Cholesterol Distribution in Renal Epithelial Cells LLC-PK1 As Determined by Cholesterol Oxidase: Evidence That Glutaraldehyde Fixation Masks Plasma Membrane Cholesterol Pools

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ABSTRACT: Treatment with cholesterol oxidases has shown that cholesterol is heterogeneously distributed in brush border membranes isolated from the apical domain of the renal and intestinal epithelial cells [Bloj, B., & Zilversmit, D. B. (1982) *J. Biol. Chem. 257*, 7608–7614; El Yandouzi, E. H., & Le Grimellec, C. (1992) *Biochemistry 31*, 547–551]. Cholesterol distribution between plasma membrane and intracellular membranes of the corresponding cells remains unexplored. The effects of *Brevibacterium* sp. cholesterol oxidase on the cholesterol content of LLC-PK1 cells, an epithelial cell line with multiple differentiated characteristics of the renal proximal tubule, were investigated. In confluent living cells grown as a monolayer on solid support, a small but significant fraction (13%) of the cholesterol was oxidized during the first hour of the oxidase treatment. Glutaraldehyde fixation prior to treatment resulted in a nearly complete (86.1 ± 1.8) oxidation of the cellular cholesterol according to first-order kinetics. Filipin labeling and oxidation at 15 °C confirmed that cholesterol was essentially confined to the plasma membrane in LLC-PK1 cells. When adding the oxidase either on the apical or on the basolateral side of cells grown on permeant support and fixed with glutaraldehyde, a comparable monophasic oxidation of cholesterol was observed, despite the presence of efficient tight junctions. Adding the oxidase to both sides simultaneously did not increase the rate of oxidation. Finally, fixation of isolated renal brush border membranes with glutaraldehyde rendered undiscernible their cholesterol pools. We conclude that glutaraldehyde fixation, a commonly used process in the analysis of cholesterol distribution in cells, can mask the existence of cholesterol pools in plasma membranes.

Whereas the nonuniform distribution of cholesterol among the membranes of nucleated cells is well-established (Colbeau et al., 1971; Yeagle, 1985; Lange, 1992), the relative importance of the plasma membrane as the principal site of cholesterol localization is still under debates (Dawidowicz, 1987; Van Meer, 1987; Lange, 1992). Thus, treatment of glutaraldehyde-fixed fibroblasts, Chinese hamster ovary cells, baby hamster kidney (BHK) cells, and hepatocytes with cholesterol oxidases results in the oxidation of 70–90% of the total cellular cholesterol, supporting the view that almost all cellular cholesterol is localized in the plasma membrane (Lange & Ramos, 1983; Freeman, 1987; Slotte et al., 1989, 1990; Lange et al., 1989). On the other hand, calculations based on stereological analyses suggest that plasma membrane might account for only 25–40% of cellular cholesterol in BHK cells and in Madin–Darby canine kidney cells (MDCK) (Van Meer, 1987). In addition, whereas morphological experiments coupled with the use of the polyene antibiotic filipin, which makes fluorescent complexes with cholesterol that deform membrane structure (Bittman & Fischoff, 1972; De Kruijff & Demel, 1974; Bolard, 1986), strongly suggest the existence of cholesterol pools in the plasma membrane of eukaryotic cells (Elias et al., 1979; Severs & Robenek, 1983), a view supported by ESR and fluorescence polarization experiments (van Blitterswijck et al., 1982; Gordon et al., 1983; Schroeder et al., 1991), the cholesterol oxidase technique so far failed to provide evidences for their presence (Lange, 1992).

Renal and intestinal epithelial cells appear as useful models for studies on cholesterol distribution in biomembranes. Their plasma membrane is constituted of two distinct domains, the apical domain which most often shows numerous microvilli (brush border) and the basolateral domain, of which the lipid composition differs. In particular, the cholesterol to phospholipid ratio (C/P ratio) of the apical domain is significantly higher than that of the basolateral domain (Brasitus & Schachter, 1980; Carmel et al., 1985; Molitoris et al., 1985) and physiopathological conditions alter its cholesterol content, leaving that of the basolateral domain unchanged (Brasitus & Schachter, 1982; Molitoris et al., 1985). In accordance with these data, cholesterol is heterogeneously distributed in renal and intestinal brush border membranes (BBM)[1] (El Yandouzi & Le Grimellec, 1992, 1993; Bloj & Zilversmit, 1982). LLC-PK1 is an epithelial cell line with multiple differentiated characteristics of the renal proximal tubule (Rabito, 1986). The cells form a continuous epithelium expressing the polarity of plasma membrane components, with abundant brush borders and well-developed tight junctions (Rabito et al., 1987), which act as a barrier to the diffusion of lipids between the external leaflets of the apical and basolateral domains of the plasma membrane (Dragsten et al., 1981). Their functional and biochemical polarity, in terms of enzymes and transport systems, justifies their use as an in vitro model of kidney proximal tubule cells (Gstraunthaler, 1988).

