Sterols and Sphingolipids Strongly Affect the Growth of Fusion Pores Induced by the Hemagglutinin of Influenza Virus†

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ABSTRACT: Cells expressing the hemagglutinin (HA) of influenza virus were fused to planar phospholipid bilayer membranes to evaluate the effects of sterols and sphingolipids in the target bilayer membranes on properties of fusion pores. Typically, in the absence of sterol, flickering pores are observed, followed by a successful pore (i.e., a pore that fully opens). The incorporation of cholesterol into the lipid bilayer had a marked effect: it greatly decreased the number of flickers, and the first pore formed was usually successful. Similar effects were produced by the sterols epicholesterol and 5β-cholestanol. In contrast, the sterols cholesteryl acetate, coprostanol, and stanolone did not affect pore flickering, and a successful pore was observed to follow the typical number of flickers. 5α-cholestanol gave intermediate results. From these results, it follows that the 3-OH of cholesterol is essential to reduce flickering, but it does not matter if the 3-OH is in an α or β configuration. The double bond is also not critical for the actions of cholesterol nor is the fact that it is a flat molecule. The sphingolipids sphingomyelin, lactosyl cerebroside, and glucosyl cerebroside tended to inhibit full pore enlargement, prolonging the stage of pore flickering. If a sphingolipid and a sterol that strongly interact were both included in the planar membrane, the pattern of flickering was the same as if neither had been included in the bilayer. However, if a sphingolipid and sterol that do not interact with each other were included in the bilayer, the reduced flickering characteristic of the sterol was observed.

A fusion pore is the object that connects two fusing membranes. Relative to the size of the membranes they connect, pores are small and dynamic, and thus it is difficult to determine their structure. Lipid composition is a factor in pore growth (*1*), probably because lipid is a component of the pore wall (*2*). Thus, varying lipid composition affords the opportunity to investigate how lipid−lipid and lipid−protein interactions within the confines of the pore affect pore properties. But fusion pores are usually studied in cellular systems where lipid composition cannot be conveniently varied. Consequently, lipid composition has not been extensively varied, and relatively little is known about how molecular aspects of lipids might contribute to pore properties.

Cholesterol can constitute as much as one-half of the lipids of a plasma membrane (*3*). It is therefore important to determine if and how this biologically abundant membrane component affects the fusion process. In the case of Semliki Forest virus, the presence of cholesterol in the target membrane is absolutely critical for fusion (*4*): cholesterol is required for low pH, the trigger for fusion, to induce conformational changes in the fusion protein (*5*, *6*), but once fusion occurs, the effect of cholesterol on the pore, if any, is not known. For fusion between influenza virus and liposomes, the inclusion of cholesterol has little effect on the extent or rate of fusion (*7*, *8*), but any effects on the fusion pore itself would escape detection in such systems.

Cholesterol can complex with sphingolipids, forming small domains into which proteins may preferentially partition (9−12). Biochemical assays indicate that the fusion protein of influenza virus, hemagglutinin (HA),[1] preferentially partitions into such domains located in HA-expressing membranes (*13*, *14*). Depleting HA-expressing cells of cholesterol or mutating HA so that HA no longer resides in these domains does not affect fusion (*15*), but it is not known whether the presence of such domains in target membranes has any effect on any aspect of fusion.

In this study, we fuse cells that express HA to planar lipid bilayer membranes to explore the effects of sterols and sphingolipids on properties of fusion pores induced by HA. This system has the virtue that pore conductances can be measured with electrical sensitivity and the lipid composition of the target planar membranes can be systematically varied. We found that cholesterol virtually abolishes pore flickering, with the first pore fully enlarging. We thus substituted other sterols for cholesterol to determine the chemical features that a sterol must possess to affect pore flickering. In contrast to cholesterol, sphingolipids can cause extended pore flickering and hinder full pore enlargement. When a sterol and a sphingolipid that strongly interact are both present, they cancel out each other's effects.

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[1] Abbreviations: AcetChol, cholesteryl acetate; Chol, cholesterol; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; EpiChol, epicholesterol; GalCer, galactosyl cerebroside; HA, hemagglutinin of influenza virus; LacCer, lactosyl cerebroside; SM, sphingomyelin.

