_3)_2SO$-d_6. The spectra were obtained at 30 °C with a Bruker 400-MHz NMR spectrometer. Signals were calibrated after the central signal from dimethyl sulfoxide was assigned to 2.52 ppm.

Results

Synthesis and Characterization of N-Parinaroyl G_{M1}. Pn G_{M1} was synthesized following the procedure shown in Figure 1 and described under Experimental Procedures. The acylation of deacetyl lyso G_{M1} was the most difficult step because PnA is very sensitive to light and oxygen, and deacetyl lyso G_{M1} is much more polar than PnA and parinaroyl anhydride, making it difficult to select a solvent that could dissolve all of the PnA, deacetyl lyso G_{M1}, and catalyst and that could also be evaporated easily. For example, neither in water nor in

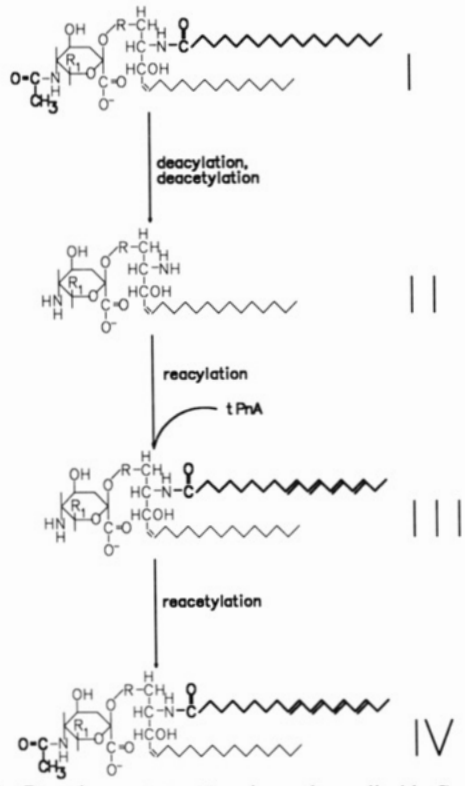

FIGURE 1: Reaction route to *N*-parinaroyl ganglioside G_{M1}. Bovine brain ganglioside G_{M1} (I) was subjected to alkaline hydrolysis as described under Experimental Procedures. This reaction released the *N*-acyl group and an acetyl moiety from the sialic acid in the head group. The deacylated, deacetylated product (II) was purified and acylated with parinaric acid as described in the text to yield intermediate III. After reaction with acetic anhydride to reacetylate the sialic acid, parinaroyl ganglioside G_{M1} (IV) was purified from the reaction mixture by using preparative TLC and HPLC as described in the text. R: Gal (β1–3)GalNAc(β1–4)Gal(β1–4)Glc–; the sialic acid is attached via an α2–3 linkage to the interior galactose residue. R_1: $-(CHOH)_2CH_3OH$.

$CH_3CN/CH_2Cl_2/CH_3OH$ (7.5/7.5/1.5 v/v) would the reaction occur. Of the solvents tested, only CH_2Cl_2 containing 5% CH_3OH allowed the reaction to occur. Special conditions were also required for the acetylation step because parinaroyl residues degrade faster in aqueous solution than in organic solvents, especially at low pH. To protect the parinaroyl group, acetylation of the oligosaccharides of G_{M1} was carried out in a two-phase solution consisting of ethyl ether and saturated $NaHCO_3$ aqueous solution.

Purified cPn G_{M1} and tPn G_{M1} were first characterized by HPTLC and absorbance spectroscopy. Three solvent systems, described under Experimental Procedures, were used in the HPTLC analyses. Only a single band, which had the same mobility as natural G_{M1}, was detectable in each solvent system. A thin-layer chromatogram of the products, as well as natural G_{M1} and deacetyl lyso G_{M1}, in one solvent system is shown in Figure 2. The absorption spectrum of cPn G_{M1} is shown in Figure 3. The similarity of the spectra of the products to those of cPnA and tPnA indicates that the products have intact parinaroyl groups.

