Enzyme-Catalyzed Oxidation of Cholesterol in Mixed Phospholipid Monolayers Reveals the Stoichiometry at Which Free Cholesterol Clusters Disappear†

J. Peter Slotte
Department of Biochemistry and Pharmacy, Åbo Akademi University, SF 20500 Turku, Finland
Received December 2, 1991; Revised Manuscript Received March 19, 1992

ABSTRACT: In this study, we have used cholesterol oxidase as a probe to study cholesterol/phospholipid interactions in mixed monolayers at the air/water interface. Mixed monolayers, containing a single phospholipid class and cholesterol at differing cholesterol/phospholipid molar ratios, were exposed to cholesterol oxidase at a lateral surface pressure of 20 mN/m (at 22 °C). At equimolar ratios of cholesterol to phospholipid, the average rate of cholesterol oxidation was fastest in unsaturated phosphatidylcholine mixed monolayers (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and egg yolk phosphatidylcholine), intermediate in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, and slowest in sphingomyelin monolayers (egg yolk or bovine brain sphingomyelin). The average oxidation rate in mixed monolayers was not exclusively a function of monolayer packing density, since egg yolk and bovine brain sphingomyelin mixed monolayers occupied similar mean molecular areas even though the measured average oxidation rate was different with these two phospholipids. This suggests that the phospholipid acyl chain composition influenced the oxidation rate. The importance of the phospholipid acyl chain length on influencing the average oxidation rate was further examined in defined phosphatidylcholine mixed monolayers. The average oxidation rate decreased linearly with increasing acyl chain lengths (from di-8:0 to di-18:0). When the average oxidation rate was examined as a function of the cholesterol to phospholipid (C/PL) molar ratio in the monolayer, the otherwise linear function displayed a clear break at a 1:1 stoichiometry with phosphatidylcholine mixed monolayers, and at a 2:1 C/PL stoichiometry with sphingomyelin mixed monolayers. These observed breaks are interpreted to indicate the highest possible C/PL stoichiometry at which cholesterol still associates with the phospholipid, and does not segregate into free cholesterol clusters in the monolayer.

Cholesterol is a membrane-active sterol, and by interacting with phospholipids it modulates the fluidity of the phospholipid acyl chains [see Yeagle (1985) and references cited therein] and also affects the ion permeability of such phospholipid membranes (Demel et al., 1971; Papahadjopoulus et al., 1971; Yeagle et al., 1977). The planar fused-ring structure, the β-hydroxy group at carbon-3, and the flexible isooctyl side chain at carbon-17 all together give cholesterol its remarkable membrane-active properties (Demel et al., 1972). The interaction between cholesterol and phosphatidylcholine in model membranes appears to be of an equimolar nature, in that complexes with 1:1 stoichiometries are formed (Lecuyer & Dervichian, 1969; Phillips & Finer, 1974; Collins & Phillips, 1982). If the cholesterol to phospholipid molar ratio (C/PL)[1] is less than 1, clusters of free phospholipid coexist with 1:1 cholesterol/phospholipid complexes (McLean & Phillips, 1982). If the C/PL molar ratio exceeds 1:1, on the other hand, free cholesterol clusters coexist with cholesterol/phospholipid complexes (Lecuyer & Dervichian, 1969). It has further been postulated that at low C/PL ratios, complexes with a stoichiometry of 1:2 could also exist in model membranes (Presti et al., 1982). Although it is possible to prepare model membranes with C/PL molar ratios higher than 1:1 (Freeman & Finean, 1975; Lundberg, 1977), these are considered to be thermodynamically unstable (Collins & Phillips, 1982).

The molecular interactions between cholesterol and different phospholipid classes are at least in part different, since the sterol appears to have a specific affinity toward sphingomyelin compared to other phospholipid classes. Model membrane studies have convincingly demonstrated that the rate of cholesterol desorption from sphingomyelin-containing membranes is markedly slower as compared to the rate of desorption from membranes containing other phospholipid classes, suggesting a special interaction between cholesterol and sphingomyelin (Clejan & Bittman, 1984; Lund-Katz et al., 1988). Monolayer experiments have further revealed that cholesterol packs more tightly with sphingomyelin as compared with other phospholipid classes (Lund-Katz et al., 1988).

The enzyme-catalyzed oxidation of cholesterol in mixed monolayers is influenced both by the lateral surface pressure and by the phospholipid composition of the mixed monolayer (Grönberg & Slotte, 1990; Slotte & Grönberg, 1990). We have in this study used cholesterol oxidase as a probe to elucidate the interaction between cholesterol and various phospholipids in mixed monolayers having (i) differing phospholipid acyl chain compositions and (ii) different C/PL molar ratios.