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EXPERIMENTAL PROCEDURES

Preparation of Cells and Formation of Planar Membranes. HAb2 cells, NIH-3T3 fibroblasts constitutively expressing HA (A/Japan/305/57 strain of influenza virus), were provided by J. M. White (University of Virginia, Charlottesville, VA) and maintained as previously described (*16*). Solvent-free horizontal planar bilayer lipid membranes were formed from a mixture of dioleoylphosphatidylcholine (DOPC)/dioleoylphosphatidylethanolamine (DOPE) (Avanti Polar Lipids Inc., Alabaster, AL) 2:1 (mol/mol) and 5 mol % of the ganglioside Gd1a (Sigma Chemical Co., St. Louis, MO) dissolved in squalene (ICN Biomedicals Inc., Aurora, Ohio) that had been purified by passing it through an activated alumina column. We refer to this lipid mixture as the "base". When indicated, 25 mol % of a sterol (Steraloids, Inc., Newport, RI) and/or 10 mol % of a sphingolipid (Avanti Polar Lipids) was included in the membrane-forming solution. The final lipid concentration was 40 mg of lipid/mL of squalene. Bilayer membranes were formed within a 150-μm diameter hole in a Teflon film bathed by a symmetrical solution of 140 mM NaCl, 2.5 mM KCl, 5 mM MgCl, 2 mM $CaCl_2$, and 1 mM HEPES, pH 7.4, maintained at 35−37 °C in a chamber mounted on an inverted microscope (Nikon Diaphot, Garden City, NY).

Fusion Pore Measurements. For fusion experiments, cells were removed from culture dishes, enzymatically treated, and suspended in phosphate buffered saline at $\sim 10^7$ cells/mL as described (*1*). Ten microliters of the concentrated cell suspension was added above the planar membrane, and the cells spontaneously sedimented to rest, within 1 min, upon the lipid membrane. Four minutes after adding the cells, fusion was triggered by lowering the pH of the top, cell-containing solution to 4.9 by injecting 25 μL of an isotonic succinate buffer directly over the bilayer. Upon fusion, the cell capacitance is electrically detected to add to the capacitance of the planar bilayer through the conductance of the fusion pore. Fusion pores were thus detected by measuring the electrical admittance of the bilayer membrane (*16*). Briefly, a 1-kHz frequency sine wave voltage of 20 mV peak-to-peak was superimposed upon a DC holding potential of 20 mV and applied across the voltage-clamped bilayer. The currents that were in-phase and 90° out-of-phase with the applied sine wave voltage, as well as the DC current, were obtained in real time, with a software-based phase detector (*17*). Pore conductances were calculated offline (*16*).

Statistical Analysis of Data. The nonparametric Kolgomorov−Smirnov test was used in all cases to test whether two distributions were statistically different. Thus, although only mean values of open times (t_o, Figures 6 and 7) of flickering pores are shown (for visual clarity), to compare the effect of lipid composition the entire open time distributions were used for analysis. Similarly, only delay times until fusion (t_{delay}) are shown for most lipid mixtures, but the entire waiting time distributions of lag times (t_{lag}) until fusion were used to conclude whether kinetics were statistically different for two lipid mixtures. The delay times, t_{delay}, shown (Figure 5) were obtained by fitting the final falling phase of waiting time distributions with a single exponential and taking the intercept with the ordinate of 1 as the t_{delay} (e.g., see Figure 4). That is, t_{delay} is the "corner" of the lag time distribution. Two distributions (for either t_o or t_{delay}) were considered the same if $p > 0.1$. Whether the number of flickers before full pore enlargement was different between two lipid compositions was assessed by comparing the distributions using a two-tailed bivariate correlation analysis [Statistics Package for the Social Sciences (SPSS), Chicago, IL]. If the correlation coefficient, r, was close to 1 with $p < 0.05$, the distributions were taken to be the same; for r near 0 and $p > 0.1$, the distributions were declared to be different. For intermediate cases, an absolute judgment was not made.

