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Articles

Cholesterol Oxidase Catalyzed Oxidation of Cholesterol in Mixed Lipid Monolayers: Effects of Surface Pressure and Phospholipid Composition on Catalytic Activity†

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Received September 13, 1989; Revised Manuscript Received December 4, 1989

ABSTRACT: The catalytic activity of cholesterol oxidase from *Streptomyces* sp. in mixed monolayers of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), *N*-oleoylsphingomyelin (O-SPM), and cholesterol (CHL) has been determined at lateral surface pressures between 10 and 30 mN/m. The highest cholesterol oxidase activity (determined at 37 °C) was observed at surface pressures around 20 mN/m in a POPC/CHL monolayer (50:50 mol %). Above and below this surface pressure, the enzyme activity decreased markedly. A similar optimal activity vs surface pressure relationship was observed also for an O-SPM/CHL monolayer (50:50 mol %). The activity of cholesterol oxidase toward cholesterol in the O-SPM/CHL monolayer was, however, less than in the corresponding POPC mixed monolayer. The surface activity of cholesterol oxidase decreased markedly when the temperature was lowered to 20 °C, and hardly any enzyme activity was observed in an O-SPM/CHL monolayer at 25 mN/m or above. With a monolayer containing POPC/O-SPM/CHL (42:18:40 mol %), maximal cholesterol oxidase activity was observed at the lowest surface pressure tested (i.e., 10 mN/m), and the catalytic activity decreased markedly with increasing lateral surface pressures in the monolayer. The results of this study show (i) that the activity of cholesterol oxidase in general is highly dependent on the lateral surface pressure in the substrate membranes and (ii) that sphingomyelin, by interacting tightly with cholesterol, can prevent or restrain the accessibility of cholesterol for oxidation by cholesterol oxidase.

The characterization of the distribution of unesterified cholesterol between structures of intact cells has been a challenging and difficult task. One approach has utilized the enzyme cholesterol oxidase, which under controlled conditions is believed to oxidize cell surface cholesterol without oxidizing cholesterol located in intracellular membranes (Gottlieb, 1977; Lange & Ramos, 1983). However, it has been found that cholesterol in native membranes of cultured cells and erythrocytes is not readily accessible for oxidation by the enzyme (Gottlieb, 1977; Patzer et al., 1978; Lange & Ramos, 1983). In order to make cell cholesterol susceptible to oxidation by cholesterol oxidase, different manipulations (e.g., phospholipase C treatment, exposure to low ionic strength buffers, or sphingomyelinase treatment) have been necessary (Patzer et al., 1978; Lange & Ramos, 1983; Slotte et al., 1989). The resistance of cholesterol in native membranes to enzymatic oxidation could be due to at least two different mechanisms, both of which could operate simultaneously.

First, the resistance to oxidation could be due to the tight interaction of cholesterol with some classes of membrane

†This research was supported by grants from the Aarne Koskelo Foundation (Helsinki) and the Council for Sciences, Academy of Finland.
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0006-2960/90/0429-3173$02.50/0

phospholipids (e.g., sphingomyelin) that have been reported (Demel et al., 1977; Wattenberg & Silbert, 1983; Barenholz, 1984; Yeagle & Young, 1986; van Blitterswijk et al., 1987; Lund-Katz et al., 1988). A tight cholesterol/phospholipid interaction could by itself prevent or prolong the enzymatic oxidation of the sterol molecules.

Second, the catalytic activity of cholesterol oxidase could be dependent on the surface pressure or molecular packing in the substrate-containing membrane structure. Such a dependency would be analogous to properties of various phospholipases, the catalytic activities of which are sensitive to changes in the packing density and lateral surface pressure of the lipid membranes (Demel et al., 1975; Yedgar et al., 1982; Verger & Pattus, 1982).

