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Membrane-bound neuraminidase from calf brain: Regulation of oligosialoganglioside degradation by membrane fluidity and membrane components

(lipophilic and hydrophilic substrates/inhibitors/general anesthetics/fluorescence depolarization)

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ABSTRACT **The degradation of lipophilic ganglioside G_{D1a} and hydrophilic sialyllactitol by membrane-bound neuraminidase (EC 3.2.1.18) from calf brain has been studied at substrate concentrations of 0.1 mM. Ganglioside G_{D1a} taken up by cell membranes is hydrolyzed faster by membrane-bound neuraminidase than are water-soluble substrates of the enzyme, sialyllactitol and des-G_{D1a}. Availability and enzymic breakdown of the disialoganglioside are enhanced by general anesthetics such as N_2O or halothane whereas the degradation of the hydrophilic substrate sialyllactitol is not affected or even is decreased by these agents. General anesthetics lower the microviscosity of membranes as indicated by studies of fluorescence depolarization with the indicator 1,6-diphenylhexatriene. Increased fluidity can result in higher lateral diffusion of ganglioside G_{D1a}, thus increasing its chances of presentation to, and interaction with, membrane-bound neuraminidase. Lipophilic derivatives of the disialoganglioside, gangliosides G_{M1} and G_{M2} and gangliotriaosylceramide G_{A2}, are strong inhibitors of the ganglioside degradation whereas water-soluble derivatives des-G_{M1}, des-G_{M2}, *N*-acetylneuraminic acid, and sialyllactose are not. A model is presented that suggests that the activity of membrane-bound neuraminidase on gangliosides of brain membranes is regulated by the viscosity of these membranes and their monosialoganglioside content.**

Membrane-bound enzymes act on various water-soluble and lipophilic substrates (1, 2). Many of these enzymes have been purified and characterized. The kinetics obtained with lipophilic substrates often deviate from the Michaelis–Menten type because the enzyme–substrate interaction takes place at interphases (1, 3). The properties and regulation of membrane-bound enzymes acting on lipid substrates that are components of the same membrane are as yet unknown. Their activity may well be influenced by their membranous environment. This, in turn, may influence the availability, and thus regulate the turnover, of the lipid substrates catalyzed by membrane-bound enzymes. A suitable object for studying such phenomena is the membrane-bound neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18), which is especially active in mammalian brain tissue. This enzyme is bound to various membranes of the cytocavitary system and reaches its highest specific activity in plasma membranes isolated from synaptosomes (4, 5). This enzyme cleaves terminal *N*-acylneuraminic acid (NeuAc) residues, predominantly from oligosialogangliosides, thereby degrading them to monosialogangliosides (6, 7).

Because substrates (8) and neuraminidase (4, 5) reside within the same membrane, the question arises as to the regulation of ganglioside degradation. In the studies reported here, we investigated the effect of changes in membrane fluidity and of putative enzyme inhibitors on the degradation of both lipophilic membrane-bound and water-soluble substrates by membrane-bound neuraminidase.

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EXPERIMENTAL PROCEDURES

Microsomes were prepared from fresh calf brain and washed with diluted buffers as described (9); they were stored in 10 mM Tris·HCl buffer (pH 7.2) at −20° until used. The methods for measuring the enzymic degradation of [^{3}H]ganglioside G_{D1a} (61 μCi/μmol) and [^{3}H]sialyllactitol (88 μCi/μmol) have been reported (9, 10). Measurements were performed in the absence of detergent if not otherwise stated. All enzymic incubation experiments were carried out for 1 hr at pH 4.2 which is optimal for the degradation of ganglioside G_{D1a} and sialyllactitol. Incubations in the presence of volatile agents such as halothane and ether were performed in closed incubation vials having no gas volume left, in order to avoid extensive evaporation. This resulted in stronger effects by small amounts of volatile agents. Incubations under gas pressures were performed as described (11). Microviscosity of membranes was calculated as a function of temperature from fluorescence depolarization measurements using the dye 1,6-diphenylhexatriene essentially according to the method of Shinitzky and Barenholz (12) and the microviscosimeter MV-1a from Elscint (Wiesbaden, Federal Republic of Germany).