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[1] Abbreviations: PBS, phosphate-buffered saline; BBM, brush border membrane vesicles; DDAVP, 1-deamino[D-Arg 8]vasopressin.

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In the present experiments, we have examined the distribution of cholesterol as well as the existence of plasma membrane cholesterol pools in LLC-PK1 cells grown as a confluent monolayer using *Brevibacterium* sp. cholesterol oxidase which oxidizes more than 90% of the cholesterol present in isolated renal BBM according to a biphasic process (El Yandouzi & Le Grimellec, 1992, 1993).

EXPERIMENTAL PROCEDURES

Materials. Cholesterol oxidase (EC 1.1.3.6) from *Brevibacterium* sp. was obtained from Beckman Instruments (Nyon, Switzerland). Sphingomyelinase from *Bacillus cereus* was obtained from Boehringer Mannheim. Filipin was from Sigma Chemical Co. (St. Louis, MO). [2-^{14}C]Mevalonic acid (sodium salt, 49 mCi/mmol) was purchased from C. E. A. (Saclay, France). Culture media were from Gibco (Paisley, Scotland). Plasticware was purchased from Costar (France). After other reagents were of analytical grade.

Cell Culture. LLC-PK1 cells (passages 212–230) were grown to confluence on either 35-mm diameter plastic Petri dishes or on 25-mm diameter permeable filters (Costar Transwell no. 3412) at 37 °C, in a 5% CO_2/95% air atmosphere. The growth medium was a 50:50 mixture of Ham's F-12 and Dulbecco's modified Eagle medium (DMEM). For cells grown on Petri dishes, the medium was supplemented with insulin (25 μm/mL), transferrin (10 μg/mL), triiodothyronine (5 nM), hydrocortisone (0.2 μM), DDAVP (0.1 μM), and cholesterol (0.1 μM). For cells grown on permeable filters, the medium contained in the lower chamber was supplemented with 5% fetal calf serum. The medium was changed on the second day and then on alternative days.

Labeling of Cellular Cholesterol. For cells grown on Petri dishes, 48 h before the experiments (between days 5 and 6), 0.5 μCi/mL of [^{14}C]mevalonate in ethanol was added to the medium of each dish. For cells grown on permeable support, the upper and lower medium of cells plated for 6–8 days on Transwell cell culture chambers were replaced by 1.6 mL of medium (without calf serum) and 2.6 mL of medium supplemented with hormones and [^{14}C]mevalonate, respectively. The monolayers were further incubated for 48 h at 37 °C.

Oxidation of Cell Cholesterol. Cells were washed two times with mannitol/Tris/$CaCl_2$ (300/12/0.5 molar ratio), pH 7.4. After 10-min preincubation in this buffer, sphingomyelinase and or cholesterol oxidase were added to a final concentration of 1 and/or 5 IU/mL, respectively. After various times of incubation, cells were chilled and rinsed, and immediately lipids were extracted and stored at −20 °C. In most experiments, cells were fixed with 1% of glutaraldehyde in mannitol buffer during 15 min at 4 °C prior to treatment with cholesterol oxidase. Unreacted glutaraldehyde was eliminated by washing twice with mannitol buffer, and the enzyme(s) was(were) added as previously.

Lipids Analysis. Lipids were extracted according to Hara and Radin (1978) with a hexane/2-propanol mixture (3:2 vol/vol) and sonication of the samples. Extracts were evaporated to dryness under a nitrogen stream and solubilized in a chloroform/methanol mixture (2:1, vol/vol). Lipids were analyzed for [^{14}C]cholesterol and [^{14}C]cholestenone by thin-layer chromatography on precoated silica gel plates, using hexane/diethyl ether/acetic acid (130:30:1.5, vol/vol) as the developing solvent. Lipid spots were detected by exposure to iodine vapor and identified by comparison with authentic standards. The appropriate spots were marked, the I_2 stain was removed, and the spots were scraped off and transferred into scintillation vials for radioactivity determination (LKB RackBeta). Cholesterol oxidation was estimated on the basis of cholesterol remaining and cholestenone formation [cholestenone/(cholesterol + cholestenone)].