Experimental Procedures

Materials. Cholesterol (99+%), bovine brain sphingomyelin (bb-SPM), egg yolk sphingomyelin (E-SPM), egg yolk phosphatidylcholine (EPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dicaproyl-*sn*-glycero-3-phosphocholine, 1,2-didecanoyl-*sn*-glycero-3-phosphocholine, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-*sn*-glycero-3-

†Supported in part by grants from the Borg Foundation, the Aarne Koskelo Foundation, the Walter & Lisi Wahl Foundation, the Oskar Öflund Foundation, the Sigrid Juselius Foundation, and the Council of Sciences (the Academy of Finland).

[1] Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; EPC, egg yolk phosphatidylcholine; E-SPM, egg yolk sphingomyelin; bb-SPM, bovine brain sphingomyelin; C/PL, cholesterol to phospholipid molar ratio.

0006-2960/92/0431-5472$03.00/0

phosphocholine were obtained from Sigma Chemicals (St. Louis, MO). The sterol was 99% pure by gas–liquid chromatography, and the phospholipids gave a single spot on thin-layer chromatography plates [Kiselgel 60 (Merck), eluted with chloroform/methanol/acetic acid/water (25:15:4:2 v/v)]. Cholesterol oxidase (*Streptomyces* sp.) was purchased from Calbiochem (CA) and was used as delivered. Buffer salts were of pro analysis grade, and the water used was double-distilled and further purified with a Millex Q system (to better than 15 MΩ/cm).

Lateral Surface Pressure versus Mean Molecular Area Isotherms. Force–area isotherms were determined for pure cholesterol monolayers, or for mixed monolayers containing varying molar ratios of cholesterol and phospholipids (either bb-SPM, E-SPM, EPC, DPPC, or POPC), with a KSV 3000 surface barostat (KSV Instruments, Helsinki). The isotherms were run in a rectangular Teflon trough (450 mm × 60 mm) on an aqueous Tris buffer (50 mM Tris-HCl/140 mM NaCl, pH 7.5) at 22 °C. Stock solutions of the lipids were made up in hexane/2-propanol and were stored desiccated at −20 °C. The lipid solution was spread on the buffer, and the monolayer was then allowed to stabilize for 3–5 min before it was compressed at a barrier speed of 10 mm²/s. Data were sampled every 2 s. At least three different runs were performed at each lipid composition, and the reproducibility was better than ±3%.

Oxidation of Cholesterol in Mixed Monolayer Membranes. The oxidation of cholesterol in pure or mixed monolayers by cholesterol oxidase was determined in a zero-order Teflon trough with Tris buffer (Verger & De Haas, 1973). The reaction compartment (2550 mm², 30 mL) was magnetically stirred (100 rpm) and thermostated to 22 °C. The lipid solution was spread on the buffer surface, and the monolayer was compressed to 20 mN/m at a rate of about 1000 mm²/min. Constant surface pressure was maintained by compensatory barrier movement (computer controlled) throughout the experiment. After the monolayer had stabilized for 5 min, cholesterol oxidase (16 milliunits/mL) was added to the reaction compartment. The total time the monolayer was exposed to air at the air/water interface was about 15 min. The rate of the enzyme-catalyzed 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. The absolute average rate of cholesterol oxidation is not exactly comparable from one experimental condition or from one batch of the enzyme to the other, since cholesterol oxidase is fairly unstable in aqueous solution event at 0 °C. Therefore, the oxidation data of this study were derived from experiments performed with one batch of enzyme and within a time span of less than 4 weeks, the enzyme being dispensed into 250-μL aliquots, which were stored at −20 °C, and were used within 2 h after being thawed to 0 °C.

Calculation of Enzyme Activity. The area over the reaction chamber in the zero-order trough was 2550 mm². The mean molecular area determinations of pure and mixed monolayers made it possible to calculate the number of cholesterol molecules that would fit on the surface over the reaction chamber. Since only the surface above the reaction chamber was exposed to cholesterol oxidase, the time needed for the overall reaction (as determined from the monolayer area expansion, cf. Figure 2) was equal to the time needed for oxidation of all cholesterol molecules above the reaction chamber. The rate of oxidation is given as the average (not maximal) rate, and is calculated to indicate the number of cholesterol molecules that were oxidized per second. Usually three experiments were performed with each mixed monolayer, and the measured oxidation times deviated less than ±15% of the calculated mean value.

