Journal of Cellular Biochemistry 21:141–150 (1983)

Role of Cholesterol in the Structure and Function of Gastric Microsomal Vesicles

Tushar K. Ray, Jyotirmoy Nandi, Andrew Dannemann, and Gerald B. Gordon

Department of Surgery and Pathology, State University of New York, Upstate Medical Center, Syracuse, New York 13210

Digitonin was used as a tool to investigate the organization and function of cholesterol in gastric microsomes. Microsomal vesicles were treated with digitonin for different time at 0–4°C under isotonic conditions. The effects of digitonin treatment of the vesicles on removal of cholesterol, ultrastructural changes, (H^+ + K^+)-ATPase activity, and gastric ATPase-dependent H^+ uptake ability were investigated. Microsomal cholesterol was extracted in an exponential manner with a $t_{1/2}$ of 32 min. There was no release of microsomal phospholipids by digitonin treatment during the same period. Digitonin treatment (30 min) produced visible "holes" in the vesicles; at the same time (H^+ + K^+)-ATPase-dependent H^+ uptake was abolished. Under the same conditions the K^+-stimulated ATPase activity, however, was moderately (about 35%) reduced, although the response of K^+ stimulation to valinomycin was obliterated. Longer digitonin treatment resulted in gradual diffusion and eventual disappearance of the "holes" with the generation of distorted cup-shaped microsomes. The data strongly suggest that membrane lipids are freely mobile and that there is a certain degree of specialization in the organization of gastric microsomal cholesterol for the proper maintenance of the membrane structure and function.

Key words: gastric microsomal vesicles, membrane cholesterol, digitonin, H^+, K^+-ATPase, vesicular H^+ transport

Cholesterol and phospholipids are the two primary lipid components of the animal cell plasma membranes. Although significant progress has been made in recent years in our understanding of the distribution and function of phospholipids in biological membranes [1], the knowledge on similar aspects of cholesterol remains relatively scanty. Furthermore, the nature and extent of orientation of cholesterol across the membranes have been reported to be different in different cell types [2–6]. Also, the role of membrane cholesterol in the function of various membrane-bound enzymes is not well understood. Thus, the topology of cholesterol and its impact on membrane function are areas that need more exploration.

The lipid composition of purified gastric microsomes derived primarily from the apical and tubulovesicular membranes of the parietal cells from a number of

Received June 30, 1982; revised and accepted December 30, 1982.

different species has been well characterized [7]. The microsomes are highly enriched in K^+-stimulated ATPase, which has been identified as the proton pump [8,9]. Recent studies from our laboratory [10,11] using purified gastric microsomes demonstrated that the cholesterol-to-phospholipid molar ratio of the lipids extracted by 15% ethanol for 60 sec at different temperatures varied to a great extent. The cholesterol molecules were predominantly extracted with increasing temperature by ethanol treatment of pig gastric microsomes [11]. The previous [11] work not only suggested a heterogeneity in the lipid topology on the microsomal surface but also demonstrated specificity of the lipids in gastric ATPase function. Furthermore, it was suggested [11] that cholesterol exists in the form of "islands" or "patches" surrounded by neighboring phospholipids within the membrane matrix.

In order to elucidate further the role of cholesterol in gastric microsomes we tested the effects of digitonin treatment on gastric microsomal structure and function. The activity of gastric K^+-stimulated ATPase and the accompanying H^+ uptake ability of the microsomal vesicles were studied before and after treatment with digitonin under controlled conditions. Under appropriate situations digitonin treatment created numerous visible "holes" on the microsomal surface within 30 min. Appearance of the "holes" correlated well with the depletion of membrane cholesterol and the abolition of vesicular H^+ uptake mediated by the gastric K^+-stimulated ATPase system. Microsomal K^+-stimulated ATPase activity was also reduced by about 35% under similar conditions. However, the valinomycin-stimulated ATPase activity, which has been estalished [6,7] as an indicator of vesicular integrity, was completely abolished. Prolonged exposure to digitonin caused gradual disappearance of the small "holes" followed by the formation of abundant cup-shaped structures. The data offer some insight into the role of cholesterol in the structure-function relationship of gastric microsomal vesicles.