Oxidation of Cholesterol in Isolated Renal Membranes. Brush border membranes vesicles from the kidney cortex of male New Zealand White rabbits were isolated as previously described (El Yandouzi & Le Grimellec, 1992) using an $MgCl_2$ precipitation method (Booth & Kenny, 1974), which gave close, right-side-out vesicles (Venien et al., 1988). In BBM preparations, the activity of alkaline phosphatase was enriched 10–15-fold over that of cortical homogenates compared to less than 0.7-fold for Na^+/K^+-ATPase and less than 0.5-fold for glucose-6-phosphatase. Membranes were suspended (4 mg of protein/mL) in 150 mM NaCl/20 mM Hepes (pH 7.4). Treatment with cholesterol oxidase (1 IU/mL) was performed in parallel on native BBM and on glutaraldehyde-fixed BBM. For fixation, membranes were treated with 1% glutaraldehyde in NaCl/Hepes buffer for 15 min at 4 °C. Unreacted glutaraldehyde was eliminated by centrifugation (20 min, 30000*g*, 5 °C) and washing in the above-mentioned buffer. BBM diluted in 5 vols of a 50 mM mannitol/2 mM Tris (pH 7.0) (final NaCl concentration: 25 mM) were preincubated for 10 min at 37 °C before adding cholesterol oxidase (El Yandouzi & Le Grimellec, 1992). 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, and the amount of cholesterol oxidized, corrected for the recovery of extraction, was determined enzymatically (Omodeo-Salé et al., 1984).

Filipin Labeling of Fixed Cells. Cells were fixed in glutaraldehyde as described above, washed in $NaBH_4$ (0.5 mg/mL in phosphate saline buffer, pH 7.4), and incubated for 15 min at room temperature with 125 μg of filipin/mL of PBS buffer (Pagano et al., 1989). The cells were then washed twice in PBS and observed by fluorescence microscopy using a 100× Dapo UV objective and ultraviolet excitation (combination cube U, IMT2 inverted microscope, Olympus). Photomicrographs were obtained using Tri-X film (ASA 400, Kodak).

Cellular Viability. Cellular viability was estimated either by the ability to exclude the vital dye trypan blue or by the determination of the release of lactate dehydrogenase (LDH), a marker of nonspecific cellular injury, as previously described (Blais et al., 1993).

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 in LLC-PK1 Cells Grown on Solid Support. To facilitate oxidation, LLC-PK1 cells grown on plastic support as a confluent monolayer were incubated in a low ionic strength medium, which contained 0.5 mM calcium for maintaining tight-junction integrity. As judged from trypan blue exclusion and LDH release, this medium did not alter the cellular viability during the time of experimentation. Adding cholesterol oxidase alone (5 IU/mL) or cholesterol oxidase plus sphingomyelinase (1 IU/mL) to the medium for incubations as long as 4 h had a limited but significant effect on the cholesterol content of the cell monolayer. As indicated in Table 1, oxidation essentially

Table 1: Oxidation of Cholesterol in Unfixed LLC-PK1 Cells[a]

	cholesterol remaining (% of control)	
treatment duration (h)	chol oxidase	chol oxidase + SMase
1	87 ± 6	81 ± 3
4	84 ± 4	72 ± 5

[a] Monolayer cultures of LLC-PK1 cells grown on plastic support were incubated with 5 IU/mL of *Brevibacterium* sp. cholesterol oxidase (chol. oxidase) alone or cholesterol oxidase plus sphingomyelinase (SMase). At the time indicated, cells were chilled and rinsed, and the remaining cholesterol was assayed as described under Experimental Procedures. Data are presented as mean ± SEM of three experiments.