The objective of this study was to determine the catalytic activity of cholesterol oxidase (from *Streptomyces* sp.) in cholesterol-containing mixed monolayers of varying phospholipid composition [1-palmitoyl-2-oleoylphosphatidylcholine (POPC)[1] and *N*-oleoylsphingomyelin (O-SPM)] and at varying surface pressures. Since the oxidation product of cholesterol, 4-cholesten-3-one (cholestenone), does not condense a phospholipid monolayer to the same extent as cholesterol does, the monolayer will expand when the enzyme converts cholesterol to cholestenone. This loss of condensation (i.e., area increase) was used as a correlate for enzyme activity at the lipid/water interphase.

Materials and Methods

Materials. POPC, O-SPM, cholesterol (CHL), 4-cholesten-3-one (CHE or cholestenone), and sphingomyelinase (SMase; from *Staphylococcus aureus*) were from Sigma Chemicals, St. Louis, MO. Cholesterol oxidase (COase; from *Streptomyces* sp.) was obtained from CalBiochem, San Diego, CA. Bovine [*N-methyl*-^{14}C]sphingomyelin (50–60 mCi/mmol) was purchased from Amersham International. Buffers, salts, and hexane were from Merck (FRG) and of the highest available purity (99% or better). Ethanol (99.5%) was from Oy Alko Ab, Finland. The water used was purified with a MilliQ organex system and had a resistivity greater than 10 MΩ/cm.

Cell Culture. Normal human lung fibroblasts were obtained from the Statens Bakteriologiska Laboratorium, Stockholm (Sweden). The cells were cultured in Dulbecco's modified Eagle's medium with 9% fetal calf serum. Cells for experiments were seeded in 30-mm-diameter cell culture dishes (at about 50000 cells/dish) and were grown in this medium (with one change) for 4 days. Cells were then labeled with [^{3}H]-cholesterol by incubating the cells for 48 h with DMEM containing [^{3}H]cholesterol-labeled fetal calf serum (9% during the labeling; Slotte et al., 1989).

Oxidation of Cell Cholesterol. Confluent and [^{3}H]cholesterol-labeled cells were further incubated in serum-free DMEM for 24 h prior to the experiments. Before oxidation of the cell [^{3}H]cholesterol with cholesterol oxidase, cells were fixed for 10 min at 0 °C with 1% glutaraldehyde in isotonic phosphate-buffered saline. Control cells were treated with phosphate-buffered saline alone. After these pretreatments at 0 °C, cells were rinsed with phosphate-buffered saline (2 × 3 mL) and incubated for 30 min at 37 °C with phosphate-buffered saline containing either 0.4 unit/mL cholesterol oxidase or 0.4 unit/mL cholesterol oxidase and 0.1 unit/mL sphingomyelinase together. After the incubation, cells were chilled, rinsed, and stored frozen at −20 °C. Lipids were extracted (hexane/2-propanol) and analyzed for [^{3}H]cholesterol and [^{3}H]cholestenone by thin-layer chromatography within 24 h, as described (Slotte et al., 1989).

The Surface Barostat. The monolayer experiments were performed on a KSV 5000 surface barostat (KSV Instruments, Helsinki) controlled by a Unisys 286 computer. A thermostated rectangular Teflon trough with an area of 712.5 cm^2 (47.5 cm × 15 cm) was used to measure the surface pressure–mean molecular area isotherms. For enzymatic experiments, a zero-order Teflon–glass trough was used (Verger & de Haas, 1973). The zero-order trough had a 30-mL thermostated reaction compartment (25.5 cm^2), a 7.1 cm^2 compartment for the platinum Wilhelmy plate (surface pressure determination), and a 68.1 cm^2 lipid reservoir. The compartments were connected with glass bridges, giving a total trough area of 101.6 cm^2.

Surface Pressure–Mean Molecular Area Isotherms. Surface pressure versus mean molecular area isotherms were measured for phospholipid films containing either cholesterol or cholestenone. The isotherms were run in a rectangular trough on an aqueous buffer (20 mM Tris-HCl/145 mM NaCl, pH 7) at 20 °C. Stock solutions of individual lipids were made up in hexane/ethanol (9:1 v/v) and were stored desiccated at −20 °C in a hexane-saturated atmosphere for a maximum of 24 h before use. Fifty microliters of a 1 mg/mL lipid solution was spread on the buffer for each isotherm. The monolayer was allowed to stabilize or 3–5 min before it was compressed at a barrier speed of 125 mm^2/s. Data were sampled every 3 s and were collected and analyzed with proprietary software from KSV Instruments (Helsinki).