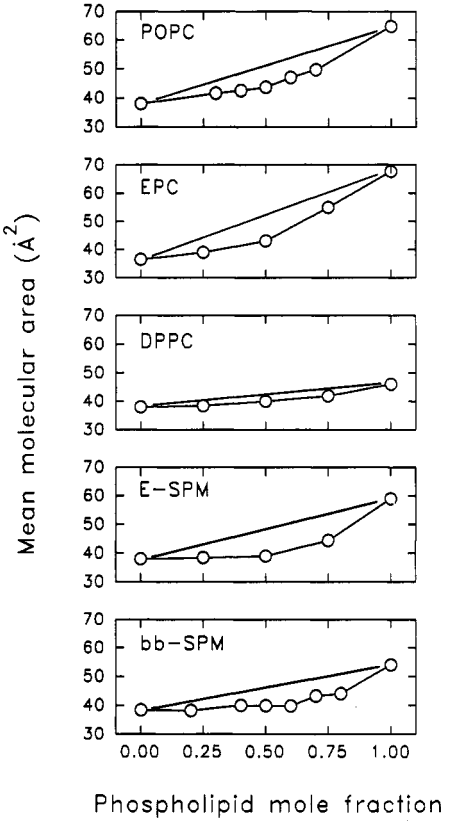

FIGURE 1: Mean molecular area requirement of pure and mixed cholesterol/phospholipid monolayers at different phospholipid mole fractions. The force–area isotherms were determined, and the mean molecular area at 20 mN/m was plotted against the molar fraction of the phospholipid in the mixture. Values are averages from three separate experiments at each concentration.

Data Analysis. Data were collected by proprietary software from KSV Instruments. The raw data files were converted to ASCII, and were further imported into TableCurve software (Jandel Scientific). With this program, nonlinear curve fits were made of the force-area isotherms, and exact values of the mean molecular areas were calculated for a lateral surface pressure of 20 mN/m. The curves presented are based on the average value from at least three different experiments analyzed in this way.

Phospholipid Assay. The accurate concentration of the various phospholipid stock solutions was determined with a phospholipid/phosphate assay kit delivered by Boehring-Mannheim.

Results

Cholesterol/Phospholipid Interactions in Mixed Monolayers. Different molar ratio mixtures of cholesterol and either POPC, EPC, DPPC, bb-SPM, or E-SPM were spread on the clean buffer surface of an isotherm trough. Force–area isotherms were obtained (not shown), and the mean molecular area for each mixture at 20 mN/m was calculated. These data, plotted against the phospholipid molar fraction, are shown in Figure 1. The curves show the well-known condensing effect of cholesterol on the monolayer packing of phospholipids (Leathes, 1925; De Bernard, 1958; Chapman et al., 1969), except for the DPPC curve. The mean molecular area values from these experiments were used to calculate the number of cholesterol molecules (in each mixed monolayer at each molar fraction) which fit on the surface of the reaction

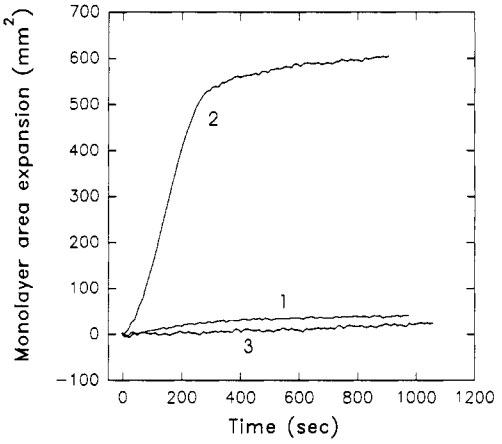

FIGURE 2: Monolayer area expansion due to oxidation of cholesterol by cholesterol oxidase. A 2550 mm^2 surface was covered by a pure sphingomyelin monolayer (line 1) or a cholesterol/sphingomyelin monolayer (0.5:0.5 mol/mol, line 2), whereafter 16 milliunits/mL cholesterol oxidase was added to the subphase (30 mL) at 22 °C. The time needed to reach a postreaction stable monolayer area was taken as the total time needed for the oxidation of cholesterol in the monolayer above the reaction chamber. Line 3 represents a monolayer of pure cholesterol at the air/water interface in the absence of added cholesterol oxidase to the subphase.

chamber in the zero-order trough (used for calculation of reaction velocities in subsequent experiments).

Oxidation of Cholesterol in Mixed Monolayers. The oxidation of cholesterol in pure sterol monolayers (Slotte, 1992), or in mixed phospholipid-containing monolayers (Gröberg & Slotte, 1990), is known to result in an oxidation-dependent monolayer area expansion. The sterol monolayer did not expand due to exposure to air (noncirculating) at the air/water interface, suggesting that the rate of spontaneous oxidation of the sterol was negligible (Figure 2, line 3). The time needed for the enzyme-catalyzed oxidation of cholesterol on the surface of the reaction chamber is equal to the time needed for the monolayer expansion to reach a postreaction stable base line (Figure 2). With the knowledge of this reaction time, and with the knowledge of the number of cholesterol molecules on the surface of the reaction chamber, one can calculate an average (not maximal) reaction rate for the overall process.