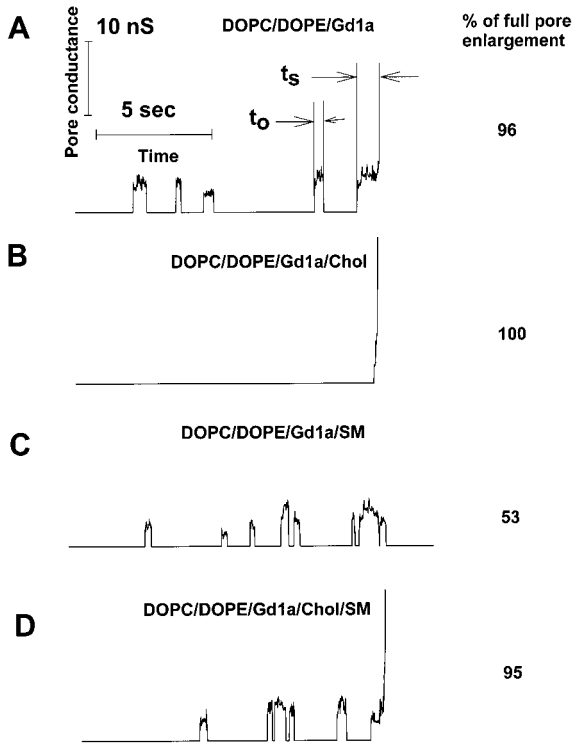

FIGURE 1: Pattern of evolution of fusion pores. (A) For the base mixture, several flickers were typically observed prior to a successful pore, which formed in 96% of the experiments. The open time of a flickering pore, t_o, and of a successful pore, t_S, are indicated. (B) When 25 mol % cholesterol was added to the base mixture, flickering was greatly reduced. The first pore was often successful. (C) Adding 10 mol % sphingomyelin to the base mixture could lead to an extended period of flickering. A successful pore only formed in 53% of these experiments. (D) Including both cholesterol and sphingomyelin in the bilayer gave responses that could not be distinguished from those with the base mixture.

RESULTS

Basic Effects of Cholesterol and Sphingolipids on Fusion Pores. HA cells were adhered to planar bilayer membranes and fusion triggered by lowering pH. At variable times after acidification, a fusion pore would form. In the absence of sterol (DOPC/DOPE/G_{D1a}, denoted "base"), fusion pores that would open and close were observed, a phenomenon known as flickering. After several flickers, a pore would irreversibly open and fully enlarge (pore conductance > 60 nS). In the case of viral infection, full enlargement must occur for release of the viral nucleocapsid into a cell's cytosol, and so we refer to all pores that fully enlarge as "successful". In our experiments, successful pores almost always occurred with the base mixture (Figure 1, panel A). If cholesterol was included in the planar membrane, fusion always occurred, but flickering was largely eliminated; the first pore tended to be successful (Figure 1, panel B). This abolishment of

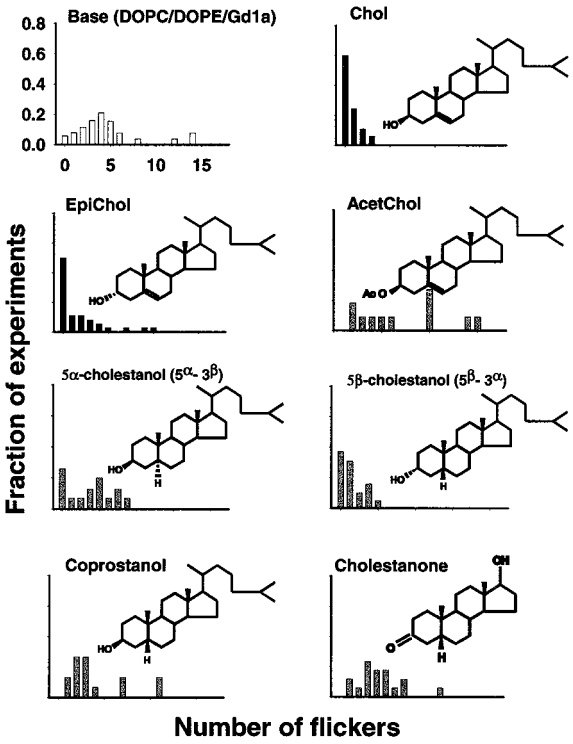

FIGURE 2: Frequency distribution for the number of flickers that occurred prior to a successful pore. These distributions are shown for the base mixture alone and when a variety of sterols were included in the bilayer at 10 mol %. Only experiments in which a successful pore was observed were used in obtaining these distributions.

flickering was observed with as little as 10 mol % cholesterol, but at this concentration the effect was not reproducible. We routinely used 25 mol % cholesterol for the present study; this concentration is in the biological range. At 25 mol %, flickering was severely and consistently reduced. Incorporating 10 mol % sphingomyelin (SM) in the base lipid mixture led to one of two results: In about one-half of the experiments, flickering was observed, and then a successful pore formed, in a manner similar to that of the base lipid mixture. That is, the SM appeared to have no effect, but in the other one-half of the experiments (Figure 1, panel C), flickering persisted for long times (up to 6 min); the experiment would be terminated after 6 min or earlier if the bilayer became leaky. When 25 mol % cholesterol and 10 mol % SM were included in the base mixture, flickering followed by a successful pore was observed in virtually all the experiments (Figure 1, panel D). The effect of adding cholesterol and SM was the same as if neither had been added.

Chemical Requirements for a Sterol to Hamper Pore Flickering. Including cholesterol in the planar membrane had such a profound effect on the open pore that we then explored which features of the four-member sterol ring and side groups were necessary to eliminate pore flickering. We performed experiments with various sterols in the planar membrane, each chosen for its slight chemical difference from cholesterol, to test the importance of particular features of cholesterol in the elimination of flickering. For every experiment, we counted the number of flickers observed before a successful pore formed and generated frequency histograms for each sterol used (Figure 2).