Proton NMR spectra of natural G_{M1}, deacetyl lyso G_{M1}, and cPn G_{M1} are shown in Figure 4. Similar to what was previously reported by Sonnino et al. (1985), the signals from the methyl protons of the alkyl chains were at 0.85 ppm and the signals from the acetyl protons of *N*-acetylneuraminic acid and *N*-acetylgalactosamine were at 1.89 ppm and 1.75 ppm, respectively. For natural G_{M1}, the signal intensity of the methyl protons was twice as high as that of protons of each

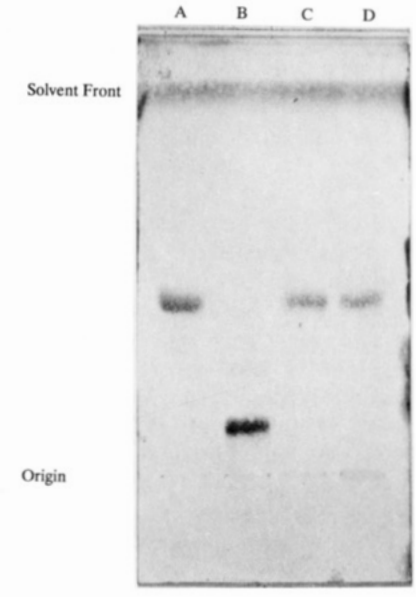

FIGURE 2: Thin-layer chromatogram of *N-cis*-parinaroyl G_{M1}, *N-trans*-parinaroyl G_{M1}, deacetyl lyso G_{M1}, and bovine brain G_{M1}. TLC plates were developed in $CHCl_3/CH_3OH/12$ mM $MgCl_2/NH_4OH$ (60/35/7.5/3 v/v/v/v). Compounds were visualized by spraying the TLC plate with 50% H_2SO_4, followed by charring at 180–200 °C overnight. (Lane A) G_{M1}; (lane B) deacetyl lyso G_{M1}; (lane C) cPn G_{M1}; (lane D) tPn G_{M1}.

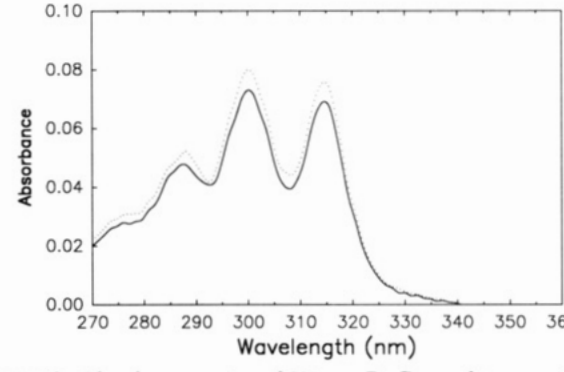

FIGURE 3: Absorbance spectra of *N-trans*-Pn G_{M1} and *trans*-parinaric acid in methanol. Parinaroyl lipids were added to 2.5 mL of methanol. The absorbance spectra were obtained by scanning from 270 to 360 nm in an SLM-Aminco DW-2 spectrophotometer; background absorbance (from a similar scan of the solvent alone) was subtracted to give the spectra shown. The solid line represents *N-trans*-Pn G_{M1} (1.83 nmol); the dotted line represents *trans*-parinaric acid (2.0 nmol).

acetyl, as natural G_{M1} contains two alkyl chains. The proton NMR spectrum of deacetyl lyso G_{M1} shows no signal at 1.89 ppm, indicating the loss of one acetyl group from natural G_{M1}. In addition, the intensity ratio of the methyl proton signal and the remaining acetyl proton signal is 1:1, indicating the loss of the fatty acyl chain from G_{M1}. In the proton NMR spectrum of the purified final product, the acetyl proton signal (1.89 ppm) of *N*-acetylneuraminic acid is again visible. The intensity ratio of the methyl proton signal to each acetyl proton signal is 2:1 again, indicating that this material has been reacylated. All these results are consistent with the notion that the final product was Pn G_{M1}.

Interaction of Choleragen with N-Parinaroyl G_{M1}. Since ganglioside G_{M1} is a high-affinity receptor for choleragen on plasma membranes of eukaryotic cells, the functional interaction of choleragen with Pn G_{M1} should be a stringent test of the structure of Pn G_{M1}. Liposomes containing 1 nmol of Pn G_{M1} and varying amounts of G_{M1} and DEPC were selected as a model membrane system. One might expect choleragen