Gangliotriaosylceramide G_{A2} (13), ganglioside G_{M2} (14, 15), ganglioside G_{M1} (16), and ganglioside G_{D1a} (9) were prepared and labeled according to published procedures. [^{3}H]des-G_{D1a} and [^{3}H]des-G_{M1} were a gift of Ziegler and Wiegandt (17). 5′-Nucleotidase, Na^+K^+-ATPase, and adenylate cyclase were measured according to published procedures (18–21).

For binding studies, ^{3}H-labeled lipids (G_{D1a} and G_{M2}) or lipid derivatives (des-G_{M1}, des-G_{D1a}, and sialyllactitol) (10 nmol) and 200 μg of microsomal protein were incubated together with 1 μmol of Tris·HCl buffer (pH 7.2) or 10 μmol of acetate buffer (pH 4.2) in a volume of 100 μl for 30 min at 37°. The mixtures were cooled to 4°, resuspended with 900 μl of water, and centrifuged for 30 min at 100,000 × *g*. Radioactivity of pellets and supernatants was measured in a liquid scintillation counter.

Abbreviations: NeuAc, N-acetylneuraminic acid; des-G_{M1} (monosialo-gangliotetraitol); Galβ1→3GalNAcβ1→4Gal[3←2αNeuAc]-β1→4[^{3}H]sorbitol; des-G_{D1a} (disialo-gangliotetraitol), Gal[3←-2αNeuAc]β1→3GalNAcβ1→4Gal[3←2αNeuAc]β1→4[^{3}H]sorbitol; G_{A2} (gangliotriaosylceramide, $GgOse_3$-Cer), GalNAcβ1→4Galβ1→4Glc1→1′ ceramide; G_{M1} (ganglioside II^3 NeuAc-$GgOse_4$-Cer), Galβ1→3GalNAcβ1→4Gal[3←2αNeuAc]β1→4Glcβ1→1′ ceramide; G_{M2} (ganglioside II^3 NeuAc-$GgOse_3$-Cer), GalNAcβ1→4Gal[3←2αNeuAc]β1→4Glcβ1→1′ ceramide; G_{D1a} (ganglioside IV^3 NeuAc, II^3 NeuAc-$GgOse_4$-Cer), Gal[3←2αNeuAc]-β1→3GalNAcβ1→4Gal[3←2αNeuAc]β1→4Glcβ1→1′ ceramide; sialyllactitol, NeuAcα2→3Galβ1→4[^{3}H]sorbitol.

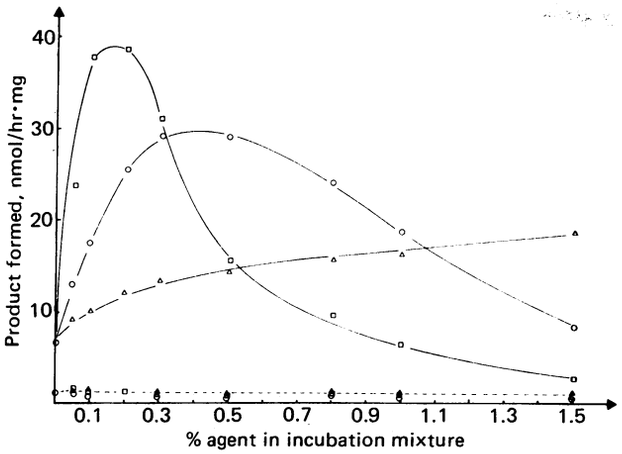

FIG. 1. Effect of increasing concentrations of different agents on the activity of neuraminidase from calf brain microsomes toward [^{3}H]ganglioside G_{D1a} (full lines) and [^{3}H]sialyllactitol (broken lines). □, Triton X-100; ○, halothane; △, ether. The 100-μl incubation mixtures contained 10 nmol of substrate ([^{3}H]ganglioside G_{D1a} or [^{3}H]sialyllactitol), 100 or 400 μg of microsomal protein, respectively, and 15 μmol of acetate buffer, pH 4.2. They were incubated at 37° for 1 hr.