METHODS

Isolation of Gastric Microsomes

Fresh pig stomachs were purchased from a local slaughterhouse. The gastric microsomal membranes were harvested by a procedure described previously [12]. All procedures were carried out at 0–4°C. Briefly, the fundic mucosa from the pig was desquamated and scraped [13] to collect a parietal cell-enriched population. The mucosal scraping was homogenized gently in a medium consisting of 250 mM sucrose, 0.2 mM EDTA, and 0.2 mM Pipes buffer (pH 6.8) using a loose pestle Dounce homogenizer. The homogenate was centrifuged at 8,000g for 5 min. The process was repeated three times. All the supernatants were pooled together and layered over 40 ml of 37% sucrose in the 84-ml capacity screw cap tubes and centrifuged at 100,000g for 5 h in a type 35 Beckman angular rotor. The microsomal membrane bands appeared at the interface of soluble supernatant and 37% sucrose. The microsomal bands were collected, diluted with homogenizing medium, and centrifuged at 100,000g for 90 min. The pellet was suspended in the homogenizing medium with a protein concentration of 0.5 mg/ml and used in our study. The proteins were assayed by the Lowry procedure [14].

Assay of ATPase and pNPPase

The ATPase was assayed as previously described [12]. Briefly, the incubation mixture contained in a total volume of 1 ml, 50 μmol Pipes buffer (pH 6.8), 1 μmol $MgCl_2$, 2 μmol Tris-ATP, 20 μg membrane protein with or without 150 μmol KCl in presence and absence of 10^{-5} M valinomycin. After a 10-min incubation at 21°C the reactions were stopped by 1 ml 12% CCl_3COOH. The P_i was assayed by the procedure of Sanui [15].

For paranitrophenylphosphatase (pNPPase), the incubation mixture contained in a total volume of 1 ml 50 μmol Pipes buffer (pH 6.8), 2μmol $MgCl_2$, 5 μmol pNPP, 10 μg membrane protein, with and without 20 μmol KCl. After 20 min of incubation at room temperature the reactions were stopped by 1 ml 1.5 N NaOH. After a brief centrifugation the supernatant was read at 410 nm.

Study of Vesicular H^+ Uptake

Vesicular accumulation of H^+ was measured at room temperature according to Lee and Forte [16] and as described previously [17]. The method uses the change in fluorescence intensity (quenching) of acridine orange which is proportional to the amount of dye taken up by the microsomes. The amount of dye taken up is a sensitive measure of intravesicular H^+ concentration. Wavelengths used were 493 → 530 nm (excitation → emission) for acridine orange in the Aminco Bowman spectrofluorometer.

Treatment With Digitonin

Digitonin was dissolved in CH_3OH:H_2O (1:1) at a concentration of 20 mg/ml. Aliquots of digitonin solution were added to microsomal suspension (1–2 mg) in 0.25 M sucrose–0.2 mM EDTA–0.2 mM Pipes buffer (pH 6.8) at 0–4°C so that the final concentration of digitonin was 0.8 mg/mg membrane protein. Treatment with digitonin was conducted for different time periods as will be specified in the Results section. At the end of the digitonin treatment, the microsomes were diluted 10-fold with ice-cold buffer as above and centrifuged at 100,000g for 60 min. The control microsomes were treated with an equal amount of CH_3OH:H_2O (1:1) without any digitonin and run in parallel. For assays such as ATPase and pNPPase activities and lipid estimation, the pellet was suspended carefully in the same ice-cold buffer mentioned above at an appropriate protein concentration.

Assay of Lipids

The lipids were extracted from the control and digitonin-treated microsomes by the method of Bligh and Dyer [18] and as described previously [19] using a nitrogen atmosphere to prevent aerial oxidation. After evaporation of the lipid extracts to dryness using nitrogen, the lipids were dissolved in 1 ml of acetone–95% ethanol (1:1, v/v). One milliliter of ethanol–water (1:1, v/v) was then added to the lipid solution and allowed to stand at room temperature for 10 min for the precipitation of the cholesterol-digitonide complex. The precipitate was harvested by centrifugation at 2,000g for 5 min, washed twice with acetone, and then dissolved in glacial acetic acid for the estimation of cholesterol by the $FeCl_3$–H_2SO_4 method [20]. The super-

natant was assayed for lipid phosphorus using Bartlett procedure [21] and cholesterol by the $FeCl_3$–H_2SO_4 method [20].