We have prepared mixed cholesterol/phospholipid monolayers with varying ratios of cholesterol to phospholipid, and exposed these to cholesterol oxidase. The resulting monolayer expansion times were converted to yield the average rate of cholesterol oxidation (as molecules oxidized per second), and the results are presented in Table I. Within the group of

Table I: Average Oxidation Rate versus Monolayer Packing Density (20 mN/m)

mixed monolayer	mean molecular area ($Å^2$)	average oxidation rate (molecules/s)
1:1 cholesterol/POPC	43.7	0.9×10^{13}
1:1 cholesterol/EPC	43.0	0.9×10^{13}
1:1 cholesterol/DPPC	40.0	0.5×10^{13}
1:1 cholesterol/E-SPM	39.0	0.2×10^{13}
1:1 cholesterol/bb-SPM	39.9	0.5×10^{13}
2:1 cholesterol/E-SPM	≈39	0.6×10^{13}
2:1 cholesterol/bb-SPM	≈40	1.7×10^{13}

phosphatidylcholines, it can be seen that the average oxidation rate (at a 1:1 C/PL molar ratio) was highest for POPC and EPC and moderately slower for DPPC mixed monolayers. Within the group of sphingomyelins, the average oxidation rate was faster with bb-SPM and slower with E-SPM, despite the similarity in mean molecular areas for these mixed monolayers. At equimolar ratios of cholesterol to phospholipids, the oxidation rate was in general slower for sphingomyelins than for phosphatidylcholines, except for DPPC and bb-SPM monolayers which yielded similar average oxidation rates.

If one looks at the average oxidation rates plotted as a function of the C/PL molar ratio (Figure 3), it is evident that with POPC, EPC, and DPPC mixed monolayers, the rate versus C/PL function exhibited a break at a 1:1 stoichiometry. This break is consistent with the idea that free cholesterol clusters disappear at and below this stoichiometry. However, with both E-SPM and bb-SPM mixed monolayers, the oxidation rate versus C/PL function exhibited a break at a 2:1 stoichiometry. These data suggest that at least in mixed monolayers, cholesterol associates with sphingomyelins with a different stoichiometry as compared with phosphatidylcholines.

The rate of cholesterol oxidation in a pure cholesterol monolayer at 20 mN/m was about 3.5×10^{13} molecules/s, which is by definition the maximal possible under these conditions. If one extrapolates the linear portion of the oxidation rate versus C/PL molar ratio function from Figure 3 to a rate corresponding to a pure cholesterol monolayer (i.e., 3.5×10^{13} molecules/s), one gets the C/PL molar ratio where the phospholipid no longer affects the rate of cholesterol oxidation in the mixed monolayer. The extrapolated values are given in Table II, and it can be seen that for both sphingomyelins the value is close to 12, whereas the value is between 4.5 and 7.6 for the phosphatidylcholines. This suggests that sphingomyelins can affect (i.e., retard) the enzyme-catalyzed oxi-

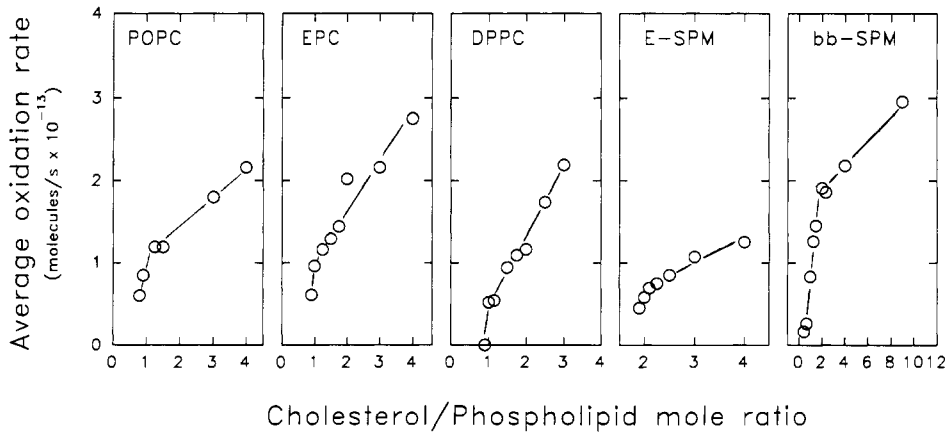

FIGURE 3: Average oxidation rate as a function of the C/PL molar ratio in the mixed monolayer. Mixed monolayers at indicated molar ratios were prepared, and the average oxidation rate was determined at 20 mN/m and 22 °C. Values are averages from at least three different experiments at each ratio. For comparison, a pure cholesterol monolayer was oxidized with an average rate of 3.5×10^{13} molecules/s.