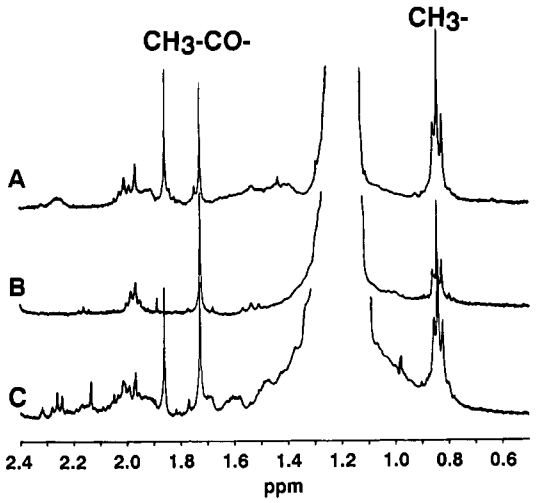

FIGURE 4: Proton NMR spectra of *N-cis*-parinaroyl G_{M1}, deacetyl lyso G_{M1}, and bovine brain G_{M1} obtained at 400 MHz and 30 °C. (A) Bovine brain ganglioside G_{M1}, prepared as described under Experimental Procedures. (B) Deacetylated, deacylated ganglioside G_{M1} (intermediate II in Figure 1), prepared as described under Experimental Procedures. (C) *N-cis*-Parinaroyl ganglioside G_{M1}, prepared and purified as described under Experimental Procedures.

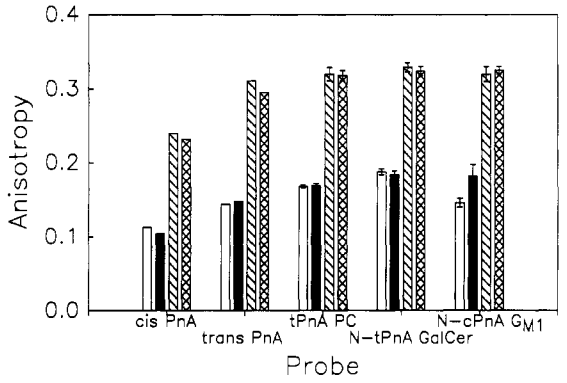

FIGURE 5: Effect of choleragen on steady-state fluorescence anisotropy of different probes. DEPC (320 nmol), G_{M1} (80 nmol), and probe (1 nmol) were dried under nitrogen, resuspended in 10–15 μL of ethanol, and injected into 2.5 mL of buffer as described under Experimental Procedures. After the liposomes were incubated for 2 min at 5 or 30 °C, fluorescence in the absence of choleragen was determined. Choleragen (2.0 nmol in HEPES buffer) was added; steady-state fluorescence anisotropy was measured and calculated as described under Experimental Procedures. Data shown are means of triplicate determinations, except for the data shown for cPnA and tPnA probes, which are the results of a single experiment; error bars indicate standard errors. Open bars represent the absence of choleragen at 30 °C, solid bars represent the presence of choleragen at 30 °C, diagonally slashed bars represent the absence of choleragen at 5 °C, and crosshatched bars represent the presence of choleragen at 5 °C.

binding to G_{M1} to affect the motion of the lipid in the membrane. Since rotational lipid motion can be monitored with steady-state fluorescence anisotropy, we measured steady-state fluorescence anisotropies of different probes (cPnA and tPnA, Pn PC, *N-trans*-parinaroyl galactocerebroside, and cPn G_{M1}) in DEPC liposomes containing 20% G_{M1} with or without choleragen (Figure 5). At 30 °C, which is above the phase transition temperature of this mixture (data not shown), only the cPn G_{M1} anisotropy was obviously changed by choleragen. The anisotropies of the other probes were unchanged by the addition of choleragen, within the range of experimental error. This indicates that choleragen was interacting with Pn G_{M1} but not with the other probes. The binding of choleragen to pN G_{M1} increased the anisotropy, which connotes a decrease in rotational motion of Pn G_{M1}. At 5 °C, which is below the