For the DOPC/DOPE/G_{D1a} base mixture, there were typically about four flickering pores before the successful pore. When cholesterol was included, the frequency of flickers was greatly decreased. While flickering could occur, the most frequently occurring value (i.e., the mode) for the number of flickers was zero. Cholesterol contains a 3β-OH, known to be important for many of cholesterol's physical effects on bilayer membranes (*18*). Using epicholesterol (EpiChol), which has a 3α-OH, led to a frequency of flickers statistically the same as that of cholesterol ($r = 0.79$, $p < 0.05$, where r is the correlation coefficient, and p is the two-tailed level of significance, see Experimental Procedures). On the other hand, for cholesteryl acetate (AcetChol) in the bilayer, considerable flickering ($r = 0.65$, $p < 0.05$), similar to that of the base lipid mixture occurred. Since AcetChol is identical to cholesterol except for an acetate in place of the 3-OH, the presence of the hydroxyl is probably essential to cholesterol's action on flickering, but it does not matter if that hydroxyl is in an α or β configuration.

The double bond in ring B between carbons 5 and 6 is important, but not essential to reduce flickering. 5α-cholestanol is identical to cholesterol except that it lacks the double bond (Figure 2). No flickering was a more common outcome than any other value for 5α-cholestanol, as was the case for cholesterol, but flickering did often occur, and when it did its distribution was similar to that of the base mixture. These similarities to both the cholesterol and the base mixture phenomenologies are reflected in the frequency distribution of flickers correlating with both the base mixture ($r = 0.61$, $p < 0.05$) and with the cholesterol mixture ($r = 0.66$, $p < 0.01$). 5β-cholestanol contains a 3α-OH and a 5β-H, whereas 5α-cholestanol contains a 3β-OH and a 5α-H. Because of these differences, 5α-cholestanol is structurally flat (as is cholesterol), whereas 5β-cholestanol is not. We therefore placed 5β-cholestanol in the planar membrane to examine whether flatness of the sterol was important to flickering. The resulting flickering was similar to that which occurred for cholesterol ($r = 0.91$, $p < 0.01$) and quite different from that for the base mixture ($r = 0.27$, $p > 0.1$). This emphasizes that the double bond is not essential to reduce flickering, and the flatness of the sterol is not important in the elimination of flickering. Coprostanol also does not have a double bond in ring B; its 3-OH and 5-H are in a β configuration, and consequently it is not flat. It gave flickering more reminiscent of the base mixture ($r = 0.71$, $p < 0.05$) than of cholesterol ($r = 0.47$, $p > 0.2$). Cholestanone has no double bond in ring B, no OH at the 3-carbon, and no side chain at carbon 17 and is flat. Flickering was statistically indistinguishable from the base mixture ($r = 0.91$, $p < 0.01$). The absence of 3-OH alone could account for this. Our data thus indicate that while flatness per se does not appear to be a critical factor for flickering, in the case of a flat sterol, a double bond in ring B may contribute to the elimination of flickering.

Interactions between Sterols and Sphingolipids Affect Pore Flickering. With sphingomyelin (SM) incorporated in planar bilayers, in the one-half of the experiments in which a successful pore was observed, the distribution of the number of flickers prior to formation of the successful pore (Figure 3, panel A) was comparable to the distribution of the base mixture ($r = 0.77$, $p < 0.01$). In the other half of the experiments (in which a successful pore did not form),

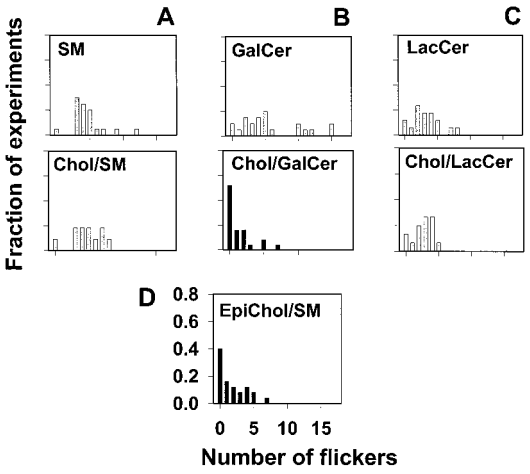

FIGURE 3: Frequency distributions for the number of flickers when sphingolipids were included alone or combined with cholesterol (or epicholesterol). (A) Sphingomyelin (SM) alone and with cholesterol (Chol/SM). (B) Galtosyl cerebroside (GalCer) without and with cholesterol (Chol/GalCer). (C) Lactosyl cerebroside (LacCer) without and with cholesterol (Chol/LacCer). (D) Epicholesterol and SM together (EpiChol/SM).