RESULTS

As shown in Fig. 1, small amounts of the nonionic detergent Triton X-100, halothane, or ether stimulated the degradation of [^{3}H]ganglioside G_{D1a} but failed to increase the breakdown of sialyllactitol even at substrate concentrations of 2 mM (data not given). Similar observations have been made with the enzymic hydrolysis of endogenous gangliosides (11). The degradation of ganglioside G_{D1a} was also enhanced by bile salts at concentrations up to 5 mM (e.g., cholate, deoxycholate, taurocholate, and taurodeoxycholate) whereas other detergents such as Lubrol, Tween 20, Span 40, and dodecylamine were ineffective in this respect. Triton X-100 (2.5 mM), deoxycholate (5 mM), and taurodeoxycholate (5 mM) were also effective in extracting the enzyme from the membrane preparation (data not shown).

It has been shown (11) that general anesthetics such as xenon and N_2O (Fig. 2 *upper*) enhance the degradation of endogenous and exogenous gangliosides by membrane-bound neuraminidase. Prolonged incubations showed furthermore that availability of [^{3}H]ganglioside G_{D1a} to membrane-bound neuraminidase is increased in the presence of 30 atm. (3000 kPa) of N_2O. The ganglioside degradation increased with time up to 95% and more of the added substrate after 24 hr., whereas under standard conditions (i.e., in the absence of N_2O), only up to 30% of the added substrate was split in the same time. In the presence of general anesthetics, the enzyme was not solubilized but remained bound to the membranes. The activating effect increases with increasing pressures of the anesthetic gases (11), whereas other gases such as H_2, N_2, Ne, and He have no effect. In contrast to their stimulation of ganglioside degradation, detergents such as Triton X-100 and anesthetic gases such as N_2O failed to enhance the breakdown of sialyllactitol at concentrations of 0.1 as well as 2.0 nM. On the contrary, the degradation of the latter was inhibited up to 50% by N_2O at a pressure of 30 atm (Fig. 2 *lower*). The same type of inhibition also was observed at sialyllactitol concentrations of 2 mM, indicating that the activity of the enzyme is decreased rather than stimulated under these conditions.

To determine whether a gas such as N_2O primarily affects the ganglioside micelles or the membranes, substrate and microsomes were separately preincubated under N_2O at 30 atm. Only the preincubation of membranes at pH 7.2 (Fig. 3) and pH 4.2 resulted in a stimulation of the subsequent degradation of [^{3}H]ganglioside G_{D1a} performed under standard conditions. This, in turn, could be partially reversed by degassing the membranes under reduced pressure prior to the assay.