Cholesterol Oxidase Activity at the Water/Monolayer Interphase. The enzymatic determinations were performed in a zero-order trough with 20 mM Tris-HCl/145 mM NaCl, pH 7, as buffer. The reaction compartment was magnetically stirred (150 rpm) and thermostated to 20 or 37 °C; 125 μL of the lipid solution (0.1 mg/mL in hexane/ethanol, 9:1 v/v) was spread onto the buffer surface. The monolayer was allowed to stabilize for 3–5 min, whereafter it was compressed to a predetermined surface pressure (barrier speed 6 mm^2/s). Constant surface pressure was maintained by compensatory barrier movement (computer controlled) throughout the experiment. After the monolayer had stabilized at the chosen surface pressure for 5 min, cholesterol oxidase (4 milliunits/mL in buffer) was added to the reaction compartment. The rate of oxidation of cholesterol in the monolayer was registered (at constant surface pressure) as a backward movement of the barrier due to an oxidation-dependent increase in the monolayer area. Data were sampled every 10 s. Three different kinetic measurements were made at each surface pressure.

To rule out the possibility that the observed area increases resulted from enzyme penetration into the lipid monolayer, experiments were performed where the interaction between cholesterol oxidase and a nonsubstrate monolayer (POPC) was measured at initial surface pressures of 10 and 15 mN/m. It was observed that the addition of 4–10 milliunits/mL cholesterol oxidase to the subphase neither increased the surface pressure of the monolayer nor changed the monolayer surface area (data not shown).

Calculation of Enzyme Activity. The oxidation of cholesterol to cholestenone was observed as an increase of the monolayer area. The enzyme activity in this paper is presented as the amount of cholesterol (taken as the percent of the total cholesterol available in the total monolayer) that was converted

[1] Abbreviations: POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; CHE, 4-cholesten-3-one (cholestenone); CHL, cholesterol; COase, cholesterol oxidase; O-SPM, *N*-oleoylsphingomyelin; SMase, sphingomyelinase.

Table I: Oxidation of Cell [^{3}H]Cholesterol with Cholesterol Oxidase

treatment	enzymes used[a]	% oxidation (±SD)
control cells		0.4 ± 0.1
no fixation	COase (0.4 unit/mL)	10.3 ± 0.5
no fixation	COase + SMase (0.1 unit/mL)	87.3 ● 0.8
fixed cells	COase	25.4 ± 1.1
fixed cells	COase + SMase	87.5 ● 0.2

[a] COase = cholesterol oxidase; SMase = sphingomyelinase. Cells were labeled with [^{3}H]cholesterol for 48 h and then incubated in serum-free medium for an additional 24 h. Washed cells were treated with 1% glutaraldehyde (fixation) or phosphate-buffered saline (no fixation) for 10 min at 0 °C and then exposed to either cholesterol oxidase (0.4 unit/mL) or a combination of cholesterol oxidase and sphingomyelinase (0.1 unit/mL) for 30 min at 37 °C. Percent oxidation is defined as the cpm in [^{3}H]cholestenone over the total cell-unesterified [^{3}H]sterol cpm. Each value is the average of $n = 4 \pm$ SD.

to cholestenone per time unit (seconds). The rate of conversion was calculated from the linear part of the area expansion curve and represented the maximal rate. Since the degree of cholesterol-induced condensation of the phospholipid monolayer varied with surface pressure and lipid composition (cf. Figure 1), the measured enzyme activities at different surface pressures were normalized to the corresponding mean molecular area isotherms at each surface pressure. The difference in the areas of corresponding cholesterol- and cholestenone-containing isotherms was used as a measure for the total oxidation of cholesterol. The area increase which resulted from the enzymatic oxidation was directly related to this measure for total oxidation.

