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Cholesterol Heterogeneity in the Plasma Membrane of Epithelial Cells

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ABSTRACT: The distribution of cholesterol in the plasma membrane of epithelial cells has been determined using renal brush border vesicles as a model. In brush borders treated with *Brevibacterium sp.* or *Nocardia erythropolis* cholesterol oxidases, a significant fraction of the free cholesterol was oxidized rapidily, without glutaraldehyde fixation, and the remaining cholesterol was oxidized at a slower rate. The size of the readily accessible cholesterol pool, however, depended on the enzyme used, varying from 16% of the total in membranes treated with *N. erythropolis* oxidase, to 27% using the *Brevibacterium sp.* enzyme. The slowly accessible pool detected by the *Brevibacterium* oxidase was suppressed upon sphingomyelinase addition. On the other hand, the restricted activity of the *Nocardia* oxidase might depend on phosphatidylcholine/cholesterol interactions. These results indicate that cholesterol distribution is heterogeneous in intact renal brush border vesicles. They suggest that, as proposed for model systems [Demel, R. A., Jansen, J. W. C. M., van Dijck, P. W. M., & van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta 465*, 1–10], preferential interactions between some classes of phospholipids and cholesterol define cholesterol pools in the plasma membrane of epithelial cells.

Existence of cholesterol domains in the plasma membrane of epithelial cells is still under debate (Van Meer, 1987). So far, only in intestinal cells has a heterogeneous distribution of cholesterol been reported. This heterogeneity was proposed to result from lipid/protein interactions, with more than two-thirds of the cholesterol associated with a membrane protein fraction (Bloj & Zilversmit, 1982). In renal epithelial cells, modification of the cholesterol content of the apical membrane (brush borders) modulates transport activities (Molitoris et al., 1985; Levi et al., 1990). The distribution of cholesterol in this membrane, however, remains unknown.

As in intestinal cells, significant differences exist between the lipid order of the apical and basolateral domains of the

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plasma membrane of renal cells, as well their corresponding cholesterol/phospholipid ratios (Brasitus & Schachter, 1980; Le Grimellec et al., 1982; Carmel et al., 1985). For intestinal cells, it has been proposed (Van Meer, 1988), that the presence of a large amount of glycolipids might account for the apparent heterogeneity of the distribution of cholesterol between the two domains of the plasma membrane. Such an explanation does not hold for the renal proximal tubular cells for which the glycolipid content is low both in the apical and in the basolateral domains (Spiegel et al., 1988). Preparations of renal brush border membrane vesicles (BBM)[1] maintain the proper sidedness (right side out) during the isolation procedure (Haase et al., 1978; Vênien et al., 1988), thus allowing lipid topology studies (Vênien & Le Grimellec, 1988). This suggested that renal BBM might be an useful model for studies on cholesterol domains in epithelial cells membranes.

In the present investigation, we have treated renal brush border membranes with cholesterol oxidases isolated from different microorganisms. The rationale for using different enzymes was that, as for phospholipases A_2 [see Waite (1987)], activity of the oxidases is highly dependent on the conditions of the assay, on the lipid composition and lateral surface pressure of the membrane (Pal et al., 1980; Lange & Ramos, 1983; Gronberg & Slotte, 1990).

Experimental Procedures

Materials. Cholesterol oxidases (EC 1.1.3.6) from *Nocardia erythropolis* (COD I), *Brevibacterium sp.* (COD II and IV), and *Pseudomonas* (COD III) were purchased from Boehringer Mannheim, Beckman Instruments, Chemical Dynamics Corporation (South Plainfield, NJ), and Sigma, respectively. Sphingomyelinase from *Bacillus cereus* was obtained from Boehringer Mannheim. Color-free TNBS was purchased from Eastman Kodak. All other reagents were of analytical grade.

