DISTRIBUTION OF CHOLESTEROL BETWEEN THE OUTER AND INNER HALVES OF THE LIPID BILAYER OF MYCOPLASMA CELL MEMBRANES

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SUMMARY: Rapid kinetic studies of filipin binding to intact cells and isolated membranes were performed with a stopped-flow apparatus to determine the distribution of cholesterol in the outer and inner surfaces of mycoplasma membranes. The initial rates of association of filipin with cholesterol in *Mycoplasma gallisepticum* and *Mycoplasma capricolum* intact cells were slower than those obtained with isolated membrane preparations. Ratios of the second-order rate constants for filipin binding to cells relative to membranes indicate that cholesterol is distributed symmetrically in membranes of *M. gallisepticum* cells whereas in *M. capricolum* ~66% of the free cholesterol is localized in the outer half of the lipid bilayer.

Mycoplasmas are useful tools for the study of the role of cholesterol in biological membranes because cholesterol is an essential component in their cell membrane (1). Mycoplasmas lack the ability to synthesize cholesterol and thus require an exogenous supply of cholesterol (1). The sterol is incorporated exclusively into the cell membrane and affects the physical state of membrane lipids, some biological properties of the cells such as osmotic fragility and permeability (2, 3), and the activity of membrane enzymes, rendering the cells susceptible to cholesterol-binding agents such as digitonin and polyene antibiotics (4, 5). Although the lipids of the mycoplasma membranes are mostly organized as a bilayer (1), no information is available

concerning the distribution of the various phospholipids and cholesterol between the outer and inner halves of the bilayer. We have previously shown that the initial rate of binding of the polyene antibiotic, filipin, to cholesterol in artificial phospholipid bilayer membranes is dependent on the accessibility, absolute concentration, and mole percent of cholesterol in the bilayer (6). In this communication we compare the initial rates of filipin binding to intact cells and isolated membranes and present data describing the distribution of cholesterol in the inner and outer halves of the lipid bilayer of two *Mycoplasma* species. This is, to our knowledge, the first report concerning cholesterol localization in the membrane of any cell capable of autonomous growth.

MATERIALS AND METHODS

Cells of *Mycoplasma gallisepticum* (strain A5969) and *Mycoplasma capricolum* (Calif. Kid) were grown at 37 °C in a modified Edward medium containing 5% horse serum. The organisms were harvested at the mid-exponential phase of growth by centrifugation at 12,000 *g* for 15 min at 4 °C and were washed once and resuspended in 10 mM sodium phosphate buffer (pH 7.2) containing 0.4 M sucrose. Membranes were prepared from a portion of the washed cell suspension by ultrasonic irradiation under nitrogen for two (*M. capricolum*) or four (*M. gallisepticum*) 15-sec periods at 0 °C in a MSE Ultrasonic Disintegrator (60 W) operated at 1.5 amp. Membrane preparations had absorbance (500 nm) values 80-90% lower than those of cell suspensions containing the equivalent membrane mass per ml. In some experiments *M. capricolum* membranes were prepared by osmotic lysis as described previously (7). Initial rates of filipin binding to membranes prepared in this manner were very similar to those of membranes obtained from sonicated cells. Membranes were collected by centrifugation at 34,000 *g* for 30 min at 4 °C. Cells and membranes were suspended in 10 mM sodium phosphate (pH 7.2) containing 0.4 M sucrose and 20 mM $MgCl_2$, treated at 37 °C for 15 min with deoxyribonuclease and ribonuclease (Sigma Chemical Co.) at a final concentration of 20 μg/ml of each nuclease, then washed and resuspended in 10 mM sodium phosphate buffer (pH 7.2) in 0.4 M sucrose and 20 mM $MgCl_2$. Total cholesterol was determined colorimetrically (8). The ratio of free to esterified cholesterol in each strain was measured by gas-liquid chromatography using a Packard Model 840 instrument equipped with a 6 ft. x 0.25 in. 3% SE-30 column. Protein concentrations were determined by the method of Lowry *et al.* (9) using bovine serum albumin as standard. Total lipid phosphorus was determined by the method of Ames after digestion of the sample with ethanolic $Mg(NO_3)_2$ (10). The molar ratios of free cholesterol to phospholipid in *M. gallisepticum* and *M. capricolum* membranes were 0.80 and 0.88, respectively.