Table II: C/PL Limit Value Giving an Oxidation Rate Similar to a Pure Sterol Monolayer[a]

phospholipid species	C/PL limit
POPC	7.6
EPC	5.0
DPPC	4.5
E-SPM	11.8
bb-SPM	12.0

[a] The linear part of the oxidation rate versus C/PL molar ratio function in Figure 3 was extrapolated to the value corresponding to the rate of oxidation in a pure cholesterol monolayer (3.5×10^{13} molecules/s), and the corresponding C/PL molar ratio was determined. This C/PL ratio defines the limit where the phospholipid no longer retards the oxidation of cholesterol in the mixed monolayer.

dation of cholesterol at much lower concentrations compared to the phosphatidylcholines. This function may also reflect a higher affinity of cholesterol for sphingomyelins as compared to phosphatidylcholines.

Effect of Phospholipid Acyl Chain Length on Cholesterol Oxidizability. It is clear from the previous results that the lateral packing density is not the only effector of oxidation rates in the mixed monolayer and it appears likely that the acyl chain composition of the phospholipids has marked effects on oxidation rates. To test for the effects of phospholipid acyl chain length on cholesterol/phospholipid association in monolayers, as probed with cholesterol oxidase, mixed monolayers (at a C/PL molar ratio of 1.5:1) were prepared with disaturated phosphatidylcholines of varying acyl chain lengths. In such mixed monolayers, it was observed that the rate of cholesterol oxidation decreased with increasing acyl chain length (Figure 4), further demonstrating the influence of the acyl chain composition on the rate of enzyme-catalyzed oxidation of cholesterol in mixed monolayers.

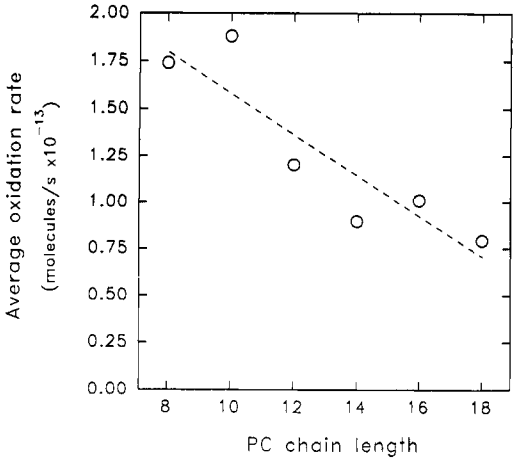

FIGURE 4: Average oxidation rate as a function of the phospholipid acyl chain length in a mixed cholesterol/phosphatidylcholine monolayer. Mixed monolayers (1.5:1 C/PL) of cholesterol and a disaturated phosphatidylcholine (either di-8:0, di-10:0, di-12:0, di-14:0, di-16:0, or di-18:0) were prepared, and the oxidation of cholesterol by cholesterol oxidase at 20 mN/m and 22 °C was determined. Values are averages from three separate experiments at each condition.

Discussion

The cholesterol oxidase susceptibility of cholesterol in mixed monolayers is by definition related to the degree of exposure of the 3β-hydroxy group at the water/lipid interface. If the exposure is optimal, like it is in pure sterol monolayers, the oxidation rate is mainly a function of the surface concentration of cholesterol (Slotte, 1992). In mixed monolayers containing phospholipids, the oxidation of cholesterol is slower compared to a pure sterol monolayer, apparently because the tight association of cholesterol with phospholipids in the monolayer limits the exposure of the 3β-hydroxy group. Therefore, factors which influence the tightness of the cholesterol/phospholipid molecular association (e.g., surface pressure and the choice of phospholipid) also will affect the degree of 3β-hydroxy group exposure and consequently the rate of enzyme activity (Grönberg & Slotte, 1990; Slotte & Grönberg, 1990).

In this study, we have kept the lateral surface pressure constant at 20 mN/m and examined the general effects of the C/PL molar ratio and the phospholipid acyl chain and head-group composition on the average oxidation rate. In equimolar mixed monolayers, the oxidation rate was observed to be faster in unsaturated phosphatidylcholine monolayers and somewhat slower in saturated phosphatidylcholine and sphingomyelin monolayers. The faster oxidation rate in unsaturated mixed monolayers probably reflects the looser lateral packing (and consequently a looser molecular interaction) seen in these membranes. In looser membranes, one would expect the 3β-hydroxy group of cholesterol to be less shielded (or more exposed), and therefore to be more readily available to cholesterol oxidase. In an analogous situation, it is known that the rate of cholesterol desorption from a membrane is markedly influenced by the tightness of cholesterol/phospholipid molecular interactions so that cholesterol desorbs more easily from unsaturated phosphatidylcholine membranes than from saturated or sphingomyelin-containing membranes (Clejan & Bittman, 1984; Fugler et al., 1985; Phillips et al., 1987; Lund-Katz et al., 1988). It therefore appears that both the oxidizability and the exchangeability of cholesterol at least to some extent depend on the lateral packing density [but see also Grönberg et al. (1991) for exceptions].