Table 1: Frequency of Successful Pores and Flicker for Different Membrane Compositions

sterol[a]	sphingolipid	percentage of full pore enlargement/ total no. of experiments	mean no. of flickers per experiment with a successful pore
		96/25	4.5
Chol		100/36	0.3
Chol	SM	95/15	4.8
Chol	GalCer	95/16	0.4
Chol	LacCer	100/16	3.9
	SM	53/15	4.9
	GalCer	65/20	6.1
	LacCer	78/18	4.3
EpiChol		100/22	0.4
EpiChol	SM	71/35	2.8
AcetChol		100/16	4.6
coprostanol		100/16	4.4
stanolone		95/17	6.1
AcetChol	SM	60/15	4.8
coprostanol	SM	62/15	5.0
α-cholestanol		90/17	3.1
β-cholestanol		100/18	1.0

[a] Sterols and sphingolipids were added to the base mixture of DOPC/DOPE/Gd1a.

flickering went on for protracted periods of times, and flickering distributions were not generated. When SM and cholesterol were included together (denoted Chol/SM) in the planar membrane (in which case a successful pore almost always formed after flickering, Table 1), the distribution of flickers (Figure 3, panel A) was similar to that for the base mixture ($r = 0.59$, $p < 0.05$). It is notable that when SM, which significantly increases flickering, and cholesterol, which significantly reduces flickering, are both added to the base mixture, their effects are canceled out.

We explored whether the addition of other sphingolipids to a cholesterol-containing bilayer would also obviate the effect of cholesterol. We tested galactosyl cerebroside (GalCer) in a planar membrane without cholesterol, and outcomes were similar to those observed with SM alone added to the base mixture: In one-third of the experiments (7 out of 20), flickering occurred over an extended time period without the full enlargement of a pore (Table 1). When full pore enlargement did occur, a broad distribution for the number of flickers (Figure 3, panel B) prior to full pore enlargement was observed, and the distribution was similar to that of the base mixture ($r = 0.65$, $p < 0.01$), but when GalCer and cholesterol were included in the bilayer (Chol/GalCer), the results were different than those for Chol/SM. The number of flickers was significantly less than for the base mixture (Table 1). Most commonly, the first pore was successful (i.e., 0 flickers); the frequency distribution of flickers was virtually the same as for cholesterol alone ($r = 0.98$, $p < 0.01$). In other words, the effects were the same as if only cholesterol was in the membrane. On the basis of data of force-area isotherms and the activity of cholesterol oxidase on monolayers containing cholesterol and sphingolipids, it is thought that cholesterol interacts more strongly with SM and with lactosyl cerebroside (LacCer) than it does with GalCer (*19*). To determine if the differences observed for SM and GalCer might be due to differences in their interactions with cholesterol, we explored the effects of LacCer on pore flickering. For 10 mol % LacCer in the planar membrane, in about 20% (4 out of 18) of the experiments, successful pores did not result (Table 1). This is similar to SM and GalCer on pore enlargement. When successful pores did form, the distribution of flickering (Figure 3, panel C) was broad and similar to the effect of SM ($r = 0.58$, $p < 0.05$) or GalCer ($r = 0.66$, $p < 0.01$) on flickering. When both LacCer and cholesterol were included in the planar membrane (Chol/LacCer), successful pores always formed after a period of flickering, and the distribution for number of flickers was broad and similar to that of Chol/SM ($r = 0.50$, $p < 0.05$). Thus, SM, which strongly interacts with cholesterol, and LacCer, which may interact, eliminates cholesterol's inhibition of flickering; GalCer, which interacts more weakly with cholesterol, does not.

Because the 3β-OH of cholesterol probably forms hydrogen bonds with the amide of SM (*20*, *21*), epicholesterol should not complex with SM as well as cholesterol, although this has not been explicitly investigated. EpiChol reduced flickering (Figure 2) and for membranes containing both EpiChol and SM, successful pores did not form in 28% (10 of 35) of the experiments (Table 1). When a successful pore did form, prior flickering was reduced as compared to the base mixture (Figure 3, panel D), with a frequency distribution of flickers the same as when only EpiChol was added to the base mixture ($r = 0.96$, $p < 0.01$). In summary, for the two mixtures in which the sterol and sphingolipid interact most strongly, flickering exhibited the pattern of the base mixture; for the two mixtures in which the sterol and sphingolipid should not strongly interact, the truncated flickering pattern of the sterol was observed. However, the ability of a pore to fully enlarge does not appear to depend simply on the strength of interaction between sterol and sphingolipid: a successful pore did not always form for EpiChol/SM but did for Chol/GalCer, and neither of these combinations strongly interact.