Electron Microscopy

Ice-cold 2.5% glutaraldehyde in 0.02 M phosphate buffer (pH 7.25) was layered over the microsomal pellet (see Treatment With Digitonin) and allowed to fix overnight at 5°C. After washing in 0.2 M phosphate buffer the pellets were cut into 1-mm^3 blocks and postfixed in veronal-buffered osmium tetroxide (1%) [22]. Dehydration was followed by embedding in Araldite [23], and sections were cut with an LKB ultramicrotomes. The thin sections were stained with uranyl acetate [24] and lead citrate [25] before viewing in a Philips EM-300 electron microscope.

Aliquots of the control and digitonin-treated microsomal suspension were also examined by negative staining using 1% sodium phosphatungstate, pH 7.0 [26].

RESULTS

The effects of digitonin treatment of gastric microsomes for different periods on the distribution of phospholipids and cholesterol associated with the membrane and the supernatant are shown in Table I. Treatment with digitonin released cholesterol from the membrane phase without any effect on the phospholipid content of the microsomes (Table I). About 70% of the microsomal cholesterol was removed within 60 min, resulting in a drastic reduction of the cholesterol to phospholipid molar ratio (Table I). The value of total phospholipids (Table I) is in agreement with Saccomani et al [27] but higher than those previously reported [6]. The prior values [6] have been traced to incomplete digestion of the phospholipids.

Previous studies [28] from this laboratory suggested that about 15% of the microsomal vesicles isolated by our procedure are leaky or ruptured. Thus, about 5 μmoles of cholesterol/100 mg protein will be contributed by the ruptured vesicles. A plot of the cholesterol content of the intact vesicles after digitonin treatment for

TABLE I. Effects of Digitonin Treatment on the Distribution of Cholesterol in Gastric Microsomes and Supernatant

	Lipids (μmoles/100 mg microsomal proteins)			
	Microsomes			
Treatment	Phospholipid (P)	Cholesterol (C)	C (mol) / P (mol)	Supernatant (cholesterol)
None (control)	80.5 ± 1.4	42.9 ± 1.3	0.53	0
Digitonin, 15 min	80.0 ± 1.9	33.3 ± 0.5	0.41	7.25 ± 1.8
Digitonin, 30 min	79.5 ± 1.1	21.9 ± 1.4	0.27	22.1 ± 1.4
Digitonin, 60 min	79.5 ± 2.2	12.1 ± 0.9	0.15	31.8 ± 1.8

Gastric microsomes (about 1 mg) were incubated with digitonin (0.8 mg/mg protein) for the designated time at 0–4°C. Control membranes were run in parallel with the same concentration of solvent (0.02 ml methanol:water: 1:1) but without any digitonin. At the end of the incubation period the membranes were diluted 10-fold with sucrose (250 mM)-Pipes (10 mM)-EDTA (0.2mM) buffer (pH 6.8) and centrifuged for 90 min at 100,000g. The cholesterol-digitonin complex also appeared with the membrane pellet. The details of the separation of the cholesterol-digitonin complex from the membrane and the assay of lipids are given in Methods. The amount of cholesterol liberated from the microsome after digitonin treatment has been expressed on the basis of microsomal protein. The data are mean ± SEM (n = 4).

various lengths of time (Fig. 1) shows that the membrane cholesterol is depleted at an exponential rate (rate constant = 0.0215/min) with a $t_{1/2}$ of about 32 min.

The data in Figure 2a demonstrate that the sensitivity of the K^+-stimulated ATPase activity to valinomycin is obliterated within 30 min of digitonin treatment. Furthermore, the K^+-stimulated ATPase activity is reduced by 36% and 60% after 30 and 60 min of digitonin treatment respectively (Fig. 2a). Under similar conditions, however, the K^+-stimulated pNPPase activity is reduced by 20% and 40% respectively (Fig. 2b). Although the K^+-stimulated pNPPase activity is believed to be a partial reaction of the gastric K^+-stimulated ATPase system [29,30], such differential effects of digitonin treatment on the two enzymatic activities are unclear at present.

The gastric K^+-stimulated ATPase system has recently been identified as the enzymatic mechanism for the active transport of protons in intact mucosa [31,32] as well as in isolated gastric microsomal vesicles [8,9]. Under appropriate conditions, as shown in Figure 3, the microsomal vesicles can accumulate H^+ mediated by the gastric K^+-stimulated ATPase system. However, within 30 min of digitonin treatment the ability of the microsomes to accumulate H^+ within the vesicles is completely abolished (Fig. 3).