Effects of Sphingomyelinase on Monolayer Area in Sphingomyelin-Containing Monolayers. The effects of sphingomyelin degradation by the action of sphingomyelinase on the monolayer area (at constant surface pressure) of pure or mixed O-SPM monolayers were determined at 20 °C in the zero-order trough similarly as described for cholesterol oxidase determinations. The monolayer was either a pure O-SPM monolayer or a mixed one containing POPC, O-SPM, and cholesterol (POPC/O-SPM/CHL, 42:18:40 mol %). In addition, experiments were performed on monolayers containing [*N-methyl*-^{14}C]sphingomyelin instead of O-SPM. The monolayer was compressed to 25 mN/m, and after a 5-min stabilization period, 20 milliunits/mL sphingomyelinase was added to the reaction compartment. The hydrolysis of sphingomyelin was observed either as a forward movement of the barrier (with O-SPM monolayers) or as the appearance of [^{14}C]phosphocholine in the aqueous phase from a POPC/[^{14}C]SPM/CHL monolayer.

Results

Oxidation of Cell Cholesterol. Cholesterol in native cells is only poorly accessible for oxidation by cholesterol oxidase (Gottlieb, 1977; Lange & Ramos, 1983). To confirm these observations and to further characterize our own enzyme batch and experimental methods, we labeled cultured cells with [^{3}H]cholesterol from serum lipoproteins (Slotte et al., 1989). With this labeling procedure, less than 2% of the cell-associated [^{3}H]cholesterol label was found as esterified cholesterol (data not shown). Exposure of the [^{3}H]cholesterol-labeled cells for 30 min to 0.4 unit of cholesterol oxidase converted only about 10% of the total free [^{3}H]cholesterol to [^{3}H]cholestenone (Table I). Fixation of the cells with 1% glutaraldehyde increased the availability of cell [^{3}H]cholesterol for oxidation only slightly. However, the degradation of cell sphingomyelin with sphingomyelinase drastically enhanced the oxidizability of cell [^{3}H]cholesterol (Table I), whether or not the cells were fixed with glutaraldehyde.