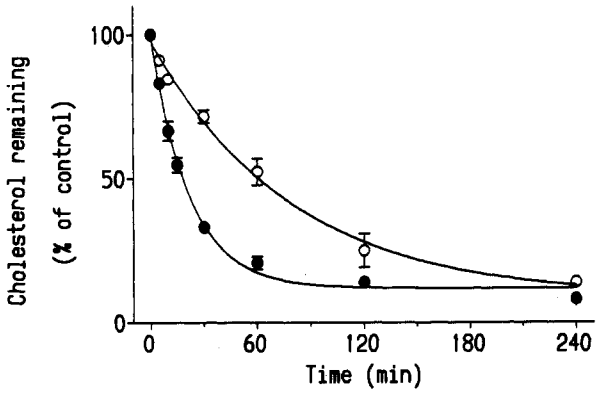

FIGURE 1: Oxidation of cholesterol in fixed LLC-PK1 cells, at 37 °C. LLC-PK1 cells grown on plastic support were incubated with 0.5 μCi/mL of [^{14}C]mevalonic acid for 48 h. Prior to treatment with cholesterol oxidase (○) or cholesterol oxidase plus sphingomyelinase (●), cells were fixed with 1% of glutaraldehyde during 15 min at 4 °C as described under Experimental Procedures. The cholesterol remaining was determined after various times of incubation with enzymes at 37 °C.

proceeded during the first hour of incubation. Because these enzymes were likely internalized via endocytic processes during such long incubations, which limits data interpretation, cells were fixed by adding glutaraldehyde prior to the enzyme treatment, a procedure commonly adopted when using cholesterol oxidase to probe the distribution of cholesterol in intact cells (Lange, 1992). Following this treatment, the addition of cholesterol oxidase resulted in the oxidation of 86.1 ± 1.8% of the cellular cholesterol in 4 h (Figure 1). Curve fitting by nonlinear regression indicated that the oxidation of cholesterol in glutaraldehyde-treated cells was best fitted by a single exponential (goodness of fit $R^2 > 0.99$; $t_{1/2} = 54$ min). The addition of sphingomyelinase to the buffer increased the rate of cholesterol oxidation, which still corresponded to a monoexponential process ($R^2 > 0.99$; $t_{1/2} = 14$ min), without affecting its maximal value (Figure 1). Although this suggested that the single cholesterol pool corresponded to plasma membrane cholesterol, movements of cholesterol from intracellular membranes with a rate constant higher than that of cholesterol oxidation could have led to the same kind of kinetics. Glutaraldehyde-treated LLC-PK1 cells, grown on solid support, were therefore incubated at 15 °C, i.e., a temperature which was reported to block the transport of intracellular cholesterol to the plasma membrane in living cells (Kaplan & Simoni, 1985). The addition of both cholesterol oxidase and sphingomyelinase resulted in a 90% cholesterol oxidation within 6 h, whose time course was best described by a single exponential (goodness of fit $R^2 > 0.95$; $t_{1/2} = 1.69$ h, Figure 2). On the other hand when adding cholesterol oxidase alone, only half of the cell cholesterol was oxidized in 3 h (Figure 2). This level remained practically stable up to 15 h, which indicated that unless sphingomyelin

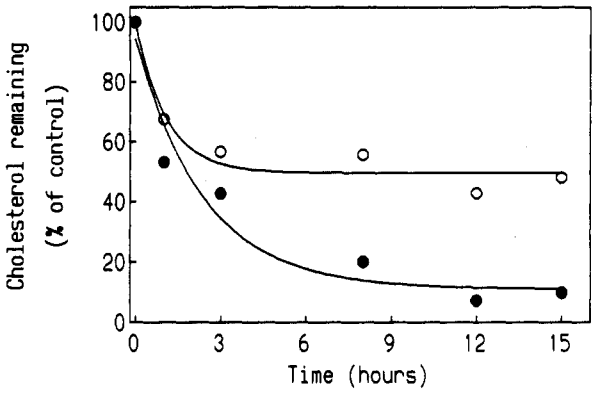

FIGURE 2: Cholesterol oxidation in LLC-PK1 cells at 15 °C. Experimental conditions and symbols are as described for Figure 1.

was degraded about 50% of the membrane cholesterol was not available for oxidation at 15 °C.

Incubation of glutaraldehyde-fixed cells with filipin, at 37 °C, resulted in a pronounced labeling of the plasma membrane, with numerous microvilli appearing as dots on the cell surface (Figure 3a). Back-exchange at the same temperature using BSA buffer resulted in a marked but incomplete withdrawal of the plasma membrane fluorescence, which allowed the visualization of a faint intracellular labeling. Occasionally, fluorescent structures, likely corresponding to the Golgi apparatus (Orci et al., 1981; Pagano et al., 1989), were observed (Figure 3b). Labeling of the cells at 15 °C gave pictures identical to those at 37 °C; however, back-exchange was practically inefficient at this temperature (Figure 3c). Treatment with cholesterol oxidase at 37 °C for 3 h resulted in a dramatic decrease in fluorescence intensity (Figure 3d). In particular, the plasma membrane of cells was no longer visible. On the other hand when incubating at 15 °C for 6 h, fluorescence of the plasma membrane, albeit significantly lower than in controls, was still marked (Figure 3e). Treatment with both cholesterol oxidase and sphingomyelinase for 6 h at 15 °C abolished the plasma membrane labeling (Figure 3f).