Morphology of the gastric microsomal vesicles under control and digitonin-treated conditions is shown in Figures 4–6. Contrary to the control microsomes (Fig. 4), the vesicles after 30 min of digitonin treatment show numerous "holes" clearly visible in the negatively stained preparations (Fig. 5). At 60 min of treatment, the "holes" appear ill-defined or diffused, and more of the distorted cup-shaped microsomal structures were found (Fig. 6).

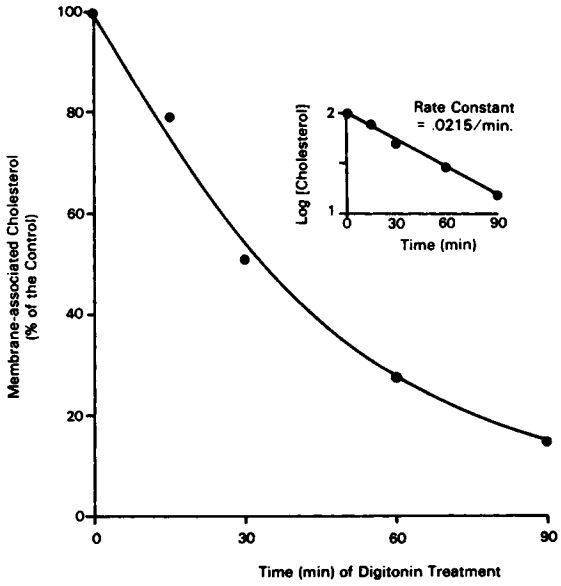

Fig. 1. Membrane-associated cholesterol after digitonin treatment of gastric microsomes for various lengths of time. Details for digitonin (0.8 mg/mg protein) treatment and assay of microsomal cholesterol are given in Table I and Methods. Since about 15% of the pig gastric microsomal vesicles were ruptured [26,36], the data in Figure 1 are presented after appropriate corrections. The data represent the amount of cholesterol associated with sealed or nonleaky membrane vesicles.

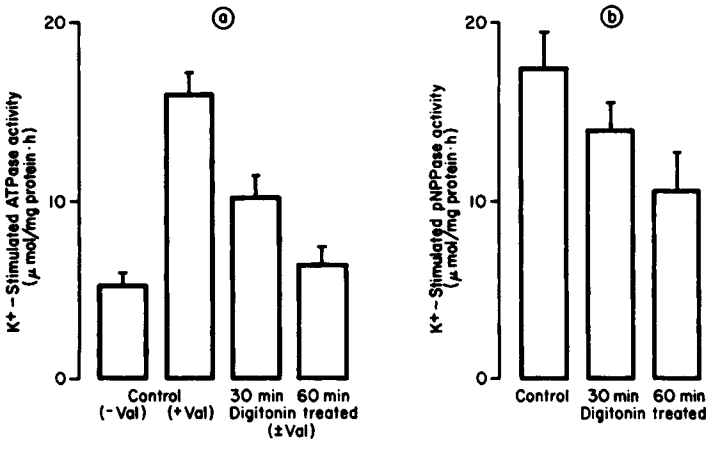

Fig. 2. a) K^+-stimulated ATPase activity in presence and absence of valinomycin asociated with control and digitonin (0.8 mg/mg protein) treated microsomes. Conditions of digitonin treatment and ATPase assay are given in Methods. Values are mean ± SEM (n = 3). b) K^+-stimulated paranitrophenylphosphatase (pNPPase) activity associated with control and digitonin-treated microsomes. Details of digitonin treatment and pNPPase assay are given in Methods.