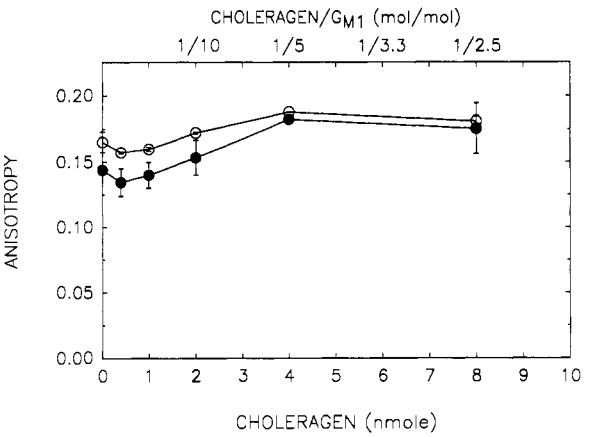

FIGURE 6: Effect of different concentrations of choleragen on steady-state fluorescence anisotropy of *N*-parinaroyl G_{M1}. Liposomes containing 380 nmol of DEPC, 20 nmol of G_{M1}; and 1 nmol of cPn G_{M1} or tPn G_{M1} were prepared as described under Experimental Procedures. Indicated amounts of choleragen in HEPES buffer were added, and the samples were incubated for 2 min at 30 °C. Fluorescence was measured and steady-state fluorescence anisotropy was calculated as described under Experimental Procedures. Data shown are means of triplicate determinations; error bars indicate standard errors. Solid circles represent cPn G_{M1}; open circles represent tPn G_{M1}.

transition temperature of the bulk lipid, addition of choleragen did not alter the steady-state fluorescence anisotropy of any of the probes used. Thus, the toxin-dependent change in molecular motion was undetectable by steady-state fluorescence anisotropy in gel-phase lipids.

The effect of different concentrations of choleragen on Pn G_{M1} was also investigated by steady-state fluorescence anisotropy measurements (Figure 6). Varying amounts of choleragen were incubated with liposomes containing DEPC and 5 mol % of G_{M1} at 30 °C. All liposome preparations contained 1.0 nmol of Pn G_{M1} probe. The anisotropy of both tPn G_{M1} and cPn G_{M1} increased as the ratio of choleragen to G_{M1} increased up to a ratio of 1 choleragen to 5 G_{M1}, indicating that, in this range, binding of choleragen to G_{M1} was dependent on the choleragen concentration. At a mole ratio of choleragen to G_{M1} of 1 to 5 or more, the anisotropy no longer increased with choleragen concentration. These data are consistent with the model that each of five polypeptides in the B subunit binds to one G_{M1} molecule (i.e., 5 G_{M1} bind to 1 choleragen molecule; Dwyer & Bloomfield, 1982; Fishman & Atikkan, 1980). It is also noteworthy that the initial anisotropy of tPn G_{M1} was higher than that of the cis isomer. Interestingly, as the choleragen concentration rose, the anisotropic difference between tPn G_{M1} and cPn G_{M1} became smaller and smaller.

Similar experiments were done with liposomes containing different fractions of G_{M1}. The results are shown in Figure 7. It is obvious that no matter how much G_{M1} was in the liposomes anisotropy of Pn G_{M1} rose as the choleragen concentration increased and stopped increasing when the mole ratio of choleragen to G_{M1} became higher than 1:5. This further confirms the conclusion that Pn G_{M1} interacts with choleragen like natural G_{M1}. It is also shown that, at the same mole ratio of choleragen to G_{M1}, higher fractions of G_{M1} in the liposomes were associated with greater increases in steady-state fluorescence anisotropy. Thus, the anisotropic increase of Pn G_{M1} resulting from the choleragen's binding was also G_{M1} concentration dependent.

Finally, the effects of choleragen and its B subunit of the steady-state fluorescence anisotropy of Pn G_{M1} were compared (Figure 8). The experiments with the B subunit yielded results

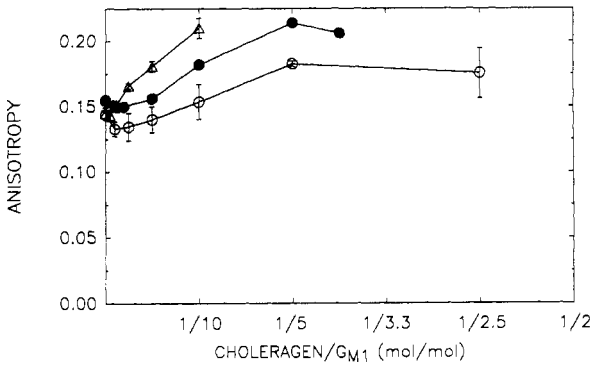

FIGURE 7: Effect of G_{M1} concentration in DEPC liposomes on steady-state fluorescence of *N-cis*-parinaroyl G_{M1}. DEPC liposomes containing 400 nmol of lipids, 1 nmol of cPn G_{M1}, and varying mole percentages of G_{M1} were prepared as described under Experimental Procedures. The liposomes were incubated with varying amounts of choleragen for 2 min at 30 °C before determination of fluorescence. Fluorescence and steady-state fluorescence anisotropy were determined as described under Experimental Procedures. Data shown are means of triplicate determinations except for the data from for DEPC liposomes containing 10% G_{M1}, which are the result of a single experiment; error bars indicate standard errors. Open circles represent 5% G_{M1}, closed circles represent 10% G_{M1}, and triangles represent 20% G_{M1}.