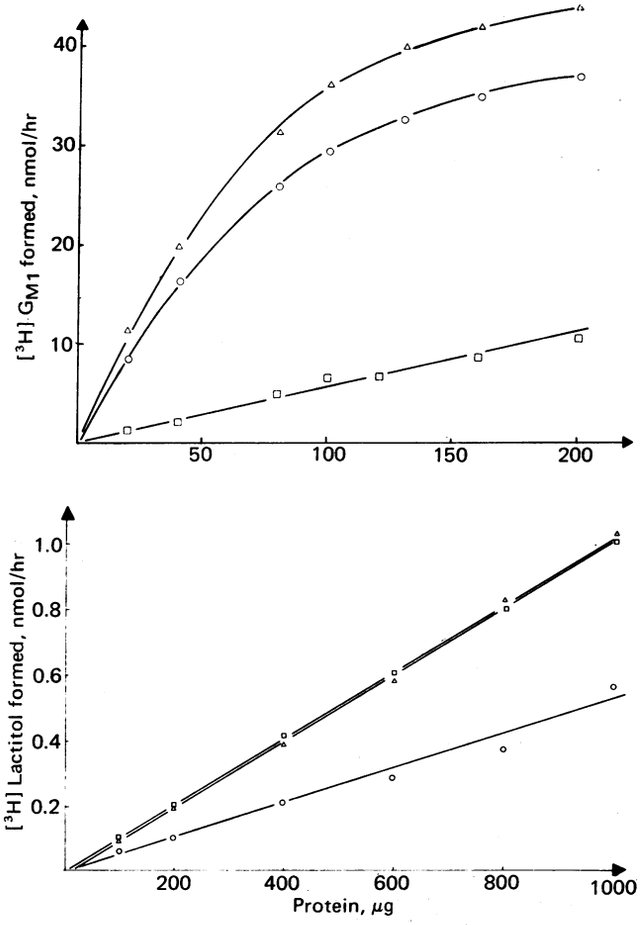

FIG. 2. Effect of Triton X-100 and nitrous oxide on the activity of increasing amounts of neuraminidase from calf brain microsomes toward [^{3}H]ganglioside G_{D1a} (*Upper*) and [^{3}H]sialyllactitol (*Lower*). Incubations were performed as described in the legend of Fig. 1 but with varying amounts of microsomal protein. □, No additions; △, 0.2% Triton X-100; ○, N_2O at 30 atm (3000 kPa).

Under standard conditions with substrate concentrations of 0.1 mM, at which still no substrate inhibition is observed, the lipophilic ganglioside G_{D1a} was cleaved about 7 times faster than sialyllactitol (Table 1) and des-G_{D1a}. Accordingly, the apparent Michaelis–Menten constant was found to be higher for sialyllactitol (1.3 mM) than for ganglioside G_{D1a} (0.1 mM). As shown in Table 1 and Fig. 4, hydrophobic degradation products of ganglioside G_{D1a} such as the gangliosides G_{M1} and G_{M2} strongly inhibited the hydrolysis of both substrates. Similar inhibition also was observed for sialyllactitol at a substrate concentration of 2 mM, whereas water-soluble substances, such as *N*-acetylneuraminic acid and des-G_{M1}, exhibited almost no effect at a comparable concentration. The ganglioside degradation was not inhibited by sialic acid up to a concentration of 50 mM, whereas the hydrolysis rate of sialyllactitol decreased to 5% under these conditions; half-maximal inhibition occurred at 1 mM. On the other hand, gangliotriaosylceramide G_{A2}, the sialic acid-free residue of ganglioside G_{M2}, inhibited only the breakdown of ganglioside G_{D1a}.