Cholesterol Accessibility in Cells Grown on Permeant Support. The observation that 80–90% of the unesterified cell cholesterol was oxidizable with first-order kinetics from the apical side of glutaraldehyde-fixed LLC-PK1 cells grown on solid support might have originated from an incomplete sealing of tight junctions and/or from an incomplete cell polarization (Ojakian et al., 1987). Accordingly, the effects of cholesterol oxidase were investigated on cells grown to confluence on permeant support, where they reach a proper polarized state. Incubation with cholesterol oxidase in the apical medium for 4 h at 37 °C resulted in a limited (13 ± 1%) oxidation of the cell cholesterol, which compared with the value obtained from cells grown on Petri dishes. Cholesterol oxidation from the basolateral side added enzyme was also limited (8 ± 2% of total cholesterol). In fixed monolayers, cholesterol oxidase introduced in the apical medium oxidized up to 85% of cellular cholesterol (Figure 4) according to a first-order process (goodness of fit $R^2 > 0.99$, $t_{1/2} = 70$ min). Cholesterol oxidation obtained when adding the oxidase to the basal side was not significantly different from this value (Figure 5). Cotreatment with sphingomyelinase increased the oxidation rate, but not the extent of oxidation, only when both enzymes were added to the apical side of cultures. Surprisingly, adding cholesterol oxidase simultaneously to the apical and basolateral baths did not