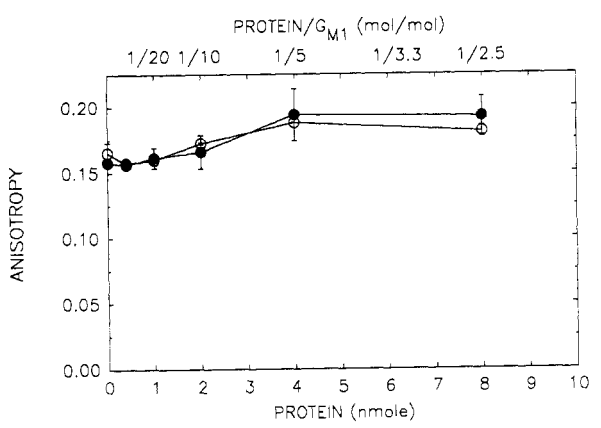

FIGURE 8: Comparison of the effect of choleragen with that of its B subunit on steady-state fluorescence anisotropy of *N-trans*-parinaroyl G_{M1}. Liposomes containing 380 nmol of DEPC, 20 nmol of G_{M1}, and 1 nmol of tPn G_{M1} were prepared as described under Experimental Procedures. Indicated amounts of choleragen or B subunit were added, and the samples were incubated at 30 °C for 2 min. Fluorescence and steady-state fluorescence anisotropy were determined as described under Experimental Procedures. Data shown are means of triplicate determinations; error bars indicate standard errors. Open circles represent choleragen; solid circles represent B subunit (choleragenoid).

similar to those obtained when intact choleragen was used. This suggests the effect of choleragen on Pn G_{M1} mainly resulted from the interaction of its B subunits with G_{M1}; this is also consistent with previous experiments using natural G_{M1} (Fishman et al., 1978; Moss et al., 1977).

Discussion

Gangliosides are specialized cell surface lipids and have more variable and complex structures than other cell surface lipids. Each molecule of ganglioside consists of a fatty acid residue, a sphingosine, and an oligosaccharide that is much larger than the head group of most phospholipids. One or more residues in the oligosaccharide are sialic acid; this sugar has a negative charge. Additionally, the sphingosine backbone has a free hydroxyl group that can serve as a hydrogen-bond acceptor or donor. These characteristics of ganglioside structure make synthesis of defined molecular species somewhat difficult. Other laboratories have synthesized ganglioside derivatives with paramagnetic and fluorescent probes or uniform fatty acid residues (Spiegel, 1985; Sonnino et al., 1985; Acquotti et al., 1986; Schwarzmann & Sandhoff, 1987; Spiegel, 1987). However, the fluorescent G_{M1} analogues synthesized have not, with the exception of the rhodamine-labeled and Lucifer Yellow labeled species described by Spiegel (1987), been characterized as functional receptors for choleragen (Spiegel, 1985). These fluorescent analogues have highly modified head groups, containing rhodamine or Lucifer Yellow dye covalently linked to the sialic acid. Despite many previous papers [e.g., Cuatrecasas (1973) and Fishman et al. (1979)] that demonstrated that slight modifications of the head-group structure of G_{M1} are sufficient to drastically decrease the affinity of the GSL for choleragen, these authors found that such highly modified G_{M1} analogues are functional choleragen receptors when they are incorporated into rat C6 glioma cell membranes. Since the probe preparations used in these experiments have been shown to contain up to 12% unreacted native ganglioside (Spiegel, 1987), an equally likely explanation for these results is that the appearance of toxin sensitivity in these cells is due to the interaction of toxin with unmodified nonfluorescent G_{M1}. This is made more likely by the observation that very small (undetectable by TLC) amounts of ganglioside G_{M1} are sufficient to sensitize rat adipocytes to the action of choleragen (Pacuszka et al., 1978).