However, the oxidation rate in a mixed monolayer is not a sole function of the lateral packing in the membrane, since the oxidation rate within the sphingomyelin group differed (higher rate with bb-SPM than with E-SPM), although the mean molecular area requirement in both monolayers was similar (Table I). One can therefore conclude that (i) the phospholipid acyl chain composition has a marked influence on the molecular interactions between cholesterol and the phospholipid molecule and (ii) the acyl chain composition may favor a molecular interaction which better shields the 3β-hydroxy group, without affecting the observable average mean molecular area requirement in the mixed monolayer.

The importance of the phospholipid acyl chain composition on the interactions with cholesterol was further demonstrated in the experiments with mixed monolayers containing phosphatidylcholines with different lengths of disaturated acyl chains. In such mixed monolayers, it was observed that the average oxidation rate decreased with increasing length of phosphatidylcholine acyl chains. This suggests that longer phospholipid acyl chains made the interaction with cholesterol more tight and therefore protected the 3β-hydroxy group of cholesterol better from the attack of the enzyme.

Since it was apparent from previous studies that cholesterol was oxidized faster in pure sterol monolayers as compared to systems with mixed phospholipid monolayers (Grönberg & Slotte, 1990; Slotte & Grönberg, 1990; Slotte, 1992), it was thought that cholesterol oxidase might actually be a useful probe with which one could titrate the stoichiometry in mixed monolayers at which clusters of free cholesterol would appear/disappear (depending on which component is diluted with respect to the other). The rationale was that in cholesterol/phospholipid mixed monolayers with high C/PL molar ratios, free cholesterol clusters would phase-separate (Ladbrooke et al., 1968; Lecuyer & Dervichian, 1969; Green & Green, 1973) and that the oxidation of these clusters would

follow kinetics of pure sterol monolayers (Slotte, 1992). Results in this study indicate that the stoichiometry where free cholesterol clusters disappeared in phosphatidylcholine-containing mixed monolayers was 1:1. This stoichiometry is similar to the stoichiometry that has been reported for bilayer model membranes containing phosphatidylcholine (Phillips & Finer, 1974; McLean & Phillips, 1982). The 1:1 stoichiometry is thought to represent the maximal thermodynamically stable mixture of cholesterol and phosphatidylcholine (Collins & Phillips, 1982). With sphingomyelin-containing mixed monolayers, the stoichiometry where free cholesterol clusters disappeared was 2:1 (C/PL). The stoichiometry reported by cholesterol oxidase appeared not to depend on the acyl chain composition of the phospholipid, since POPC, EPC, and DPPC mixed monolayers gave similar stoichiometries, but rather appeared to depend on the phospholipid class (PC versus SPM). In another study where cholesterol/phospholipid stoichiometries were examined using the probe *N*-phenyl-1-naphthylamine (Reiber, 1978), it was reported that the maximal C/PL molar ratio obtained in vesicle membranes was 2:1 for bb-SPM and egg phosphatidylcholine whereas the ratio was 1:1 for DPPC, DPPE, and cerebroside. Our results with egg phosphatidylcholine (1:1 C/PL) are not consistent with the results of Reiber (2:1 C/PL; Reiber, 1978). This discrepancy may result from the use of mixed monolayers in the present study compared to sonicated vesicles in the latter.

It is not readily apparent why sphingomyelin would display a different stoichiometry from phosphatidylcholine with regard to associating with cholesterol. The phosphocholine head group is similar in both phospholipid classes. The free allylic hydroxy group at the 3-position in sphingomyelin is lacking in the phosphatidylcholine structure, but this functional group appears not to give sphingomyelin any special features with regard to associations with cholesterol, since substitutions at this position do not affect the interactions of sphingomyelin with cholesterol to any marked extent (Kan et al., 1991a; Grönberg et al., 1991). The amide-linked acyl chain in sphingomyelin, which phosphatidylcholine lacks, may also be insignificant as an explanation for the different stoichiometries displayed by sphingomyelin and phosphatidylcholine toward cholesterol, since the introduction of an amide-linked acyl chain into the 2-position of a phosphatidylcholine molecule does not make it sphingomyelin-like with regard to cholesterol exchange kinetics (Kan et al., 1991b). On the other hand, factors which do not affect cholesterol desorption from membranes may affect the stoichiometry of cholesterol/phospholipid association.