Gangliosides are glycosphingolipids (as are the cerebrosides), a subclass of sphingolipids. Because the other sphingolipids tested strongly affected the pattern of flickering and successful pores, one might expect that gangliosides would do so as well. In control experiments, we therefore increased the concentration of the ganglioside G_{D1a} in the base mixture from 5 to 15% and found that the pattern was not affected: a successful pore always formed and the

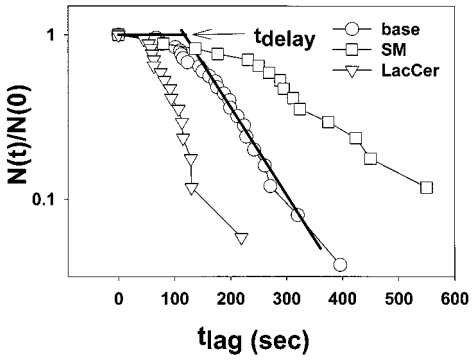

FIGURE 4: Waiting time distributions for times (t_{lag}) between acidification and the formation of the first pore are shown for the base (circles), SM (squares), and LacCer (inverted triangles). The falling phase of the distributions were fit by single exponentials, as illustrated for the base mixture. The intercept of the line of this exponential and the ordinate 1 is shown and was defined at the delay time.

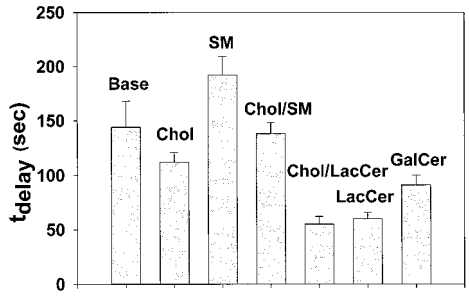

FIGURE 5: Delay times (t_{delay}) between acidification and pore formation for various lipid mixtures. Delay times were obtained as illustrated in Figure 4. The error bars indicate standard errors of the mean.

number of flickers that preceded this pore was statistically the same as for the base mixture (data not shown). Thus, the behavior of added gangliosides to the base mixture was not the same as adding other sphingolipids. This may be because gangliosides are bound to HA [i.e., they are HA receptors (*22*)] whereas the other sphingolipids are not. The effects of the various sterols and sphingolipids, alone and in combination, on the number of flickers prior to full pore enlargement and on the probability that a pore enlarges, are summarized in Table 1.

Kinetics of Formation and Characteristics of Fusion Pores. Kinetics of formation of fusion pores can be determined (*16*) from the times (t_{lag}) between acidification and the opening of the first pore of each experiment (waiting time distributions, Figure 4). We used the delay times of the entire waiting time distributions (t_{delay}, see Experimental Procedures) to characterize the effects of sterols and sphingolipids on fusion kinetics (Figures 4 and 5). Cholesterol did not affect kinetics of fusion (Figure 5); SM may have slightly slowed fusion (Figures 4 and 5), although the effect was not statistically significant. An inspection of t_{delay} for the different sterols and sphingolipids (Figure 5) shows that only LacCer significantly speeded fusion (~60 vs 140 s for base, Figures 4 and 5), independent of whether Chol was present (Figure 5). That is, the sterols and sphingolipids did not usually affect the kinetics of fusion.

In a similar manner, we characterized the kinetics of closing of flickering pores by their open time distributions (Figure 6, panel A). Of the sterols, only coprostanol had a

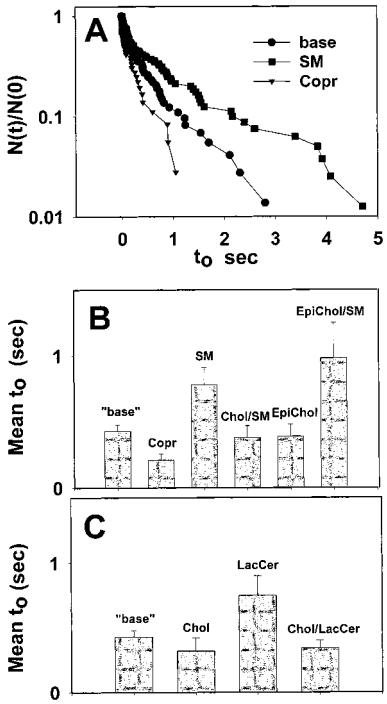

FIGURE 6: Open times of flickering pores. (A) The full distribution of open times, t_o, is given for the base mixture (circles) alone and with SM (squares) or coprostanal (inverted triangles). (B) The mean open time for the base and for various sterols alone or in combination with SM are shown. Error bars are standard errors of the mean. (C) Mean open times for membranes containing LacCer with or without cholesterol.