Filipin complex (lot no. 8393-DEG-11-8, Upjohn Co., Kalamazoo, Mich.) was purified by crystallization from a concentrated slurry in chloroform

as described before (11). The purity of the preparation was ~80% as determined by comparing the molar absorbancy of the material with that of a pure sample ($5.0 \cdot 10^4$ $M^{-1}cm^{-1}$ at 338 nm). Stock solutions of the antibiotic were prepared in dimethylformamide, and aliquots were added to 10 mM sodium phosphate buffer (pH 7.2) containing 0.4 M sucrose and 20 mM $MgCl_2$. The final concentration (after mixing with cells or membranes) of filipin was 6.2 μM, except in experiments where the antibiotic concentration was varied. The final concentration of dimethylformamide was 0.3% (v/v).

Kinetic measurements of filipin binding to cells and membranes were performed at 10 °C on a stopped-flow spectrophotometer (Model D-130, Durrum Instrument Corp., Palo Alto, Calif.) operated in the absorbance mode and equipped with a Tektronix 5103N storage oscilloscope. The initial rates of change in absorbance at 358 nm, $(dA/dt)_0$, were measured from the slopes of the linear portion of the oscillogram in the time period of 150 to 400 msec after mixing of equal volumes of cells or membranes with filipin. The initial rate of disappearance of free filipin, $-(dF/dt)_0$, was computed as follows:

$$(dF/dt)_0 = (1/\varepsilon l)(dA/dt)_0$$

where ε and l are the molar absorbance of filipin at 358 nm and the pathlength of the stopped-flow cuvet (2 cm), respectively. An optical disturbance occurring in the initial *ca.* 150 msec precluded initial rate measurements at faster times. Although the origin of the initial instability has not been determined, comparable "dead" times for stopped-flow mixing and measurement of other membrane preparations have been reported (12-13). A 100% transmittance signal at 358 nm (light-to-dark voltage) corresponded to 10 V when water was present in the stopped-flow cuvet. A monochromator slit width of 1 mm was used. Kinetic studies of filipin binding to cell suspensions were conducted within about 2 hr of harvesting of the organisms. To examine the possibility that cells undergo shearing in the stopped-flow apparatus during the course of the kinetic experiment equal volumes of cells and buffer were mixed at 10 °C and absorbance changes at 358 nm were determined. With both *Mycoplasma* species tested almost no absorbance changes were detected after the initial 150-msec disturbance period had elapsed, indicating that cell shearing does not make a significant contribution to the signal corresponding to filipin-cholesterol formation. Since it is known that incubation of filipin with cholesterol-containing vesicles and cells may result in lipid reorganization and membrane disruption (5, 14, 15), we sought to minimize the possibility of filipin-induced lysis by conducting measurements at low cholesterol/filipin molar ratios, very short reaction times, and low temperature (10 °C). Indeed, when filipin was mixed in the stopped-flow apparatus with cell suspensions containing various cholesterol concentrations no absorbance changes were detected at 450 nm (a wavelength which is a sensitive indicator of cell lysis, but one where filipin does not absorb), indicating that the antibiotic did not cause lysis under the conditions used.

RESULTS AND DISCUSSION

The study of the rapid interaction of the polyene antibiotic, filipin, with cholesterol represents a novel approach to the problem of the distribution of cholesterol between the two halves of the lipid bilayer of biological membranes. The capacity to perform rapid kinetic

measurements of filipin-cholesterol complexation enables one to minimize the possibility that movement of cholesterol molecules from the inner to the outer half of the lipid bilayer ("flip-flop") would lead to an overestimate of the asymmetry ratio of outer/inner cholesterol. Indeed, if the "flip-flop" times for cholesterol measured in bilayer vesicles (16) or in influenza virus membranes (17) are characteristic of mycoplasma membranes as well, it is more than five orders of magnitude slower than the time scale used for filipin binding.