Membrane Preparation. Brush border membrane vesicles (BBM) from the kidney cortex of male New Zealand white rabbits (2–2.5 kg body weight) were isolated as previously described (Le Grimellec et al., 1982) using an $MgCl_2$ precipitation method (Booth & Kenny, 1974), which gave right side out vesicles (Vênien et al., 1988). Membranes were suspended (4 mg of protein/mL) in 150 mM NaCl/ 20 mM Hepes (pH 7.4) and either used the same day or kept at −80 °C until used (within 1 week). In BBM preparations, the activity of alkaline phosphatase was enriched 13-fold over that of the homogenate compared to less than 0.7-fold for Na^+,K^+-ATPase and less than 0.5-fold for glucose-6-phosphatase.

Treatment of BBM with Cholesterol Oxidases. The day of the experiment, 1 volume of the BBM preparation was added to 5 volumes of a 50 mM mannitol/2 mM Tris (pH 7.0) solution (final NaCl concentration: 25 mM). After a 10-min preincubation at 37 °C, cholesterol oxidase was added to a final concentration of 1–2 IU/mL. After various times of incubation (1–240 min), two aliquots (200 μg of protein) were taken. The reaction was stopped by extracting the lipids immediately.

Lipid Analysis. Lipids were extracted by using the method of Bligh and Dyer (1959). Extraction was repeated three times to obtain a maximum recovery (Carmel et al., 1985). Extracts were pooled, dried under nitrogen, and solubilized in a chloroform/methanol mixture (2:1 v/v). Separation of neutral lipids was performed by thin layer chromatography on precoated silica gel plates (Merck), using benzene/diethyl ether/ethanol/acetic acid (50:40:2:0.2 v/v) as developing solvent. After brief exposure to iodine vapor, the spots were identified by comparison with authentic standards, and lipids were extracted from silica by addition of 12 mL of chloroform/methanol (1:1 v/v). The solvent was evaporated under nitrogen, and the lipids were solubilized in 1 mL of 2-propanol. The amount of cholesterol oxidized, corrected for the recovery of extraction, was determined by measuring the remaining cholesterol (Omodeo-Salé et al., 1984).

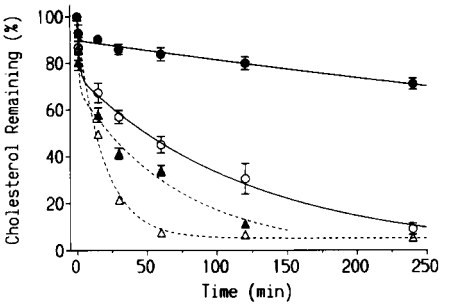

FIGURE 1: Oxidation of cholesterol in renal brush border membranes by different cholesterol oxidases. BBM (0.6–0.8 mg of protein) were incubated for 10 min at 37 °C in 50 mM mannitol/2 mM Tris (pH 7.0) containing 25 mM NaCl before addition of 1 IU/mL cholesterol oxidase. At the time indicated, aliquots of the mixture were taken, and the cholesterol remaining was assayed as described under Experimental Procedures. (●) COD I (N = 5); (○) COD II (N = 3); (▲) COD III (N = 3); (△) COD IV (N = 2). N corresponds to the number of different BBM preparations assayed. Data are presented as mean ± SEM.

Labeling by TNBS. Amino groups were chemically labeled by incubating the BBM in 2 mM TNBS/20 mM Hepes/150 mM NaCl (pH 8.0) at 4 or 37 °C, in the dark (Grunberger et al., 1982). The reaction was stopped by addition of 1 M HCl (final pH 2.5). Unreacted TNBS was eliminated by centrifugation and washing. The pellet was resuspended in pH 2.5 buffer and extracted as above. Trinitrophenyl derivatives of PE and PS were separated by thin layer chromatography on precoated silica gel plates (Whatman K5), using chloroform/methanol/water/acetic acid (65:25:4:1 v/v) as the developing solvent. Individual components were detected by exposure to iodine vapors and identified by comparison with authentic standards. The spots were scraped off and transferred into acid-washed test tubes. The phosphorus content of phospholipids classes was determined according to Mrsny et al. (1986).