The results presented in Table 1 indicate that the interaction

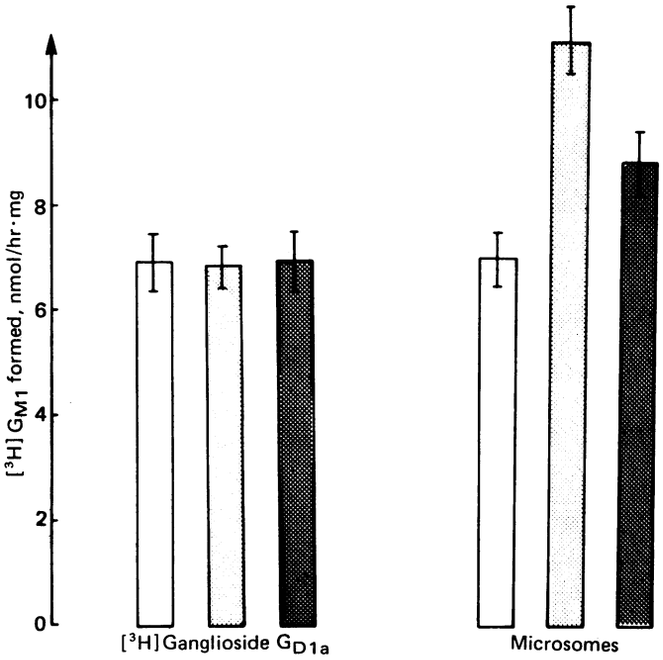

FIG. 3. Preincubation of [^{3}H]ganglioside G_{D1a} and microsomes under N_2O. [^{3}H]Ganglioside G_{D1a} (10 nmol) or microsomes (100 μg of protein) were incubated at 0° under 30 atm of N_2O in 10 mM Tris buffer (pH 7.2) for 10 min. Pressure was released and the preincubated material was used for an assay of neuraminidase activity (9) at pH 4.2 in the absence of detergent. □, Open bars, preincubation without N_2O; □, light stippled bars, preincubation under 30 atm of N_2O; □, dark stippled bars, preincubation under 30 atm of N_2O followed by degassing under reduced pressure (2 mm Hg) for 10 min at 0° prior to the enzyme incubation.

between substrates and membrane-bound neuraminidase is stronger for lipophilic than for hydrophilic compounds. This is in agreement with binding studies that showed that lipophilic ganglioside G_{D1a} at a concentration of 0.1 mM, as used in the enzyme assay, and also G_{M2} are bound to the membranes by more than 95% whereas water-soluble compounds such as sialyllactitol and the ceramide-free derivatives of ganglioside G_{D1a} and G_{M1}, des-G_{D1a} and des-G_{M1}, are not. The binding of lipophilic substrates and inhibitors of membrane-bound neuraminidase should result in an increased concentration of these substances in the membranes compared to water-soluble compounds.

Studies of fluorescence depolarization indicated that the microviscosity of liposomes and biological membranes is lowered by general anesthetics (Fig. 5). Fluorescence depolarization

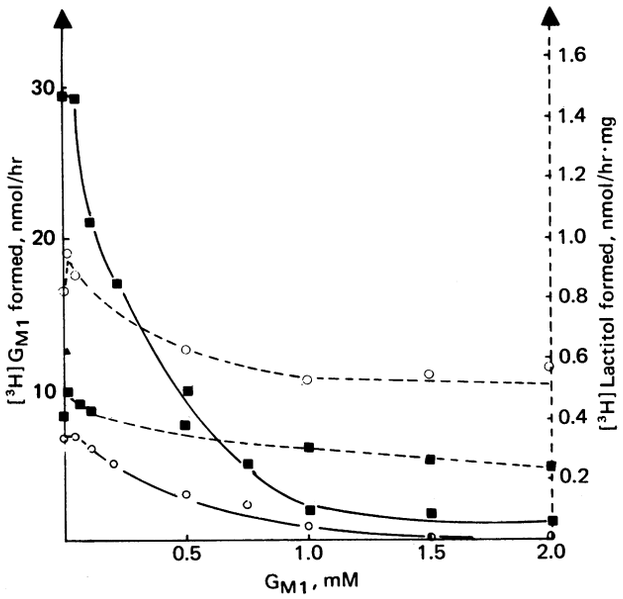

FIG. 4. Inhibition of membrane-bound neuraminidase by monosialoganglioside G_{M1}. The 100-μl incubation mixture contained 10 nmol of substrate [[^{3}H]ganglioside G_{D1a} (solid lines) or [^{3}H]sialyllactitol (broken lines)], 100 or 400 μg of microsomal protein, respectively, and 15 μmol of acetate buffer, pH 4.2. The mixtures were incubated at 37° for 1 hr under standard conditions (open symbols) and under 30 atm of N_2O (solid symbols).

in sphingomyelin liposomes and microsomes prepared from calf brain was apparent in the presence of 0.05% halothane and increased with higher concentrations of this agent. Preliminary observations suggest that this is also the case in the presence of N_2O. The increased membrane fluidity produced by lipid-soluble anesthetics should enhance the lateral diffusion of ganglioside G_{D1a} and thus its interaction with the membrane-bound enzyme. This is in agreement with the observation that higher concentrations of ether are needed to increase membrane fluidity as well as the degradation rate of ganglioside G_{D1a}.