The interaction of filipin with cholesterol-containing mycoplasma cells and membranes results in absorbance changes similar to those described for filipin-cholesterol complexation in other membranes (14). No reaction was observed in the stopped-flow spectrophotometer when filipin was mixed rapidly with *Acholeplasma laidlawii* cells and membranes lacking cholesterol; furthermore, filipin does not react with cholesterol esters in aqueous suspension (18). Since the initial velocity of filipin association with vesicle-bound cholesterol was dependent on the accessibility of cholesterol for reaction at the bilayer surface (6), the rate ratio of filipin association with cells relative to membranes is a measure of cholesterol localization in the mycoplasma cell membrane. Fig. 1 shows the initial rate of association of filipin with cholesterol in *M. gallisepticum* cells and membranes. Initial rates, rather than entire reaction profiles, were determined because measurements at very short times minimize the ability of filipin to diffuse into the membrane and react with cholesterol localized in the cytoplasmic surface of the membranes. Under the conditions of a molar excess of membrane-bound cholesterol relative to antibiotic, the initial rates of filipin-binding to cells and membranes are linearly related to the concentration of free cholesterol. The order of the reaction with respect to cholesterol is obtained by plotting the logarithm of the initial rate of

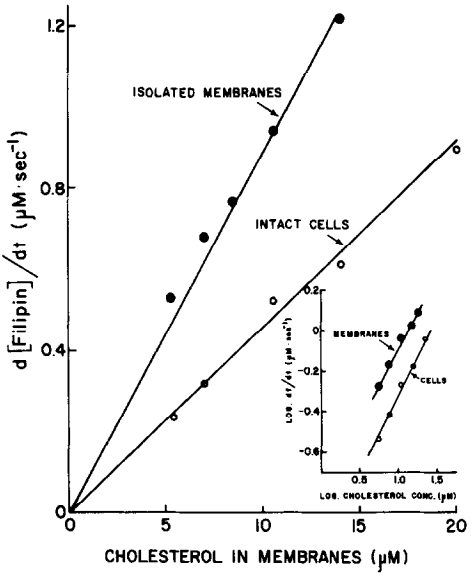

Fig. 1. Initial rate of filipin binding to *M. gallisepticum* cells (o) and membrane preparation (•) at different cholesterol concentrations. *Insert*, a plot of the logarithm of the initial rate *vs.* the logarithm of the concentration of cholesterol. The indicated cholesterol concentrations are final concentration of free cholesterol.

association *vs.* the logarithm of the cholesterol concentration, where filipin concentration remains constant. The slopes of the lines plotted in the insert to Fig. 1 are 1.0, showing that the binding reaction is first order with respect to cholesterol in both cells and membranes. To obtain the order with respect to filipin, the initial rates of association were determined at several antibiotic concentrations (2-8 μM), with the concentration of cholesterol remaining constant (10 μM). At low concentrations of antibiotic, $(dA/dt)_0$ increased linearly with filipin concentration. The slope of a log-log plot of these data is 1.0, demonstrating that the reaction is first order with respect to filipin. The rate expression for the initial velocity of disappearance of free filipin is thus $-(dF/dt)_0 = k[F_0][C_0]$, where F_0 and C_0 represent

Table 1. Second-order rate constants for filipin binding to intact cells and isolated membranes of *M. gallisepticum* and *M. capricolum*

Organism	k (liter/mole/sec)		
	cells	membranes	cell/membrane ratio
M. gallisepticum	$7.8 \cdot 10^3 \pm 0.1 \cdot 10^3$	$15.3 \cdot 10^3 \pm 0.4 \cdot 10^3$	0.51
M. capricolum	$7.5 \cdot 10^3 \pm 0.2 \cdot 10^3$	$11.4 \cdot 10^3 \pm 0.3 \cdot 10^3$	0.66

Values of k were calculated from each experimental point as described in the text. Each value of k is the mean ± the standard error of determinations made with at least five cholesterol concentrations. At each cholesterol concentration, 6 to 8 measurements of the initial rate were made.