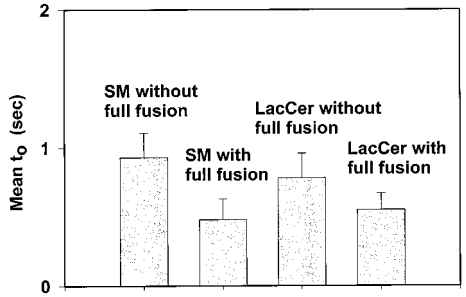

FIGURE 7: Mean open times of flickering separated for experiments according to whether a successful pore did or did not appear. Open times of flickering pores were shorter when a successful pore subsequently occurred.

statistically significant effect, shortening the open times, t_o ($p > 0.05$). In contrast, the presence of the sphingolipids SM (Figure 6, panels A and B) and LacCer (Figure 6, panel C) caused significantly longer t_o, whereas GalCer did not affect t_o (data not shown). In other words, for membranes containing SM or LacCer, pores stayed open for longer times, and therefore kinetics of closing was slower than for the base mixture. When Chol was added to the SM (Figure 6, panel B) or LacCer (Figure 6, panel C) membranes, the effect of the sphingolipid on t_o was abolished. In contrast, adding EpiChol to a SM membrane (Figure 6, panel B) did not eliminate the effect of the sphingolipid. The open times of Figure 6 were accumulated for experiments regardless of whether a successful pore ultimately occurred. Experiments were then divided into those in which a successful pore did occur versus those in which no successful pore formed (Figure 7). It became clear that the distribution of open times of flickering pores depended on whether a successful pore

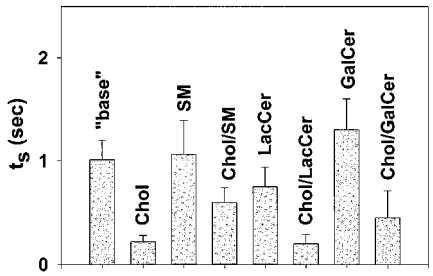

FIGURE 8: Mean times between the opening of a successful pore and its full enlargement, t_S. Comparisons between the base mixture and the base mixture plus cholesterol and/or sphingolipids, alone or in combinations.

formed in that experiment: for either SM or LacCer in the bilayer, the open times of flickering pores were greater if a successful pore did not subsequently form (Figure 7).

We also analyzed the kinetics of full pore enlargement by measuring the time, t_S, between the opening of a successful pore and its full enlargement (Figure 8). The presence of Chol greatly decreased t_S ($p > 0.05$), but none of the sphingolipids, SM, LacCer, or GalCer, significantly altered t_S (Figure 8). When cholesterol was included together with any of these sphingolipids in the planar membrane, the value of t_S was less than for base (Figure 8), although the difference was statistically different only for LacCer. In other words, even though SM and LacCer obviated the ability of cholesterol to prevent pore flickering (Figure 3), the smaller values of t_S indicate that they did not completely eliminate cholesterol's promotion of full pore enlargement. The parameters that promote the closure of a flickering pore may not be the same as those that promote full enlargement of a successful pore.

DISCUSSION

Ability of a Sterol to Reduce Pore Flickering is Graded. This is the first study to systematically investigate how properties of fusion pores can be controlled by sterols and sphingolipids. We have discovered that whether an HA-mediated fusion pore closes or fully enlarges is strongly sensitive to the presence of sterols and sphingolipids. The effect is so strong that the presence of cholesterol reduced pore flickering almost to the point of elimination. When cells expressing HA are fused to planar bilayer membranes comprised of the base mixture, pore flickering is much more pronounced than when red blood cells (RBCs) are the target (*15*, *16*). It is possible that the substantial concentration of cholesterol in RBCs (*23*) contributes to the reduced flickering in the HA−RBC system. We do not yet understand how cholesterol reduces flickering, and indeed it is not yet generally understood what causes a pore to flicker. There are at least three distinct explanations for what may be happening to the pore when it closes: As fusion pores are roughly toroidal in shape (*1*), flickering could be caused by a fissioning of the neck of the pore; if this is the case, the cell and planar bilayer would become fully separated again. Alternatively, flickering would result if fusion reverted to a hemifused state; membrane, but not aqueous, continuity would be maintained. Third, flickering could be caused by the pore lumen shrinking to an immeasurably small size; here the wall would remain with the possibility of reenlargement.

The effect of cholesterol on flickering could be through changes in a global membrane property or through local interactions with lipids residing within the wall of a fusion pore or by interacting with HA itself. In principle, one should be able to test the latter case. Cholesterol should not have the same effects on pores generated by other fusion proteins that it has on pores generated by HA if cholesterol interacts directly with HA. If the sterols and/or sphingolipids exert their effects through a change in a physical parameter, it is not clear what that parameter might be. The sterols and sphingolipids cannot have exerted their effects on pore properties through changes in spontaneous curvature: All planar membranes of this study had the same lipid composition within both monolayers so their spontaneous membrane curvature was always zero. The spontaneous curvature of the walls of a toroidal pore therefore should not have changed as a result of altered lipid composition.