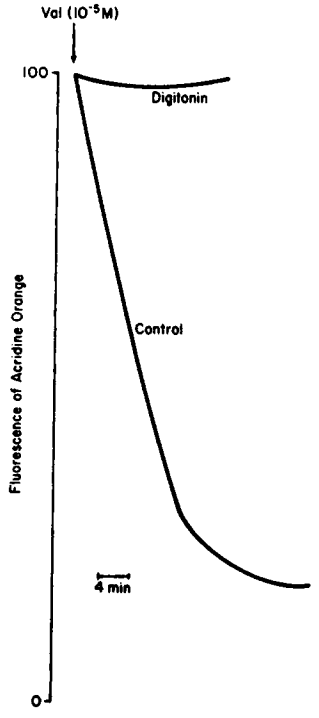

Fig. 3. Gastric K^+-stimulated ATPase mediated uptake of acridine orange by control and digitonin-treated microsomal vesicles. Conditions of digitonin (0.8 mg/mg protein) treatment are given in Methods. The incubation system for acridine orange uptake contained in a total volume of 2 ml 25mM Pipes buffer (pH 7.0), 0.5 mM EDTA, 0.01 mM acridine orange, 1 mM $MgCl_2$, 150 mM KCl, 1 mM ATP, and 0.1 mg membrane protein. The reactions were initiated at room temperature with 10^{-5} M valinomycin (val), and the fluorescence quenching of acridine orange was monitored as described in Methods.

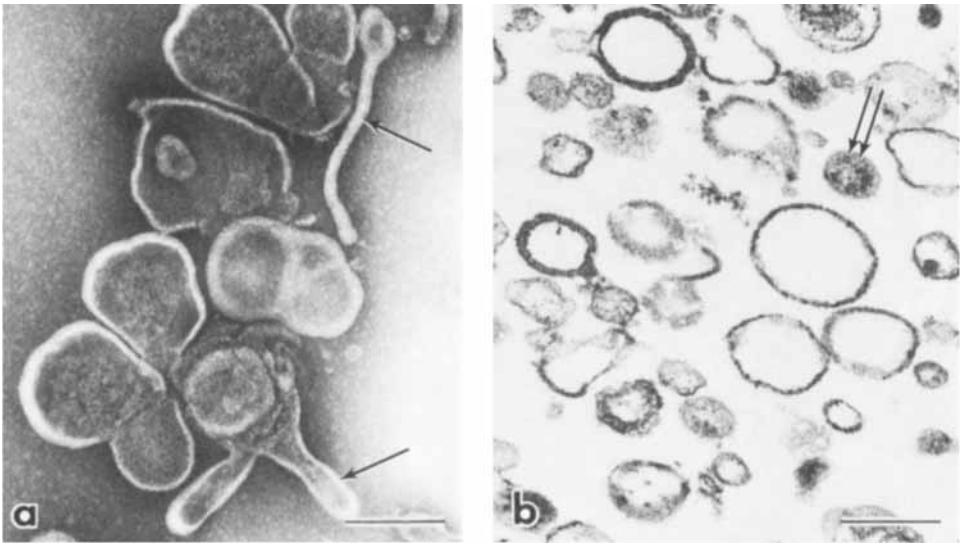

Fig. 4. Electron micrographs of isolated pig gastric microsomal vesicles incubated at 0–4 °C with 2% methanol for 1 h without any digitonin (control). Details are given in Methods. a) Preparation negatively stained with 1% sodium phosphotungstate. Note some tubular structures (single arrow) in a predominantly nontubular vesicular form. b) Preparation plastic-embedded and sectioned. Vesicles of various sizes are apparent. Some dense bodies (double arrow) are always associated with the vesicular preparation. Bars 0.2 μm, ×90,000.