Retention of the biological properties of natural G_{M1} is critical for future applications of G_{M1} probes to the investigation of ganglioside structure and function. We selected the binding of choleragen to G_{M1} as a functional test of our Pn G_{M1} probe for two reasons. First, choleragen binds to G_{M1} with a high affinity and high specificity, which makes choleragen binding a sensitive assay for functional G_{M1}. Second, the function of G_{M1} as a plasma membrane receptor for choleragen has been studied more than any other biological function of G_{M1}. By monitoring steady-state fluorescence anisotropy, we showed that Pn G_{M1} was specifically bound by choleragen and that the stoichiometry of the interaction between choleragen and G_{M1} was 1:5. These results indicate that Pn G_{M1} retains the biological activity of natural G_{M1} and that the molecular behavior of Pn G_{M1} is detectable by steady-state fluorescence anisotropy. In addition, the results imply that Pn G_{M1} can correctly reflect the molecular behavior of natural G_{M1}.

Analysis of the anisotropy of Pn G_{M1} also allows us to ascertain some details about the molecular motion of G_{M1} during its interaction with choleragen. The binding of choleragen to Pn G_{M1} increased the anisotropy of Pn G_{M1} without changing the anisotropy of other phospholipid or glycosphingolipid probes under the same conditions. This implies that the motion of G_{M1} was decreased without disturbing other lipids and suggests that the rotational motion of G_{M1} bound to choleragen is different from that of other lipids not bound to choleragen. Choleragen has also been shown to bind to the G_{M1} oligosaccharide in a stoichiometry of about 1:5 (Fishman et al., 1987; Schwarzmann et al., 1987), suggesting that the oligosaccharide is the main site interacting with choleragen. The fluorophore of Pn G_{M1} is deeply embedded in the central hydrophobic region of the membrane. Thus, one might ask how the interaction between choleragen and the oligosaccharide part of G_{M1} affects the motion of its hydrocarbon chain. Previous studies have suggested that the thermotropic behavior of gangliosides is markedly influenced by the properties and composition of their oligosaccharides and is less affected by their hydrocarbon chains (Maggio et al., 1981, 1985, 1986). However, intermolecular hydrogen bonds be-

tween adjacent GSL may be one of the most important factors (Pascher, 1976; Boggs, 1980). Perhaps the interaction of choleragen with G_{M1} concentrates G_{M1} under the choleragen and changes the intermolecular organization of G_{M1}, enhancing hydrogen bonding between G_{M1} molecules so as to decrease the rotational motion of the molecules. Previous work [reviewed by Thompson and Tillack (1985)] has indicated that ganglioside G_{M1} is miscible with phosphatidylcholine in fluid-phase bilayers, implying that the increased order associated with hydrogen bonding between the ganglioside molecules should not be evident. Binding of choleragen to a single ganglioside molecule, followed by recruitment of additional ganglioside molecules from the fluid bilayer, might then be followed by increased local order (due to interganglioside hydrogen bonding) in these small clusters. The increased anisotropy observed (Figures 5–8) thus would reflect increased restriction of the motion of the parinaroyl chain in these small clusters.

Other possible explanations for the observed changes in anisotropy could include self-quenching of the Pn G_{M1} fluorescence in the clusters. This effect should be eliminated by the use of the low probe/lipid ratios used in the experiments described. Furthermore, in all experiments the fluorescence intensity routinely increased or remained constant after choleragen was added. Another possibility might be that the Pn G_{M1} was only associated with the unlabeled ganglioside and that the choleragen did not bind directly to the fluorescent analogues. Both the stoichiometry of binding (Figures 6–8) and the fact that anisotropy changes were observed with the ganglioside probe only (Figure 5) would argue against this possibility.

Our data show that tPn G_{M1} and cPn G_{M1} had similar anisotropies after they were saturated by choleragen. This is in contrast to the difference in anisotropy between the two probes in the absence of choleragen. Steady-state fluorescence anisotropy connotes restriction in rotation of fluorophores. One hypothesis consistent with our data is that the angle of rotation of cPn G_{M1} is greater than the angle of rotation of tPn G_{M1} in the absence of choleragen. Thus, the anisotropy of the tPn G_{M1} probes is greater. Choleragen binding then restricts the angle of rotation of cPn G_{M1} more than it restricts the angle of rotation of tPn G_{M1}. Alternatively, choleragen may affect the rate of rotation or the fluorescence lifetime of the two probes differentially. Further experiments are needed to explain this phenomenon.