Enzyme and Protein Determination. Activities of the marker enzymes were determined as previously described (Le Grimellec et al., 1982). Protein concentration was determined by the method of Lowry et al. (1951), after precipitation with 10% trichloroacetic acid, using bovine serum albumin as a standard.

Results

Oxidation of Membrane Cholesterol by Enzymes from Various Origins. By analogy with phospholipase whose hydrolytic efficiency depends on the origin of the enzyme, we investigated the cholesterol accessibility of renal brush border membranes to cholesterol oxidases from *N. erythropolis* (COD I), *Brevibacterium sp.* (COD II and COD IV), and *Pseudomonas* (COD III). Incubation of the vesicles at 37 °C with an excess of enzyme (1–2 IU/mL) resulted in an oxidation of membrane cholesterol for the four COD's tested. However, the extent and the time course of the oxidation process were highly dependent of the enzyme used, ranging from 30% ox-

[1] Abbreviations: PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; TNBS, trinitrobenzene sulfonate; BBM, brush border membrane vesicles; COD, cholesterol oxidase.

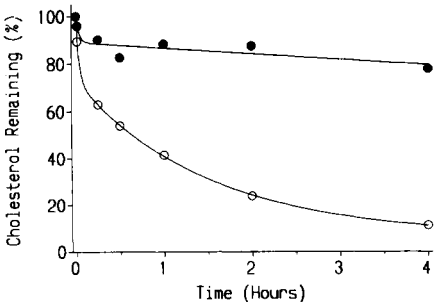

FIGURE 2: Effect of COD I and COD II on the same batch of BBM preparation. Experimental conditions and symbols are those described for Figure 1.

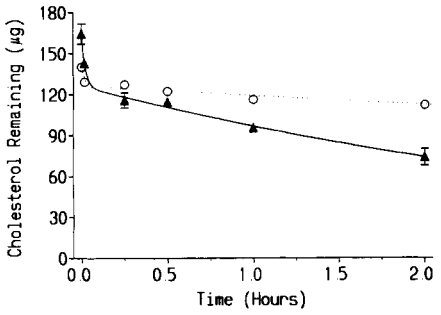

FIGURE 3: Influence of membrane cholesterol concentration on cholesterol oxidability in renal BBM. Two membrane preparations with "high cholesterol levels" (>160 μg/mg of protein) were treated by COD I under conditions similar to those of Figure 1. (▲) "high cholesterol"; (○) "normal cholesterol".

idation in 4 h for COD I to 90% oxidation in 1 h for COD IV (Figure 1). Addition of fresh cholesterol oxidase at t = 1 h did not modify the oxidation of cholesterol by COD I and COD II. Treatment in parallel of the same batch of membranes with COD I and COD II confirmed that the differences in cholesterol oxidation were related to intrinsic properties of the enzymes and not to variability between membrane preparations (Figure 2). Furthermore, for COD's I–IV, oxidation proceeded rapidly within the first minute, during which it could reach as much as 20% of total cholesterol (COD III). This suggested a heterogeneous accessibility of BBM cholesterol to the enzymes. Curve fitting by nonlinear regression indicated that for COD's I–III, oxidation was best described by the sum of two exponentials (goodness of fit $R^2 > 0.99$). It is noteworthy that these data were obtained from BBM preparations whose cholesterol content was in the usual range (130–150 μg/mg of membrane protein, mean value = 140 ± 3, n = 13) for this species (Vénien & Le Grimellec, 1988). During the course of the experiments with COD I, two BBM preparations of higher cholesterol concentration (164 and 173 μg/mg of membrane protein) were treated by the enzyme. As shown by Figure 3, the efficiency of the oxidation process was significantly increased for these two preparations.