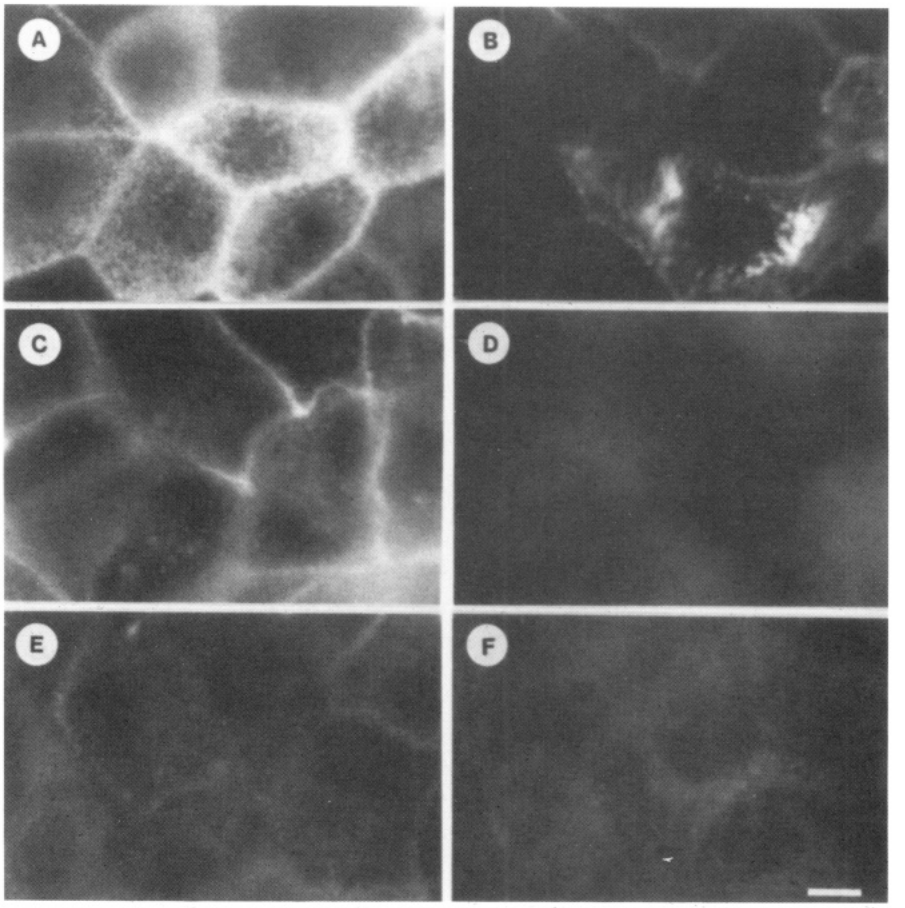

FIGURE 3: Labeling of LLC-PK1 cells with filipin. Fixed cells were washed in $NaBH_4$ and incubated for 15 min at room temperature with 125 μg of filipin/mL of phosphate saline buffer as described under Experimental Procedures. (a) Incubation of fixed cells at 37 °C; (b) back-exchange using BSA buffer at the same temperature or (c) at 15 °C; (d) treatment of cells with cholesterol oxidase at 37 °C for 3 h or (e) at 15 °C for 6 h; (f) treatment of cells with cholesterol plus sphingomyelinase at 15 °C for 6 h. Bar: 10 μm.

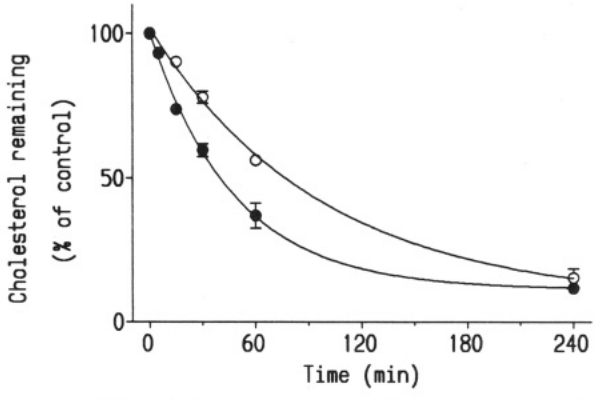

FIGURE 4: Effect of cholesterol oxidase added on the apical side of LLC-PK1 cells grown on permeant support. Cells grown to confluence on permeable filters (Costar transwell) were fixed by adding glutaraldehyde. Cholesterol oxidase alone (○) or cholesterol oxidase plus sphingomyelinase (●) were introduced in the apical medium.

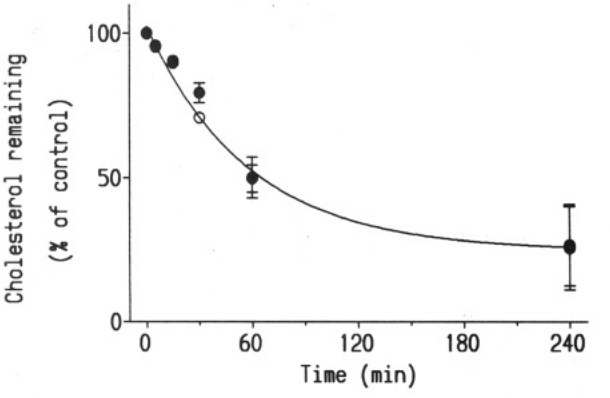

FIGURE 5: Effect of cholesterol oxidase added on the basolateral side of LLC-PK1 cells grown on permeant support. Experimental conditions and symbols are as described for Figure 4.

increase the extent of oxidation (Table 2). None of the above treatments increased the cell permeability to trypan blue.

Effect of Glutaraldehyde Fixation on Renal BBM. Oxidation of cholesterol in nonfixed isolated renal brush border membrane vesicles (BBM) is biphasic (El Yandouzi & Le Grimellec, 1992). In an attempt to understand the different behavior of apical membranes of LLC-PK1 in situ, the effect of fixation on the cholesterol distribution in renal BBM was investigated. As shown by Figure 6, fixation with glutaraldehyde markedly increased the oxidation rate of cholesterol so that the characteristic biphasic cholesterol oxidation of native BBM was no longer observed, without affecting significantly the extent of the oxidation (85 ± 6 vs 87 ± 4%).