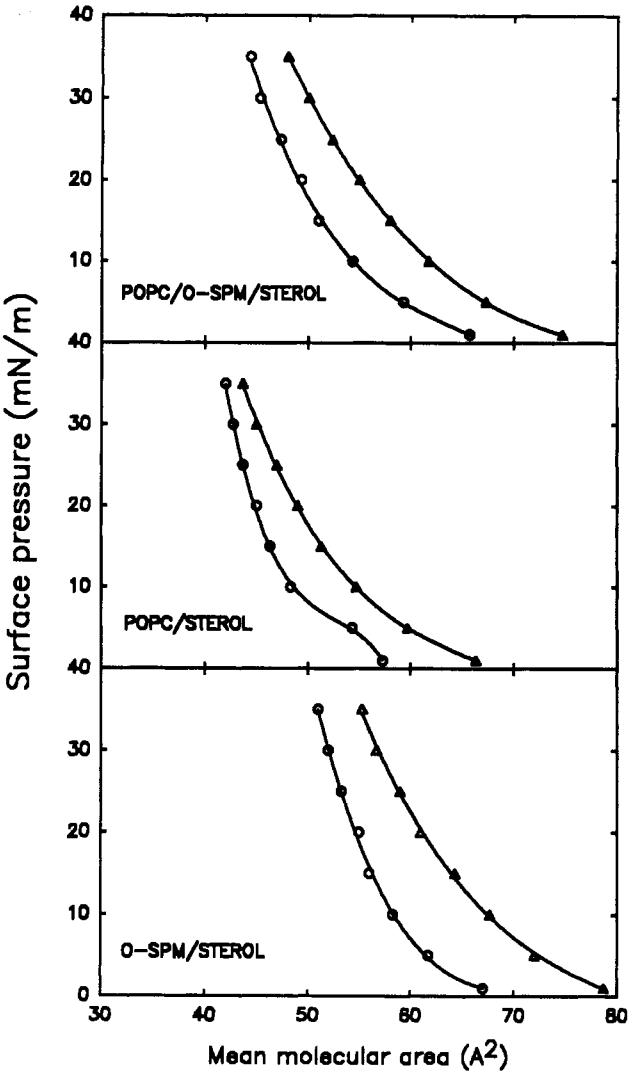

FIGURE 1: Surface pressure vs mean molecular area isotherms for mixed monolayers of POPC/O-SPM and CHL (○) or CHE (△) (42:18:40 mol %), POPC and CHL (○) or CHE (△) (50:50 mol %), or O-SPM and CHL (○) or CHE (△) (50:50 mol %) at 20 °C. The subphase was 20 mM Tris-HCl (pH 7.0) with 145 mM NaCl.

To elucidate the effects of sphingomyelin degradation on cholesterol oxidizability in general, we used the monolayer technique to study the cholesterol oxidase catalyzed oxidation reaction. One of the important biological properties of cholesterol is its ability to pack closely with phospholipids and to cause a condensation of the overall packing in the monolayer or bilayer (Demel & De Kruyff, 1976; Kawato et al., 1978). Since cholestenone does not possess the 3β-hydroxy group, we thought it likely that cholestenone also would not condense a phospholipid bilayer to the same extent as cholesterol itself does. This loss of condensation could then be used as a correlate for the cholesterol oxidase activity in the monolayer.

Isotherms. To determine the effects of cholesterol and cholestenone on the molecular packing in mixed monolayers with POPC or O-SPM, isotherms were run at 20 °C. We used three different monolayer systems: POPC/O-SPM/(CHL or CHE) (42:18:40 mol %), POPC/(CHL or CHE) (50:50 mol %), and finally O-SPM/(CHL or CHE) (50:50 mol %). With all compositions, the mean molecular area at identical lipid concentration per surface area was less when cholesterol was present in the monolayer as compared to cholestenone as the sterol constituent (Figure 1). The change in the degree of condensation when cholestenone was substituted for cholesterol was less marked at higher surface pressures (Figure 1). The

Table II: Mean Molecular Areas for Phospholipid Monolayers Containing Cholesterol or Cholestenone

surface pressure (mN/m)	monolayer composition vs mean molecular area[a]								
	POPC/S[b] (50:50)[c]			O-SPM/S (50:50)			POPC/O-SPM/S (42:18:40)		
	CHL	CHE	%	CHL	CHE	%	CHL	CHE	%
10	48.3	54.7	+13.3	58.3	67.7	+16.1	54.3	61.7	+13.6
15	46.3	51.3	+10.8	56.0	64.3	+14.8	51.0	58.0	+13.7
20	45.0	49.0	+8.9	55.0	61.0	+10.9	49.3	55.0	+11.6
25	43.7	47.0	+7.6	53.3	59.0	+10.7	47.3	52.3	+10.6
30	42.0	43.7	+5.4	52.0	56.7	+9.0	45.3	50.0	+10.4

[a] Given as angstroms squared. [b] S stands for sterol; CHL = cholesterol, and CHE = cholestenone. [c] Given as mole percent. Surface pressure versus mean molecular area isotherms were determined at 20 °C for mixtures of POPC or O-SPM (or a combination thereof) and cholesterol or cholestenone.

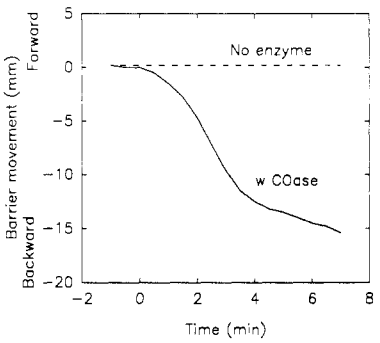

FIGURE 2: Effects of cholesterol oxidase addition (4 milliunits/mL) on the surface area of a POPC/O-SPM/CHL (42:18:40 mol %) monolayer at constant surface pressure (15 mN/m, 20 °C). A negative barrier movement (backward) represents an expansion of the monolayer area.