The present model membrane studies suggest that one sphingomyelin molecule may bind to itself two cholesterol molecules and still shield the 3β-hydroxy group, thereby retarding its oxidation. One can ask whether a similar stoichiometry can exist in the plasma membrane of cells. Sphingomyelin degradation studies with intact cells have indicated that plasma membrane cholesterol is translocated away from the plasma membrane (into the cells), as a result of sphingomyelin hydrolysis (Slotte et al., 1989, 1990; Pörn & Slotte, 1990). If one looks, for example, at the human skin fibroblast model, about 80% of endogenously labeled [^{3}H]-sphingomyelin (and also sphingomyelin mass) can be degraded by exogenously applied sphingomyelinase. Since fibroblasts contain about 13 nmol of sphingomyelin mass/mg of cell protein, this degradation takes away about 10 nmol of sphingomyelin mass (Slotte & Bierman, 1988). The degradation of sphingomyelin mass in fibroblasts may lead to the translocation of about 40% of the cell-unesterified cholesterol away from the plasma membrane (Pörn & Slotte, 1990). Forty percent of 75 nmol of unesterified cholesterol/mg of cell protein (Slotte & Bierman, 1988) is about 30 nmol. Since the degradation of about 10 nmol of sphingomyelin mass can lead to the translocation of about 30 nmol of unesterified cholesterol, it is clear that a stoichiometry higher than 1:1 could actually exist in the plasma membrane of HSF, provided that no secondary effects resulting from the sphingomyelin degradation lead to a translocation of non-sphingomyelin-bound cholesterol away from the cell surface.

Acknowledgments

The skillful assistance of Petya Dacsev-Valtakari, Ann-Len Östman, and Janita Lövgren is gratefully acknowledged.

Registry No. POPC, 26853-31-6; DPPC, 63-89-8; cholesterol, 57-88-5; 1,2-dicaproyl-*sn*-glycero-3-phosphocholine, 34506-67-7; 1,2-didecanoyl-*sn*-glycero-3-phosphocholine, 3436-44-0; 1,2-dilauroyl-*sn*-glycero-3-phosphocholine, 18194-25-7; 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, 18194-24-6; 1,2-distearoyl-*sn*-glycero-3-phosphocholine, 816-94-4.

References

Chapman, D., Owens, N. F., Phillips, M. C., & Walker, D. A. (1969) *Biochim. Biophys. Acta 183*, 458–465.

Clejan, S., & Bittman, R. (1984) *J. Biol. Chem. 259*, 10823–10826.

Collins, J. J., & Phillips, M. C. (1982) *J. Lipid Res. 23*, 291–298.

De Bernard, L. (1958) *Bull. Soc. Chim. Biol. 40*, 161.

Demel, R. A., Bruckdorfer, K. R., & Van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta 255*, 321–330.

Demel, R. A., Bruckdorfer, K. R., & Van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta 258*, 311–320.

Freeman, R., & Finean, J. B. (1975) *Chem. Phys. Lipids 14*, 313–320.

Fugler, L., Clejan, S., & Bittman, R. (1985) *J. Biol. Chem. 260*, 4098–4102.

Green, J. R., & Green, C. (1973) *Biochem. Soc. Trans. 1*, 365–368.

Grönberg, L., & Slotte, J. P. (1990) *Biochemistry 29*, 3173–3178.

Grönberg, L., Ruan, Z., Bittman, R., & Slotte, J. P. (1991) *Biochemistry 30*, 10746–10754.

Kan, C.-C., Ruan, Z., & Bittman, R. (1991a) *Biochemistry 30*, 7759–7766.

Kan, C.-C., Ruan, Z., & Bittman, R. (1991b) *Biochim. Biophys. Acta 1066*, 95–101.

Ladbrooke, B. D., Williams, R. M., & Chapman, D. (1968) *Biochim. Biophys. Acta 150*, 333–340.

Leathes, J. B. (1925) *Lancet 208*, 853.

Lecuyer, H., & Dervichian, D. G. (1969) *J. Mol. Biol. 45*, 39–57.

Lundberg, B. (1977) *Chem. Phys. Lipids 18*, 212–220.

Lund-Katz, S., Laboda, H. M., McLean, L. R., & Phillips, M. C. (1988) *Biochemistry 27*, 3416–3423.

McLean, L. R., & Phillips, M. C. (1982) *Biochemistry 21*, 4053–4059.

Papahadjopoulos, D., Nir, S., & Ohki, S. (1971) *Biochim. Biophys. Acta 266*, 561–583.

Phillips, M. C., & Finer, E. G. (1974) *Biochim. Biophys. Acta 356*, 199–206.

Phillips, M. C., Johnson, W. J., & Rothblat, G. H. (1987) *Biochim. Biophys. Acta 906*, 223–276.

Pörn, M. I., & Slotte, J. P. (1990) *Biochem. J. 271*, 121–126.

Presti, F. T., Pace, R. J., & Chan, S. I. (1982) *Biochemistry 21*, 3831–3835.

Reiber, H. (1978) *Biochim. Biophys. Acta 512*, 72–83.