DISCUSSION

The present experiments demonstrate that in polarized renal epithelial LLC-PK1 cells treated with glutaraldehyde, 86% of the cellular cholesterol is accessible as a single pool to cholesterol oxidase, added either to the apical or to the basolateral side of the cells at 37 °C. Oxidation at 15 °C and filipin experiments further supported that the plasma membrane was, by far, the most important site of cholesterol localization in LLC-PK1 cells. They also strongly suggest

Table 2: Effect of Simultaneous Cholesterol Oxidase Addition to Apical and Basolateral Side of LLC-PK1 Cells[a]

incubation time (min)	cholesterol oxidase addition	cholesterol remaining (% of control)
30	apical	79.7 ± 2.9
	basolateral	72.0 ± 1.5
	both	68.7 ± 3.7
60	apical	55.7 ± 1.1
	basolateral	51.8 ± 6.0
	both	52.0 ± 1.1
240	apical	15.4 ± 3.2
	basolateral	23.4 ± 12.6
	both	15.2 ± 4.0

[a] LLC-PK1 cells grown on permeable filters were fixed with 1% of glutaraldehyde during 15 min at 4 °C as described under Experimental Procedures. Cholesterol oxidase (5 IU/mL) was added to the apical or to the basolateral bath or to both baths simultaneously.

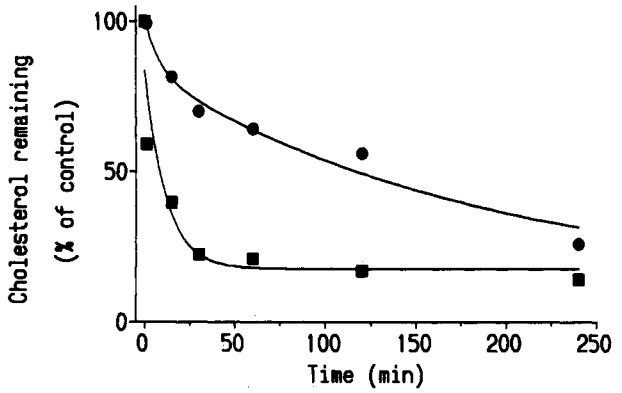

FIGURE 6: Effect of glutaraldehyde fixation on cholesterol oxidation in renal brush border membranes. Treatment with cholesterol oxidase (1 IU/mL) was performed in parallel on native BBM (●) and on glutaraldehyde-fixed BBM (□). For fixation, BBM were treated with 1% of glutaraldehyde for 15 min at 4 °C as described under Experimental Procedures. At the time indicated, aliquots for the mixture were taken, and the cholesterol remaining was determined.

that glutaraldehyde fixation prior to cholesterol oxidase addition precluded the detection of cholesterol pools present in the plasma membrane of LLC-PK1 cells.

Treatment of glutaraldehyde-fixed cells with cholesterol oxidase results in the oxidation of 80–90% of total unesterified cholesterol in fibroblast (Lange & Ramos, 1983; Lange et al., 1989), Chinese hamster ovary cells (Lange & Ramos, 1983), and macrophages (Tabas et al., 1988). The value obtained for LLC-PK1 cells falls within that range. The cholesterol pool susceptible to the oxidase is generally identified as the plasma membrane cholesterol (Gottlieb, 1977; Lange & Ramos, 1983). Several arguments indicate that this must also be valid in LLC-PK1 cells: (a) In all the experimental conditions assayed, the cellular integrity was maintained as shown by the absence of significant LDH release by living cells and by the trypan blue exclusion test in fixed cells, so the enzyme only had access to the membrane surface. (b) Half-times for cholesterol oxidation as short as 15 min, in the presence of sphingomyelinase, could be determined for cells grown on plastic dishes, i.e., much shorter than the $t_{1/2}$ of cholesterol spontaneous transfer between membranes (Philips et al., 1987). (c) Lowering the temperature to 15 °C, which even in nonfixed cells, inhibits the transfer of cholesterol from intracellular membranes to plasma membrane (Kaplan & Simoni, 1984), still allowed the oxidation of 90% of the unesterified cholesterol in the presence of sphingomyelinase. The increase in $t_{1/2}$ obtained when lowering the temperature from 37 to 15 °C was compatible with the data obtained under similar conditions in red blood cells (Lange et al., 1981). (d) Whereas filipin treatment of control cells gave fluorescence micrographs with a very high intensity at the plasma membrane level, no more labeling of the plasma membrane was detectable after incubation for 3 h with the oxidase. The remaining very faint labeling corresponded to intracellular membranes. Taken together, these data strongly suggested that, as in many other cell types (Lange, 1992), unesterified cellular cholesterol was essentially present in the plasma membrane of LLC-PK1 cells.