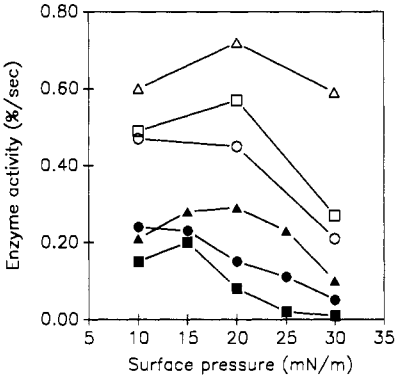

FIGURE 3: Effects of surface pressure, temperature, and monolayer lipid composition on cholesterol oxidase activity. Open symbols represent results at 37 °C and closed symbols results at 20 °C. The monolayer was made up of either POPC/CHL [50:50 mol % (△, ▲)], O-SPM/CHL [50:50 mol % (□, ■)], or POPC/O-SPM/CHL [42:18:40 mol % (○, ●)]. Values are averages of three independent experiments for each monolayer composition at the two temperatures.

change in condensation when cholestenone was substituted for cholesterol was also markedly dependent on the phospholipid composition of the monolayer. This is clearly observable if POPC/sterol and O-SPM/sterol monolayers are compared (Table II). The loss of condensation was greater with O-SPM than with POPC, which would indicate that cholesterol packed more favorably with O-SPM than it did with POPC.

Cholesterol Oxidase Activity. The loss of phospholipid condensation, i.e., the expansion of the monolayer area at constant surface pressure, was used to study the activity of cholesterol oxidase toward cholesterol in monolayers of varying lipid composition and surface pressures. Figure 2 shows a typical graph where the effects of cholesterol oxidase at 20 °C on barrier movement are plotted as a time function for a POPC/O-SPM/CHL monolayer. At constant surface pressure (15 mN/m in Figure 2) in the absence of any cholesterol oxidase, the barrier movement was close to zero (no expansion or contraction of the monolayer). Addition of 4 milliunits/mL cholesterol oxidase to the reaction compartment immediately resulted in an expansion of the monolayer, which was observed as a backward movement of the barrier (Figure 2).

The degree of monolayer expansion at different constant surface pressures was converted to represent the amount of cholesterol that was enzymatically converted to cholestenone. The results of cholesterol oxidase activity at various surface pressures and monolayer compositions are given in Figure 3. With a POPC/CHL monolayer, maximal enzyme activity was observed at about 20 mN/m (both 20 and 37 °C). The enzyme activity decreased both below and above this monolayer surface pressure. With a O-SPM/CHL monolayer at 37 °C, a similar optimal enzyme activity versus surface pressure was observed. The absolute enzyme activity was, however, significantly less with O-SPM/CHL than it was for a POPC/CHL monolayer. The enzyme activity at 37 °C also decreased more rapidly at surface pressures above 20 mN/m in an O-SPM/CHL monolayer compared to the POPC/CHL system. At 20 °C, the cholesterol oxidase activity in an O-SPM/CHL monolayer displayed maximal activity at 15 mN/m, but the activity decreased rapidly with increasing surface pressure and was undetectable at 30 mN/m. The behavior of cholesterol oxidase toward cholesterol in a POPC/O-SPM/CHL monolayer at 37 °C resembled that of a pure O-SPM/CHL monolayer. At 20 °C, the oxidase activity in the POPC/O-SPM/CHL monolayer was maximal at the lowest surface pressure tested (i.e., 10 mN/m), and decreased linearly with increasing surface pressure.

Sphingomyelin Degradation by Sphingomyelinase. To examine the effects of sphingomyelin degradation on molecular packing in monolayers of either pure O-SPM or a mixture of POPC/O-SPM/CHL (42:18:40 mol %), sphingomyelinase was added to the reaction chamber, and the monolayer expansion/retraction was followed as a time function. At a surface pressure of 25 mN/m (20 °C), addition of sphingomyelinase caused a rapid hydrolysis of O-SPM, as seen in Figure 4. Since the mean molecular area of ceramide is less than the corresponding area for sphingomyelin (Yedgar et al., 1982; own data not shown), degradation of O-SPM resulted in a retraction of the monolayer (forward barrier movement). However, with a monolayer of POPC/O-SPM/CHL (42:18:40 mol %) at 25 mN/m, no decrease in the monolayer area (i.e., barrier movement) could be observed after addition of sphingomyelinase to the reaction compartment (Figure 4). Actually, the monolayer area started to increase very slightly after about 10 min of the sphingomyelinase addition.