For COD's I–III, the kinetic analysis of cholesterol oxidation obeyed the model of Bloj and Zilversmit (1976) in which a lipid is distributed between two pools differing by their accessibility to the probing agent (Table I). The size of the readily accessible pool(a) varied from 16 (COD I, "normal cholesterol levels") to 33% (COD III). For each enzyme, the oxidation rate (K_0) exceeded the rate of migration from the less to the readily accessible pool (K_{ab}) by more than an order of magnitude, which indicated that the limiting step was not the oxidation but the passage between the two pools. For the two "high cholesterol" BBM treated with COD I, the size of the accessible pool increased from 16 to 24%. In contrast, the best fit for cholesterol oxidation by COD IV was obtained

Table I: Kinetic Parameters for the Oxidation of Cholesterol in Renal Brush Border Membrane Vesicles[a]

	pool size (%)		rate constants (h^{-1})			half time
expt	a	b	K_0	K_{ab}	K_{ba}	($t_{1/2}$) (min)
COD I (NC)[b]	16 ± 2	84	3.1	0.05	0.24	144
COD I (HC)[c]	24 ± 3	76	46.6	0.27	0.85	37
COD II	27 ± 3	73	40.4	0.50	1.35	22
COD III	33 ± 4	67	52.8	0.86	1.74	16
COD IV	93 ± 7	(7)	3.6			

[a] The biphasic curves of cholesterol oxidation were fitted by the sum of two exponentials, $K_1e^{-g_1t} + K_2e^{-g_2t}$ (Bloj & Zilversmit, 1976). In this table, a and b correspond to the pool size of the readily and less accessible pools, respectively. K_0 is the rate constant of cholesterol oxidation, while K_{ab} represents the rate constant for the cholesterol movement from the less to the readily accessible pool. [b] NC, normal cholesterol BBM. [c] HC, high cholesterol BBM.

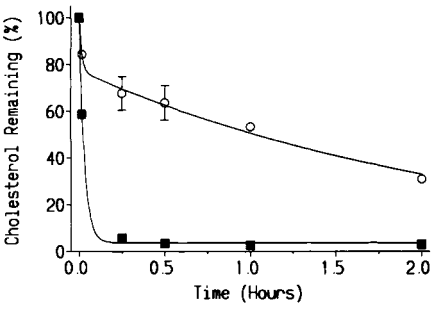

FIGURE 4: Effect of sphingomyelinase on cholesterol oxidation by COD II. Following the 10-min incubation period, 1 IU/mL COD II alone (○) or in combination (■) with 0.5 IU/mL sphingomyelinase (*B. cereus* Boehringer) was added to the BBM suspension, and the remaining cholesterol was determined after various times.

considering a single pool that accounted for more than 90% of BBM cholesterol.

Origin of the Differences in Cholesterol Oxidases Efficiency. A first, but partial, explanation to the different behavior of the oxidases was found by adding the enzymes to liposome suspensions made of either BBM total lipid extracts or of 65% PC, 25% PE, and 10% PA (mol/mol) or of 65% SM, 25% PE, and 10% PA (mol/mol). Incubation of the liposomes for 2 h at 37 °C in the presence of the different oxidases, followed by lipid extraction and TLC revealed that COD III produced large amounts of lysophospholipids and diacylglycerol, essentially from PC and PE, whereas COD IV degraded part (less than 40%) of the SM. On the other hand, no lipase(s) activity was found to be associated with COD I and COD II that were therefore used for further experiments.

Another possible explanation to the differences between the COD's might have resulted from a loss in membrane integrity, i.e., a variable opening of the vesicles according to the enzyme used. This possibility was assessed by determination of the fraction of membrane PE trinitrophenylated by TNBS in control and COD-treated vesicles. It was previously shown that treatment of the sealed right side out BBM by TNBS under nonpermeant conditions for 30 min corresponded to the labeling of PE present on the outer membrane leaflet (Vénien & Le Grimellec, 1988). In the present experiments, the amount of PE labeled, following a 4 h COD I or COD II treatment, by TNBS represented 35% of total PE, a value comparable to that obtained from untreated BBM (33%) run in parallel. This strongly argued against an opening of the vesicles associated with the COD effects. Because the only obvious difference between COD II and COD IV was the contamination by a sphingomyelinase activity, we investigated the effect of addition of sphingomyelinase to BBM on the oxidation by COD II. As shown by Figure 4, the simultaneous