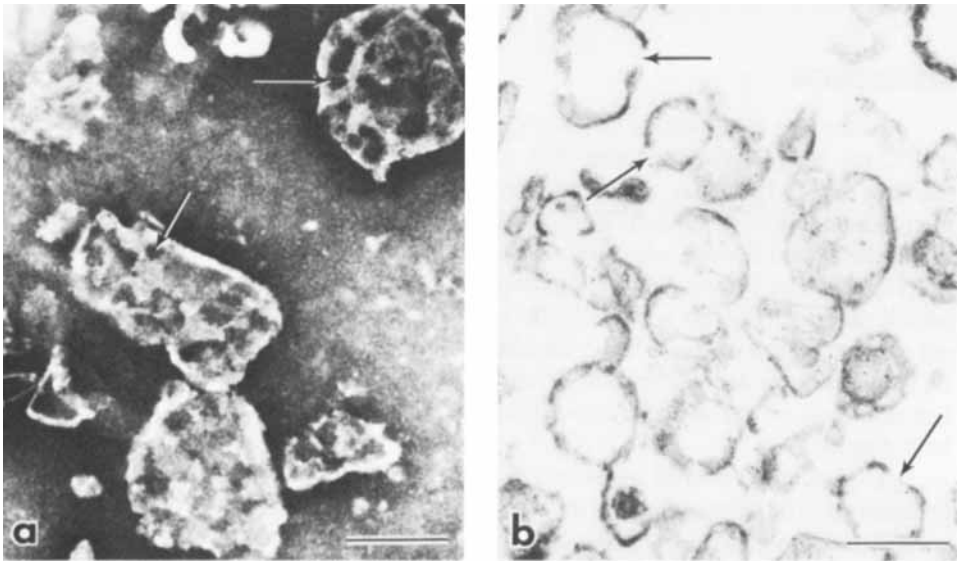

Fig. 5. Electron micrographs of pig gastric microsomes after treatment with digitonin (0.8 mg/mg protein) for 30 min at 0–4 °C. Details of digitonin treatment are given in Methods. a) Negatively stained preparation. Note the abundance of "holes" (arrow) appearing on the vesicular surface. b) Cross-sectioned material after plastic embedding. Note the frequent membrane discontinuities (few pointed by arrow) which probably represent the "holes" in the negatively stained (a) preparation. Bars 0.2 μm, ×90,000.

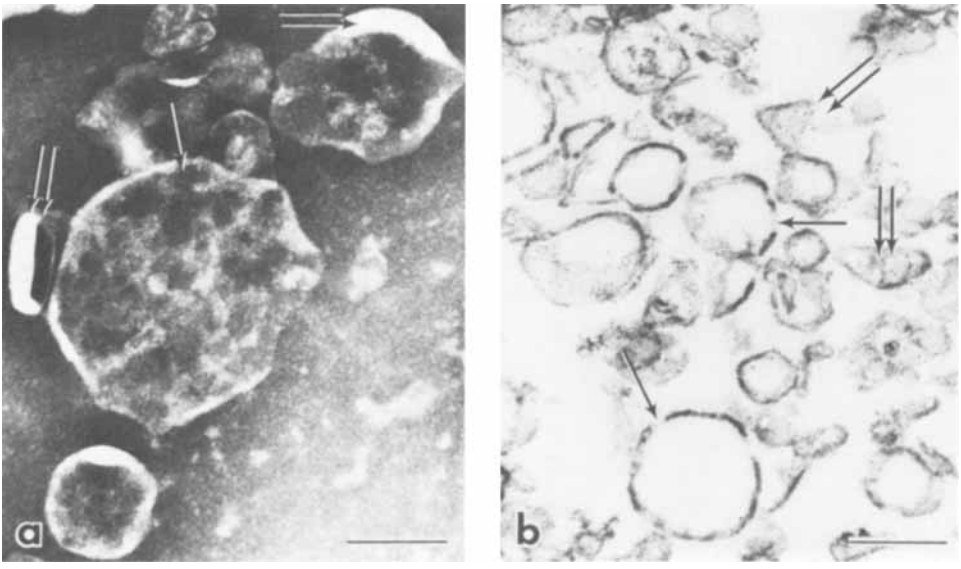

Fig. 6. Electron micrographs of pig gastric microsomal vesicles after 60 min of digitonin treatment. Details of digitonin treatment are given in the legend to Figure 5 and in Methods. a) Negatively stained preparation showing diffused and often coalesced (single arrow) region on vesicular surface (compare with Fig. 5a). Many cup-shaped structures (double arrow) are present. Note some thickened areas in those cup-shaped membranes which probably appear as deformed vesicles with sharply angulated (double arrow) structures when viewed in thin-sectioned (Fig. 6b) preparations. Discontinuities in membranes (single arrow) are also apparent in the thin sections. Bars 0.2 μm, ×90,000.

DISCUSSION

Under the condition of this study, digitonin treatment of gastric microsomes at 0–4°C for 30 min releases about 50% of the cholesterol from the membrane into the medium (Table I). Under similar conditions the vesicular H^+ uptake mediated by the microsomal (H^+ + K^+)-ATPase system is abolished (Fig. 3), even though the digitonin-treated microsomes retained about 65% of the K^+-stimulated ATPase activity (Fig. 2). The inability of the digitonin-treated microsomes to retain H^+ within the vesicle interior (Fig. 3) appears to be primarily due to leakiness of the vesicles and not to inactivation of the K^+/H^+ exchange pump. The appearance of visible "holes" on the membrane surface within 30 min of digitonin treatment (Fig. 5) and concomitant abolition of the valinomycin-stimulated ATPase activity (Fig. 2) are consistent with that idea.