To further verify that the sphingomyelin in the POPC/O-SPM/CHL monolayer was degraded by the action of sphingomyelinase, a monolayer was made to contain 0.1 μCi of [^{14}C]sphingomyelin instead of O-SPM. The degradation of the [^{14}C]sphingomyelin could be followed as the release of

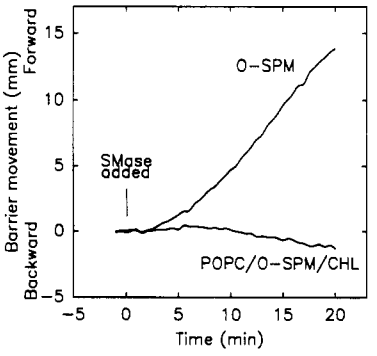

FIGURE 4: Effects of sphingomyelin degradation by sphingomyelinase (20 milliunits/mL) on the monolayer surface area at constant surface pressure (25 mN/m, 20 °C). The monolayers contained either pure O-SPM or POPC/O-SPM/CHL (42:18:40 mol %). A positive barrier movement (forward) represents a retraction of the monolayer area.

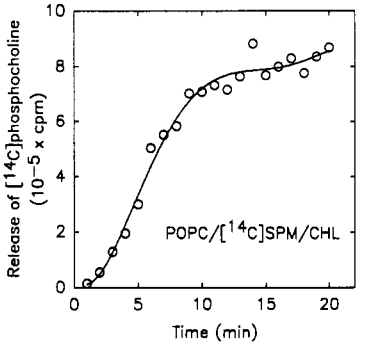

FIGURE 5: Degradation by sphingomyelinase of [^{14}C]sphingomyelin in a POPC/[^{14}C]SPM/CHL (42:18:40 mol %) monolayer at constant surface pressure (25 mN/m, 20 °C). The release of [^{14}C]phosphocholine was determined from the aqueous subphase of the reaction chamber.

[^{14}C]phosphocholine to the aqueous subphase in the reaction compartment. It was observed that at 25 mN/m and 20 °C, all the degradable [^{14}C]sphingomyelin was hydrolyzed within 10 min after addition of 20 milliunits/mL sphingomyelinase (Figure 5). The [^{14}C]sphingomyelin added to the monolayer represented about 160 000 cpm (0.1 μCi with 70% counting efficiency). A release of about 70 000 cpm as [^{14}C]-phosphocholine (during the first 10 min) from the monolayer represents a 44% degradation of the monolayer [^{14}C]-sphingomyelin. Since the *total* monolayer surface area at 25 mN/m was 63 cm^2, of which the reaction compartment covered 25.5 cm^2 (i.e., 40% of the total monolayer area at 25 mN/m), it can be concluded that all of the sphingomyelin on the reaction chamber surface was degraded by the action of sphingomyelinase. However, this also implies that the sphingomyelin in the part of the monolayer that covered the Wilhelmy plate chamber and the reservoir chamber was not degraded during the course of the experiment.

Discussion

The aim of this study was to try to explain why cholesterol in membranes of native cells is such a poor substrate for the cholesterol oxidase enzyme. Since it was known that sphingomyelin degradation resulted in a marked increase in the oxidizability of membrane cholesterol (Slotte et al., 1989; this study), it was hypothesized that sphingomyelin itself could protect cholesterol from being oxidized by cholesterol oxidase. Another possibility could be that the surface pressure in native membranes is so high that cholesterol oxidase cannot reach or react with its substrate in the membrane.