DISCUSSION

The data obtained so far lead us to assume that the lipid-soluble ganglioside G_{D1a} first binds to the membranes, where it is concentrated, and then interacts with the membrane-bound enzyme (Fig. 6). However, at the moment it cannot be decided whether the lipophilic substances are also inserted into the

Table 1. Activity of membrane-bound neuraminidase in the presence of various inhibitors

Inhibitor, 0.6 mM	[^{3}H]Ganglioside G_{D1a} as substrate*		[^{3}H]Sialyllactitol as substrate†	
	Standard condition	Under N_2O (30 atm)	Standard condition	Under N_2O (30 atm)
None	6.8	29.6	0.85	0.42
NeuAc	6.8	29.6	0.7	0.48
Des-G_{M1}	6.8	28.5	1.0	0.38
Sialyllactose	6.5	29.6	—	—
G_{A2}	5.4	22.2	1.0	0.48
G_{M2}	3.9	15.9	0.66	0.41
G_{M1}	2.3	7.2	0.61	0.35
G_{D1a}	—	—	0.55	0.22

Mixtures as specified in the legend to Fig. 1 (substrate, 0.1 mM) were incubated in the presence of the inhibitors and assayed (9, 10) in the absence of detergents.

* Values shown are [^{3}H]G_{M1} formed, nmol/hr·mg; SD = 5%.

† Values shown are [^{3}H]lactitol formed, nmol/hr·mg; SD = 3%.