In summary, it can be concluded that Pn G_{M1} not only has properties similar to those of natural G_{M1} but also can be used to elucidate the molecular behavior of G_{M1} when it is functioning as a receptor. Specifically, Pn G_{M1} will reflect the molecular behavior of natural G_{M1}, unlike other probes such as diphenylhexatriene or free parinaric acid. On the basis of overlap between the excitation wavelength region of the parinaroyl group and the emission wavelength region of tryptophan residues, Pn G_{M1} could be a very good probe for the study of protein/ganglioside interaction through resonance energy transfer. It is also probable that Pn G_{M1} will be useful for studying the molecular basis of other biological functions of gangliosides, such as modulation of growth control (Bremer et al., 1986; Hanai et al., 1988) and other cell/cell interactions.

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Kinetics of [^{3}H]Muscimol Binding to the $GABA_A$ Receptor in Bovine Brain Membranes†

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ABSTRACT: The binding of the GABA receptor agonist [^{3}H]muscimol to membrane preparations from bovine cerebral cortex has been investigated in equilibrium and kinetic experiments. Equilibrium binding curves are biphasic and suggest that [^{3}H]muscimol binds to both high-affinity (K_d approximately 10 nM) and low-affinity (K_d approximately 0.5 μM) sites. Binding to each class of sites is inhibited by GABA and by the specific $GABA_A$ receptor antagonist bicuculline. The kinetics of [^{3}H]muscimol binding have been measured by using both manual filtration assays and an automated rapid filtration technique which permits the measurement of ligand dissociation on subsecond time scales. Association and dissociation curves are biphasic at all concentrations of [^{3}H]muscimol studied, even under conditions of low receptor saturation when no significant occupancy of the low-affinity sites would be expected. These results cannot be simply explained by the presence of two populations of binding sites in the membrane preparations but suggest the existence of two forms of the monoliganded receptor. Dissociation constants for these two forms have been estimated to be 16 and 82 nM at 23 °C. At higher ligand concentrations, kinetic measurements have suggested that the binding of [^{3}H]muscimol to low-affinity sites is accompanied by a slow conformational change of the receptor–ligand complex.

γ-Aminobutyric acid (GABA)[1] is a major inhibitory neurotransmitter in the central nervous system, and the response to its binding to the $GABA_A$ receptor is a hyperpolarization of the postsynaptic membrane resulting from an increased permeability to chloride ions (Curtis & Johnson, 1974; Krnjevic, 1974). In addition to its importance in neurotransmission, the $GABA_A$ receptor is also the target for a number of clinically important drugs including the benzodiazepines and barbiturates [reviewed in Haefely et al. (1979), Tallman et al. (1980), Olsen (1982a), and Squires (1988)]. There is therefore considerable interest in the study of the ligand binding properties of the GABA receptor and attempts to correlate these binding characteristics with effects on receptor function.

There have been many studies of the binding of radioactive GABA and its analogues to membrane preparations from mammalian brain (Enna & Snyder, 1975, 1977; Beaumont et al., 1978; Wang et al., 1979; Olsen et al., 1981; Browner et al., 1981; Skerritt et al., 1982; Supavilai et al., 1982; Olsen & Snowman, 1982, 1983; Burch et al., 1983; Yang & Olsen, 1987). At equilibrium, a heterogeneity in the binding of GABA analogues has been observed, and models involving multiple independent sites (Wang et al., 1979; Olsen et al., 1981; Browner et al., 1981; Burch et al., 1983) or interconvertible states of a single site (Olsen et al., 1981; Olsen & Snowman, 1982, 1983; Burch et al., 1983) have been proposed. In these studies, the higher affinity component of GABA binding was characterized by a dissociation constant of 10–20 nM with one or more lower affinity components being observed in the range of 100 nM–1 μM (Olsen, 1982b; Falch & Krogsgaard-Larsen, 1982). It has not been unambiguously demonstrated that the high- and low-affinity states arise from the binding of agonist to one type of receptor protein. There is strong evidence for receptor heterogeneity, and recently, cDNAs encoding three distinct $GABA_A$ receptor α-subunits have been isolated from bovine brain (Schofield et al., 1987; Levitan et al., 1988). These α-subunits are differentially expressed in the central nervous system, and, when expressed with the β-subunit in *Xenopus* oocytes, they produced receptor subtypes which could be distinguished by their sensitivity to GABA (Levitan et al., 1988). Recently, Fuchs et al. (1988) have also reported the isolation of three α-subunits from rat brain which could be distinguished by their mobility on SDS–polyacrylamide gels. The observed heterogeneity in

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; GABA, γ-aminobutyric acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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