This study clearly demonstrated that the activity of cholesterol oxidase was sensitive to changes in the lateral surface pressure of the substrate membranes. Optimal activity for cholesterol oxidase was observed at surface pressures around 20 mN/m. This value is markedly lower than the corresponding surface pressure found in intact erythrocyte membranes, which has been reported to be 31–35 mN/m (Demel et al., 1975).

It appears, however, that lipid composition and molecular packing also have important effects on the apparent activity of cholesterol oxidase in monolayers. The relative activity of cholesterol oxidase toward cholesterol was markedly lower in a sphingomyelin environment than in a POPC milieu. This is in good agreement with oxidation studies in vesicle membranes, where it was shown that [^{3}H]cholesterol in sphingomyelin vesicles was only slowly oxidized by cholesterol oxidase, whereas [^{3}H]cholesterol in POPC vesicles was readily available for oxidation (Slotte et al., 1989).

Although POPC and O-SPM both are choline-containing phospholipids, they still appear to interact differently with cholesterol. This may be explained by their different hydrogen bonding capacities, which may allow for tighter packing for sphingomyelin with cholesterol in a mono- and bilayer compared to phosphatidylcholine (Schmidt et al., 1977; Lange et al., 1984). Not only does sphingomyelin appear to protect cholesterol from being oxidized by cholesterol oxidase, but it also retards the transfer of cholesterol between lipid structures (Fugler et al., 1985; Bhuvaneswaran & Mitropoulos, 1986; Yeagle & Young, 1986; Lund-Katz et al., 1988; Bar et al., 1989). The resistance of cholesterol to both oxidation and exchange when it is solubilized in a sphingomyelin environment strongly suggests that the sphingomyelin/cholesterol interaction is thermodynamically more stable or has a longer lifetime as compared with interactions of cholsterol with other classes of phospholipids.

When intact cells are treated with phospholipase C (Moore et al., 1977; Patzer et al., 1978) or sphingomyelinase (Slotte et al., 1989), the cholesterol oxidase reactivity of membrane cholesterol increases sharply. What is the likely explanation for this sharp increase in oxidizability? The experiments in this study showed that degradation of sphingomyelin in a monolayer containing POPC/O-SPM/CHL (42:18:40 mol %), in a ratio that resembled the ratio found in erythrocyte membranes, did not result in a lowered surface pressure of the monolayer.

The lack of a forward barrier movement in response to sphingomyelin degradation in a POPC/O-SPM/CHL monolayer probably resulted from two opposing forces. First, conversion of sphingomyelin to ceramide by sphingomyelinase should result in a monolayer with a smaller area (at constant surface pressure) since the mean molecular area of ceramide is less than that for sphingomyelin (Yedgar et al., 1982). However, degradation of sphingomyelin also would be expected to result in a loss of the cholesterol-induced condensation of the sphingomyelin/cholesterol interaction (Demel & De Kruyff, 1976), thus leading to an expansion of the monolayer area (constant surface pressure). Since the monolayer area did not change very much following the degradation of sphingomyelin, the two opposite forces probably were of about equal magnitude with this lipid composition.

We also have measured the area change in an erythrocyte lipid extract as a function of sphingomyelin degradation by sphingomyelinase and observed that the monolayer area actually increased at constant surface pressure, even though the sphingomyelin in the reaction compartment was completely degraded (Grönberg and Slotte, unpublished observations). This strongly suggests that the loss of condensation was of

greater importance than the loss of molecular area when sphingomyelin was replaced by ceramide in this biological lipid extract. It is therefore very unlikely that sphingomyelinase treatment of intact cells resulted in a lowered lateral surface pressure of the plasma membranes. A decrease in the lateral surface pressure was therefore probably not the mechanism which increased the oxidizability of cholesterol in sphingomyelin-depleted cells. Instead, the increased cholesterol reactivity probably resulted from the loss of protection against oxidation that sphingomyelin appears to provide in native, untreated membranes.

ACKNOWLEDGMENTS

We express our appreciation to Professor Jarl B. Rosenholm at the Department of Physical Chemistry for giving us access to the KSV surface barostat during the course of these studies. We also thank Jouko Peltonen, M.Sc., for valuable discussions during the course of this study.

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