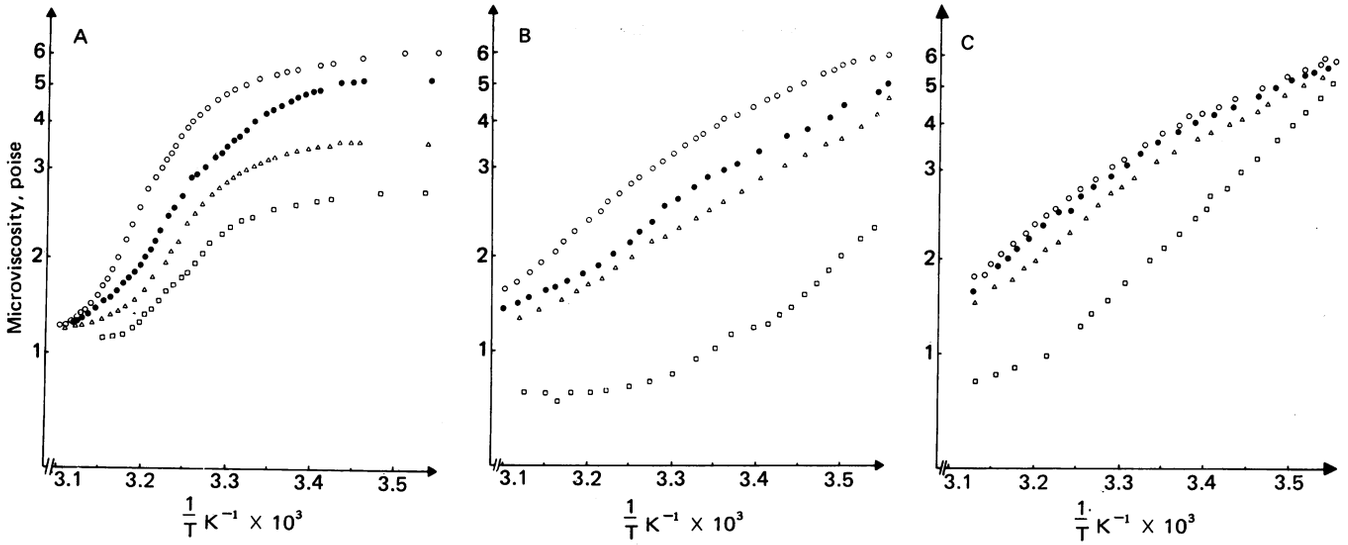

FIG. 5. Microviscosity in liposomes of sphingomyelin (*A*) and in calf brain microsomes (*B* and *C*). Microviscosity was calculated from fluorescence depolarization of 1,6-diphenylhexatriene (12). (*A*) The mixtures contained 1 mg of sphingomyelin and 2 nmol of 1,6-diphenylhexatriene in 2 ml of 10 mM phosphate buffer, pH 7.2. ○, No additions; ●, plus 0.05% halothane; △, plus 0.08% halothane; □, plus 0.10% halothane. (*B*) The mixtures contained 1 mg of microsomal protein and 2 nmol of 1,6-diphenylhexatriene in 2 ml of 10 mM phosphate buffer, pH 7.2. ○, No additions; ●, plus 0.05% halothane; △, plus 0.10% halothane; □, plus 0.30% halothane. (*C*) The mixtures contained 1 mg of microsomal protein and 2 nmol of 1,6-diphenylhexatriene in 2 ml of 10 mM phosphate buffer, pH 7.2. ○, No additions; ●, plus 0.10% ether; △, plus 1.0% ether; □, plus 3.0% ether.

membrane. Uptake of monosialogangliosides G_{M1} and G_{M2} and concentration of these compounds within the membranes may also explain why these lipophilic substances inhibit the enzyme more strongly than do water-soluble derivatives. Furthermore, the uptake of disialoganglioside G_{D1a} by and its concentration in the membranes may explain why its apparent K_m value is by far lower than that for sialyllactitol. Furthermore, this is compatible with the inhibitory effect of the sialic acid-free gangliotriaosylceramide G_{A2} on the hydrolysis of ganglioside G_{D1a}, if one assumes the gangliotriaosylceramide is also inserted