In LLC-PK1 cells as in other epithelial cells, tight junctions act as a barrier to the diffusion of lipids between the external leaflets of the apical and basolateral domains of the plasma membrane (Dragsten & al., 1981; Van Meer & Simons, 1986). It was previously shown that, in accordance with the difference in cholesterol concentration between the apical and basolateral plasma membrane domain of renal cells and with the independent control of this concentration between these two domains, cholesterol is heterogeneously distributed in isolated brush border membranes (El Yandouzi & Le Grimellec, 1992, 1993). The observation that whatever the side, apical or basolateral, of the oxidase addition only one plasma membrane cholesterol pool was detectable from kinetics analysis of oxidation in glutaraldehyde-fixed cells was therefore surprising. Several lines of evidences strongly suggested that, in LLC-PK1 cells, glutaraldehyde fixation prevented the detection of cholesterol pools present in the plasma membrane: (a) Upon addition of the enzyme to the apical side of nonfixed cells grown on filters or on dishes, 13% of cell cholesterol was oxidized in 4 h. Taking into account that in proximal tubular cells the apical domain represents about half of the plasma membrane surface (Welling & Welling, 1975), this value compared with the size of the cholesterol pool accessible to the oxidase (25–30%) in isolated renal brush border membranes (El Yandouzi & Le Grimellec, 1992, 1993). The lower oxidation obtained when adding the oxidase on the basolateral side could correspond to its lower cholesterol content (Carmel et al., 1985; Molitoris & Simon, 1985) and/or to its different phospholipid composition (Carmel et al., 1985). Using model membranes, it was shown that cholesterol availability to cholesterol oxidase markedly depends on both the cholesterol concentration and the phospholipid composition of liposomes (Patzer et al., 1978; Pal et al., 1980; Grönberg & Slotte, 1990). Despite these results on living cells, cholesterol oxidation obeyed first-order kinetics in corresponding fixed cells. (b) In isolated renal brush border membranes where cholesterol oxidation followed a characteristic biphasic pattern, glutaraldehyde treatment, likely by modifying protein/lipid and lipid/lipid interactions since it presumably lowers the pK of the aminogroups on aminophospholipids, markedly increased the rate of oxidation by the oxidase rendering the two cholesterol pools undiscernible. (c) In glutaraldehyde-fixed cells, adding the oxidase to the apical and the basolateral side simultaneously did not further increase the oxidation rate determined from conditions where the enzyme was given only on one side. The simplest explanation for this last observation, a diffusion of the oxidase from one side to the other through the tight junctions, was ruled out by the experiments done in the presence of sphingomyelinase. In accordance with previous work on fibroblasts (Slotte et al., 1989), sphingomyelinase increased the rate of cholesterol oxidation in LLC-PK1 cells grown on solid support. Preferential interactions between sphingomyelin, the predominant phospholipid of the apical membrane of renal proximal tubule cells (Carmel et al., 1985; Venien & Le Grimellec, 1988), and cholesterol likely explained these data (Demel et al., 1977; Patzer et al., 1978; Yeagle &

Young, 1986; Slotte et al., 1990). Moreover, experiments at 15 °C strongly suggested that approximately half of the cell cholesterol was involved in such sphingomyelin/cholesterol interactions. On the other hand, for fixed cells grown on permeant support, sphingomyelinase increased the rate of cholesterol oxidation only when given on the apical side. This data, explainable by the much lower content in sphingomyelin of the basolateral domain of the plasma membrane of renal cells (Carmel et al., 1985), indicated that the junctions had remained tight and impermeable to macromolecules. Accordingly, the lack of additivity of cholesterol oxidase when given on both sides of fixed cells suggested that the enzyme in the apical and basolateral bath had access to the same successive cholesterol pools. In other words, the first-order kinetics observed for cholesterol oxidation in glutaraldehyde-fixed cells only revealed the rate-limiting step of the reaction, i.e., the cholesterol transfer between membrane pools. Rapid flip-flop (Lange et al., 1981) and lateral diffusion of cholesterol might account for such a behavior. Existence of multiple cholesterol pools that became successively accessible to the cholesterol oxidase was previously advanced as an explanation of the oxidation pattern observed with various cholesterol oxidases in isolated renal brush border membranes (El Yandouzi & Le Grimellec, 1992). Whereas it has become increasingly evident that cholesterol is heterogeneously distributed in biological membranes (Schroeder et al., 1991), experiments using cholesterol oxidase on fixed cells have not allowed the detection of the existence of plasma membrane cholesterol pools (Lange, 1992). The present data strongly suggest that these different findings might be due, at least partly, to the effects of glutaraldehyde on membrane properties.

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