the filipin and cholesterol concentrations at t = 0 and k is the second-order rate constant. To allow quantitative comparisons, values of k (Table 1) were calculated for each experimental point shown in Fig. 1 using the relationship $k = -(1/F_0)(1/C_0)(dF/dt)_0$. Similarly, second-order rate constants were computed for each experimental point in a study of the dependence of the initial rate on free cholesterol concentration in *M. capricolum* cells and membranes (Table 1). The ratio of $k_{cells}/k_{membranes}$ indicates that cholesterol is symmetrically distributed between the two surfaces of the cell membrane of *M. gallisepticum* (A5969). In contrast, free cholesterol appears to be asymmetrically distributed in the cell membrane of *M. capricolum* (Calif. Kid), with two-thirds of the free cholesterol localized in the external surface.

Although we cannot rigorously exclude the possibility that rearrangement of lipids occurred in the membrane upon its isolation, two experimental approaches directed to this question revealed no differences. First, the freedom of motion of the spin-labeled fatty acids in intact *A. laidlawii* and *M. hominis* cells was the same as that in membranes isolated from spin-

labeled intact cells (19). Second, phospholipase C from *Bacillus cereus* failed to hydrolyze the phospholipids of intact cells or isolated membranes of *M. hominis*, although phospholipids extracted from the cells and dispersed in aqueous suspension were susceptible to enzyme action (20). Thus, unlike the situation in red blood cells (21), no evidence is presently available that indicates the occurrence of lipid reorganization during the lysis of mycoplasma cells.

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REFERENCES

1. Razin, S. (1973) Advances in Microbial Physiology (Rose, A.H., and Tempest, D.W., eds.), Vol. 10, pp. 1-80, Academic Press, London.
2. Rottem, S., Yashouv, J., Ne'eman, Z., and Razin, S. (1973) Biochim. Biophys. Acta 323, 495-508.
3. Rottem, S., Cirillo, V.P., de Kruiff, B., Schinitzky, M., and Razin, S. (1973) Biochim. Biophys. Acta 323, 509-519.
4. Rottem, S., and Razin, S. (1972) J. Bacteriol. 110, 699-705.
5. Kinsky, S.C. (1970) Annu. Rev. Pharmacol. 10, 119-142.
6. Bittman, R., Chen, W.C., and Blau, L. (1974) Biochemistry 13, 1374-1379.
7. Rottem, S., Stein, O., and Razin, S. (1968) Arch. Biochem. Biophys. 125, 46-56.
8. Zlatkis, A., and Zak, B. (1969) Anal. Biochem. 29, 143-148.
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
10. Ames, B.N. (1966) Methods in Enzymology (Neufeld, E.F., and Ginsburg, V., eds.), Vol. 8, pp. 115-118, Academic Press, New York.
11. Whitfield, G.B., Brock, T.D., Ammann, A., Gottlieb, D., and Carter, H.E. (1955) J. Amer. Chem. Soc. 77, 4799-4801.
12. Bittman, R., and Blau, L. (1972) Biochemistry 11, 4831-4839.
13. Owen, J.D., and Eyring, E.M. (1975) J. Gen. Physiol. 66, 251-265.
14. Bittman, R., Chen, W.C., and Anderson, O.R. (1974) Biochemistry 13, 1364-1373.
15. De Kruijff, B., Gerritsen, W.J., Oerlemans, A., Demel, R.A., and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 339, 30-43.
16. Poznansky, J., and Lange, Y. (1976) Nature 259, 420-421.
17. Lenard, J., and Rothman, J.E. (1976) Proc. Natl. Acad. Sci. 73, 391-395.
18. Schroeder, F., Holland, J.F., and Bieber, L.L. (1972) Biochemistry 11, 3105-3111.
19. Rottem, S. (1975) Biochem. Biophys. Res. Commun. 64, 7-12.
20. Rottem, S., Hasin, M., and Razin, S. (1973) Biochim. Biophys. Acta 323, 520-531.
21. Zwaal, R.F.A., Roelofsen, B., and Colley, C.M. (1973) Biochim. Biophys. Acta 300, 161-174.