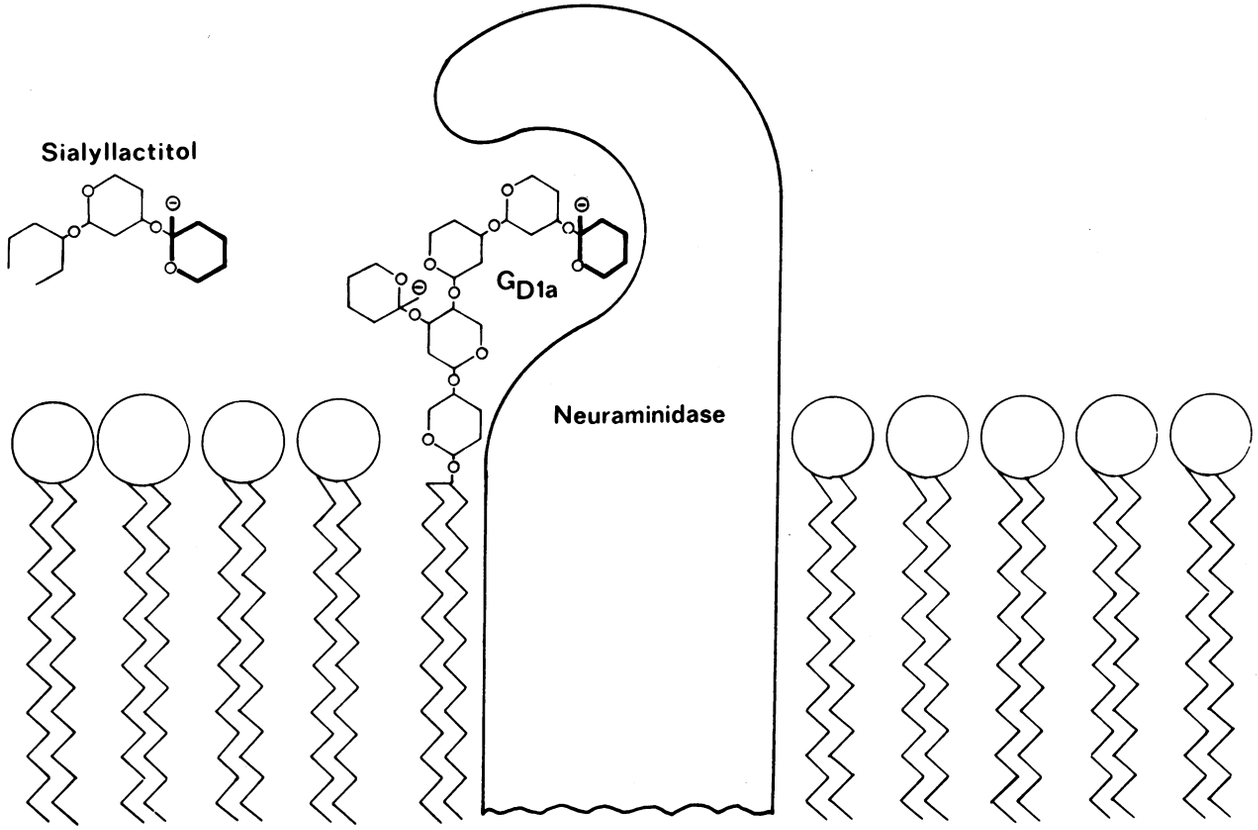

FIG. 6. Proposed model for the interaction of hydrophilic sialyllactitol and lipophilic disialoganglioside G_{D1a} with membrane-bound neuraminidase.

into the membranes. Here, the neutral glycosphingolipid may compete with ganglioside G_{D1a} for a hydrophobic binding site of the membrane-bound neuraminidase. An analysis according to Lineweaver and Burk reveals a competitive type of inhibition. However, because gangliotriaosylceramide G_{A2} lacks sialic acid, it would not be bound to the active center of the enzyme and therefore would fail to inhibit the degradation of the hydrophilic sialyllactitol. At this moment, however, an alternative explanation—e.g., the existence of isoenzymes of membrane-bound neuraminidase—cannot be ruled out. On the other hand, a competitive inhibition of the hydrolysis of sialyllactitol by NeuAc is expected. That NeuAc up to 50 mM does not inhibit the G_{D1a} degradation may again be explained by the concentration of the ganglioside in the membrane and an additional hydrophobic interaction between ganglioside and enzyme.

In contrast to sialyllactitol, which remains in the water phase of the incubation mixture, the degradation rate with the lipophilic ganglioside G_{D1a} should be highly dependent on the microviscosity of the membranes, which is affected by general anesthetics. An increased membrane fluidity should enhance the lateral diffusion of ganglioside G_{D1a} and thus its presentation to and interaction with the membrane-bound enzyme.

The data obtained with sialyllactitol as substrate in the presence of N_2O indicate that the neuraminidase is inhibited under these conditions. A similar decrease of activity, up to 40% of membrane-bound enzymes acting on water-soluble substrates, was observed with 5′-nucleotidase, Na^+,K^+-ATPase and adenylate cyclase. It has been shown that xenon is inserted into hydrophobic areas of proteins (22) which may result in an inhibition of their catalytic activity. On the other hand, membrane fluidity is increased by lipid-soluble anesthetics (Fig. 5; refs. 23–25) which, in turn, enhance the lateral diffusion of ganglioside G_{D1a} within the membrane and thus the availability of the membrane-based substrate to the membrane-bound neuraminidase. The increased lateral diffusion of the lipophilic substrate within the membrane under these conditions may well overcompensate the partial enzyme inactivation, giving rise to the observed stimulation of ganglioside degradation. Therefore, it is assumed that the degradation of oligosialogangliosides by membrane-bound neuraminidase in brain membranes is regulated by the viscosity of these membranes and their monosialoganglioside content. The activity of this enzyme, in turn, should be involved in the control of membrane structure and function. It can be expected that similar mechanisms control the turnover of other lipophilic membrane components as far as it is catalyzed by membrane-bound enzymes. The study of these phenomena of membrane enzymology should yield a better understanding of membrane structure and of the turnover and function of membrane components.

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