addition of sphingomyelinase and COD II resulted in the oxidation of more than 90% of the cholesterol within 15 min. The amount of sphingomyelin hydrolyzed during the same time (83%) was comparable to that obtained with sphingomyelinase alone, i.e., significantly lower than that obtained in open vesicles (Vénien & Le Grimellec, 1988). Following the sphingomyelinase plus cholesterol oxidase treatment, the percentage of PE labeled by TNBS at 4 °C, although increased (55%), remained significantly lower than the one obtained under permeant conditions (90%), suggesting that simultaneous treatment with both enzymes increased the passive permeability properties of BBM.

Discussion

Through the use of cholesterol oxidases, the present experiments indicate the existence of cholesterol pools in isolated renal brush border membranes. They also demonstrate that the size of the pools detected depends on the origin of the oxidase used. Finally, they suggest that lipid–lipid interactions may define cholesterol pools in the plasma membrane of epithelial cells.

Cholesterol Pools in Renal Brush Border Membranes. For three of the enzymes (COD's I, II, and III), oxidation was best fitted by the sum of two exponentials. This biphasic character was not attributable to a mixture of vesicles showing different orientations: renal BBM form close vesicles, relatively homogeneous in size and oriented 90% right side out (Haase et al., 1978; Vénien et al., 1988). It was also not due to either an inactivation of the enzymes or to an opening of the vesicles during the time course of experiments. Accordingly, these data strongly suggested the existence of a heterogeneous distribution of cholesterol in native renal BBM, i.e., in BBM nontreated by glutaraldehyde.

Accessibility of cholesterol to cholesterol oxidase(s) has been related to the membrane content in phospholipids bearing the choline head-group (Patzer et al., 1978; Pal et al., 1980; Slotte et al., 1989). In vesicles composed mainly of choline phospholipids, cholesterol became accessible to cholesterol oxidase only when its concentration exceeded 42–46 mol % (Pal et al., 1980). For renal BBM, polar lipids in the outer membrane leaflet are constituted of 75% sphingomyelin (which correspond to 0.21 μmol of phospholipids/mg of protein), 7% phosphatidylcholine (Vénien & Le Grimellec, 1988), and of less than 5% glycolipids (Spiegel et al., 1988). Oxidation of BBM cholesterol by noncontaminated oxidases therefore suggested that the outer membrane leaflet contains at least 0.20–0.24 μmol of cholesterol/mg of protein, i.e., more than half of the BBM cholesterol (0.36 μmol/mg of protein). Such a distribution would explain why, in renal proximal membranes (a) the cholesterol content of the brush border membrane domain exceeds that of its basolateral counterpart (Carmel et al., 1985; Molitoris et al., 1985) and (b) diet can affect the cholesterol content of the brush borders, leaving the basolateral content unchanged (Molitoris et al., 1985). Cholesterol heterogeneity, also reported for intestinal brush border membranes (Bloj & Zilversmit, 1982), might be a characteristic of highly polarized cells where it could serve in the control of functions of apical membranes (Le Grimellec et al., 1988).