Although we cannot yet determine the mechanism by which cholesterol alters flickering, we can begin to appreciate the chemical aspects of cholesterol that are critical for its actions on HA-mediated pores. We know that the 3-OH is essential for its effect because AcetChol did not affect flickering (Figure 2), but whether the hydroxyl is in the α or β configuration is irrelevant since epicholesterol and cholesterol were equally effective. The equivalence of cholesterol and epicholesterol on flickering runs contrary to many of their known differences in effects upon lipid bilayer membanes, such as cholesterol's, but not epicholesterol's, ability to cause a collapse of membrane area or reduction of membrane permeability (*18*). The double bond in a sterol's ring B contributes to the elimination of pore flickering but is not essential (Figure 2). The flatness of a sterol is also not critical to reduce flickering. Differences in reduced flickering caused by the different sterols therefore shows that the ability of a sterol to reduce flickering is not all or none but is graded, with the degree of reduction dependent upon the sterol's precise chemical structure. The incorporation of cholesterol, epicholesterol, or 5α-cholestanol into phospholipid membranes promotes a tightly packed and ordered state of the saturated lipid dipalmitoylphosphatidylcholine (*24*), and all three sterols reduced flickering, although 5α-cholestanol did so to a lesser degree than did the other two. In contrast, incorporation of coprostanol or cholestanone inhibits the formation of these domains of ordered states (*24*), and neither reduced pore flickering. The mechanism by which sterols induce the formation of domains of ordered states for the saturated lipid may thus be related to their ability to suppress pore flickering.

Interactions between Sterols and Sphingolipids. Once the effects of an individual sterol or sphingolipid upon pore properties have been determined, it can be qualitatively predicted to a considerable degree, based on a knowledge of whether the two components strongly interact with each other, how the sterol and sphingolipid in combination will affect properties of open pores. Cholesterol and SM can interact to the point of clustering into domains (*11*, *12*). This ability increases with concentration in a manner that depends on the other phospholipids present. At our 25 mol % cholesterol and 10 mol % SM in a DOPC/DOPE background, the concentrations would appear to be a little low to form complexes (*25*), although the physical chemistry of the lipid mixtures we employed have not been explicitly tested. Perhaps cholesterol and SM become concentrated as a

complex within the wall of a fusion pore. In any case, Chol and SM do tend to associate with each other. For Chol/SM and Chol/LacCer [which also form clusters at high concentrations in monolayers (*19*)], the flickering pattern of the base mixture appeared (Figure 3). In contrast, Chol/GalCer [which does not form clusters, even at high concentrations (*19*)] did not give rise to significant flickering (Figure 3). Thus, if cholesterol strongly interacts with the sphingolipid, they tend to cancel each other out. If they do not strongly interact, the effect of cholesterol dominates and the flickering is largely eliminated. Similarly, epicholesterol and SM are not expected to strongly interact (*20*, *21*), and flickering was largely eliminated for EpiChol/SM (Figure 3), but in this case, a successful pore did not always form (Table 1). Gangliosides did not exhibit the behaviors of the other glycosphingolipids. Although incorporating 5% gangliosides in the planar membranes greatly facilitated fusion, increasing the concentration to 15% did not significantly alter the properties of open pores: the number of flickers, t_o, and t_S were not affected (data not shown).

In biological cells, domains of sphingomyelin and cholesterol (referred to as "rafts") are small, less than 70 nm in diameter (*26*), but this is appreciable as compared to the area of the wall of a 2-nS pore. Thus, if clusters of sterol and sphingolipids form within the target membrane, they might not be able to freely enter the confined area of the pore wall because of their size. Only lipids that are structurally part of the pore wall can affect pore evolution. An alternate possibility is that sterols and sphingolipids may strongly interact with each other within the pore wall, and when they do so they do not interact with other components of the pore wall and thereby do not exert their individual influences. Our data indicate that prior to pore formation, a sphingolipid and sterol that strongly interact need not cancel each other out. For example, including LacCer in the planar membrane leads to fast fusion kinetics (Figure 5). Kinetics was still fast for Chol/LacCer (Figure 5).

In conclusion, we have shown that sterols and sphingolipids have marked effects on pore growth. One would expect that the larger the pore becomes, the more lipidic it must be, and therefore the more its growth is dependent on the behavior of its lipid composition. In this study, we have found that during the period that a pore maintains a relatively stable size, cholesterol and lipids, normally present in membranes, strongly influence whether the pore will close or fully open.

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