Slotte, J. P. (1992) *Biochim. Biophys. Acta 1123*, 326–333.
Slotte, J. P., & Bierman, E. L. (1988) *Biochem. J. 250*, 653–658.
Slotte, J. P., & Grönberg, L. (1990) *J. Lipid Res. 31*, 2235–2242.
Slotte, J. P., Hedström, G., Rannström, S., & Ekman, S. (1989) *Biochim. Biophys. Acta 985*, 90–96.
Slotte, J. P., Härmälä, A.-S., Jansson, C., & Pörn, M. I. (1990) *Biochim. Biophys. Acta 1030*, 251–257.
Verger, R., & De Haas, G. H. (1973) *Chem. Phys. Lipids 10*, 127–136.
Yeagle, P. L. (1985) *Biochim. Biophys. Acta 822*, 267–287.
Yeagle, P. L., Martin, R. B., Lala, A. K., & Bloch, K. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4924–4926.

On the Mechanism of a Mammalian Neuronal Type Nicotinic Acetylcholine Receptor Investigated by a Rapid Chemical Kinetic Technique. Detection and Characterization of a Short-Lived, Previously Unobserved, Main Receptor Form in PC12 Cells†

Norio Matsubara‡ and George P. Hess*
Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, 217 Biotechnology Building, Cornell University, Ithaca, New York 14853-2703

Received June 14, 1991; Revised Manuscript Received December 31, 1991

ABSTRACT: The mammalian nicotinic acetylcholine receptor in PC12 cells has many properties characteristic of the neuronal receptors involved in key chemical reactions that are responsible for signal transmission between cells of the nervous system. This report describes initial investigations of the mechanism of this receptor using a rapid chemical kinetic technique with a time resolution of 20 ms, which represents a 250-fold improvement over the best time resolution (5 s) employed in previous studies. Carbamoylcholine, a stable analogue of the neurotransmitter acetylcholine, was the activating ligand used, and the concentration of open transmembrane receptor–channels in PC12 cells was measured by recording whole-cell currents at pH 7.4, 21–23 °C, and a transmembrane voltage of −60 mV. Two receptor forms that account for 80% and 20% of the receptor-controlled current were detected; the main receptor form, accounting for 80% of the whole-cell current, desensitized completely before the first measurements had been made in previous studies. Only the main receptor form has been investigated so far using the new method. The constants of a mechanism that accounts for the concentration of the open transmembrane receptor–channel over a 100-fold range of carbamoylcholine concentration were evaluated: the dissociation constant of the site controlling channel opening (K_1 = 2.0 mM), the channel-opening equilibrium constant (Φ^{-1} = 5.0), and the dissociation constant of an inhibitory site to which carbamoylcholine binds (K_R = 6.5 mM). These evaluated constants allow one to calculate P_o, the conditional probability that at a given concentration of carbamoylcholine the receptor–channel is open. P_o was also determined in the presence of 2 mM carbamoylcholine by an independent method, the single-channel current-recording technique, and the agreement between the P_o values obtained in two independent ways is within experimental error. This result indicates that the time resolution of the chemical kinetic technique employed was sufficient to evaluate the constants pertaining to the active state of the receptor, which forms a transmembrane channel, before its conversion to desensitized receptor forms with different properties. Previous kinetic measurements with a time resolution of 5 s showed that many compounds, such as anesthetic-like molecules, nerve growth factor, and substance P, modify the function of the neuronal receptor in PC12 cells or react specifically with the neuronal but not with the muscle receptor, for example, some toxins. The results presented here indicate, however, that in previous experiments the properties of only inactive (desensitized) receptor forms were observed; these forms have ligand-binding properties quite different from those of the active receptor forms. The effects all these compounds have on the active receptor form that leads to channel opening and signal transmission are, therefore, still unanswered and interesting questions for future research.

The neuronal nicotinic acetylcholine receptor is one of many membrane-bound proteins involved in the key reactions that determine whether signals are transmitted between cells of the nervous system (Kandel & Schwartz, 1985). Ultimately signal transmission depends on the concentration of open transmembrane channels formed in these reactions. Understanding the mechanism that allows the concentration of open receptor–channels to be adjusted by a variety of factors, and thus whether or not a signal is transmitted between nerve cells, continues to be a major challenge for all the neurotransmitter receptors so far identified (for example, those for acetylcholine, glycine, glutamate, and γ-aminobutyric acid). Our aim was to determine what one can learn about a receptor mechanism

† This work was supported financially by a grant (NS 08527) awarded by the National Institutes of Health to G.P.H. N.M. was supported for 2 years by a postdoctoral fellowship from the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation and a consortium of industries.

* Author to whom correspondence and reprint requests should be addressed.
‡ Present address: Department of Material Science, Faculty of Science, Himeji Institute of Technology, 2167 Shosha, Himeji, Hyogo 671-22, Japan.

0006-2960/92/0431-5477$03.00/0