Origin of Cholesterol Pools. For "normal cholesterol" membrane preparations, the readily accessible pool viewed by COD II represented 27% of total cholesterol. On the other hand, with COD IV, a COD II-type oxidase contaminated by a sphingomyelinase activity, cholesterol oxidation was best described by a single exponential. Sphingomyelin degradation also induces a nearly complete oxidation of the cell cholesterol in glutaraldehyde-fixed fibroblasts (Slotte et al., 1989). Moreover, recent data on baby hamster kidney cells in culture indicate that the sphingomyelin content of the plasma membrane is one of the major determinants of its cholesterol concentration (Slotte et al., 1990). As a basis for such a behavior, experiments on model systems have shown that the strength of interactions between cholesterol and phospholipids decreases in the order SM > PC > PE (Demel et al., 1977; van Dijck, 1979; Yeagle & Young, 1986). Taken together, these data suggested that, in renal brush border membranes, interactions between cholesterol and sphingomyelin determined the slowly accessible pool detected by COD II. Validity of this hypothesis was supported by the observation that, following addition of sphingomyelinase to COD II, oxidation proceeded according to a single cholesterol pool model. Using COD III, an oxidase contaminated by a phospholipase C acting essentially on PE and PC, the size of the readily accessible pool was comparable to that of COD II. On the other hand, the readily accessible pool detected by COD I represented only 16% of membrane cholesterol. By analogy with the data obtained in model systems (Patzer et al., 1978; pal et al., 1980), this suggested that not only SM/cholesterol but also PC/cholesterol interactions might have limited the oxidation of BBM cholesterol by COD I. Accordingly, preferential lipid–lipid interactions might be at the origin of the heterogenous distribution of cholesterol pools in renal BBM. The reasons for the differences in efficiency of COD I and COD II are not known. However, they might be related to the fact that, as for the phospholipases (Demel et al., 1975), the activity of cholesterol oxidase(s) is highly dependent on the lateral surface pressure in the substrate membrane (Grönberg & Slotte, 1990).

Finally, oxidation by COD I was increased toward values similar to that of the COD II's in "high cholesterol" renal BBM. This effect of increasing the cholesterol content of a membrane preparation on its susceptibility to cholesterol oxidase resembles that described for vesicular stomatitis virus (Moore et al., 1977) and red blood cells (Lange et al., 1980, 1984). Our data support the hypothesis that the conflicting results on the accessibility of intestinal BBM cholesterol to COD I (Bloj & Zilversmit, 1982: Thurnhofer et al., 1986) were related to the different cholesterol content of the preparations (Thurnhofer et al., 1986).

Registry No. Cholesterol, 57-88-5.

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Antagonistic and Synergistic Peptide Analogues of the Tridecapeptide Mating Pheromone of *Saccharomyces cerevisiae*†

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ABSTRACT: Biologically inactive, truncated analogues of the *Saccharomyces cerevisiae* α-mating factor (WHWLQLKPGQPMY) either antagonized or synergized the activity of the native pheromone. An amino-terminal truncated pheromone [WLQLKPGQP(Nle)Y] had no activity by itself, but the analogue acted as an antagonist by competing with binding and activity of the mating factor. In contrast, a carboxyl-terminal truncated pheromone [WHWLQLKPGQP] was not active by itself nor did the peptide compete with α-factor for binding to the α-factor receptor, but it acted as a synergist by causing a marked increase in the activity of α-factor. The observation that residues near the amino terminus may be involved in signal transduction whereas those near the carboxyl terminus influence binding allows us to separate binding and signal transduction in the yeast pheromone response pathway. If found for other hormone–receptor systems, synergists may have potential as therapeutic compounds.

Communication between cells is mediated by an elegant information system which involves the specific recognition of effector molecules by membrane-bound receptors and subsequent signal transduction to the intracellular machinery. The molecular interactions between messenger molecules and their receptors and the pathway of signal transduction are two of the most intensively studied areas of cellular biology. Such phenomena occur throughout nature and can be examined in lower eukaryotes such as *Saccharomyces cerevisiae*.

Sexual conjugation in *S. cerevisiae* is dependent upon diffusible peptide pheromones, the a-factor and the α-factor [for recent reviews, see Cross et al. (1988) and Herskowitz (1988)], which are recognized by membrane-bound receptors (Burkholder & Hartwell, 1985; Hagen et al., 1986; Nakayama et al., 1985). On the basis of the sequence of the genes encoding receptors, significant structural similarity is predicted among the yeast α-factor receptor (*STE2* gene product) and a-factor receptor (*STE3* gene product) and receptors as diverse as rhodopsin and those for β-adrenergic and cholinergic agents

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