Exponential removal of gastric microsomal cholesterol by digitonin with a rate constant of 0.0215/min (Fig. 1) and consequent generation of leaky vesicles (Fig. 3) with numerous holes (Fig. 5) could be interpreted by membrane models having regions of sequestered cholesterol and/or homogenously distributed cholesterol on the microsomal surface. Recent reports from several laboratories using fillipin as a probe for cholesterol demonstrated somewhat similar lesions in the erythrocyte ghosts [33], chicken liver cell plasma membranes [33], Rous sarcoma cell membrane [33], and malpighian tubule [34]. Although the present study cannot provide us with any

definite information on the organization of cholesterol, the data do suggest some degree of specialization in the topology of cholesterol within the gastric secretory membranes. Further investigations are needed to define the precise topographic arrangement of cholesterol in gastric microsomal vesicles. It is noteworthy that a symmetrical distribution of cholesterol in the two halves of the M gallisepticum has recently been suggested [35].

It should be noted that the "holes" after a 60-min digitonin treatment appeared more diffused than those at 30 min. In addition, there were an abundance of cup-shaped microsomes at 60 min (Fig. 6) compared to 30 min (Fig. 5), at which time such structures were very rare. This could be explained if there is an increased lateral phospholipid movement to keep the vesicular structure stable in the face of increased removal of membrane cholesterol. Although it is known that the lateral diffusion of phospholipids within the plane of the bilayer occurs rather rapidly [36], the massive depletion of membrane cholesterol induced by digitonin might enhance the process to a great extent. The intrinsic surface tension and hydrophobicity of the phospholipids surrounding the "holes" would tend to pull the neighboring phospholipids until a stable structure is achieved with the resultant fusion of the "holes" and formation of cup shapes. Such a concept would be compatible with reports of digitonin-induced disruption or lysis of the plasma membranes of several cell types [37,38], including formation of leaky gastric glands [39]. Furthermore, fillipin, which like digitonin binds the cholesterol, has recently been demonstrated to cause discontinuity in the kidney podocyte plasma membranes [40], suggesting discrete and heterogenous membrane domains.

It should be noted that the activities of gastric microsomal K^+-stimulated ATPase and pNPPase were reduced by about 35% and 20% respectively during the first 30 min of digitonin treatment and by about 60% and 40% after 60 min (Fig. 2). Since the cholesterol-inactivated enzymes could not be reactivated (unpublished data) by the endogenous activator protein [12] and no phospholipid was released by digitonin (Table I), the observed reduction in enzyme activities appears very likely to be due to some rearrangement or alteration of the phospholipids within the microenvironment of the K^+-ATPase complex. Gastric microsomal K^+-ATPase system has been demonstrated to be dependent on phosphatidyl choline (PC), phophatidyl ethanolamine (PE) being about 50% less effective and sphingomyelin (Sph) and phosphatidyl inositol (PI) being completely ineffective [11]. The major phospholipid constituent of purified pig gastric microsomes are PC, PE, and Sph [7,27]. Therefore, any change in phospholipid environment of the gastric ATPase after perturbation of the bilayer structure with digitonin could result in such reduced activity. It is noteworthy that the microsomal K^+-stimulated pNPPase activity was less vulnerable to digitonin perturbation than the K^+-stimulated ATPase activity (Fig. 2), although the former activity is known to be a manifestation of the latter [12,29,30]. Similar differential effects on the two gastric phosphatase activities have also been previously noted after treatment with phospholipase A_2 [27] and sulfhydryl agents [41]. However, the reasons for such differential effects are unclear at present.

ACKNOWLEDGMENTS

We thank Mrs Lisa Lashway for typing the manuscript and Mrs Maureen Bareza for the electron microscopic specimen preparation. We also thank Dr Rolla B. Hills

for his helpful criticism. This work was supported in part from the Surgical Research Fund, Upstate Medical Center. Dr Ray is a recipient of the Research Career Development Award, AM 00623.

REFERENCES

1. Solomon AK, Karnovsky M (eds): "Molecular Specialization and Symmetry in Membrane Function." Cambridge, Massachusetts: Harvard University Press, 1978.
2. Opden Kamp JAF: Ann Rev Biochem 48:47–71, 1979.
3. Schroeder F: FEBS Lett 135:127–130, 1981.
4. Poznandky MJ, Lange Y: Biochim Biophys Acta 505:256–264, 1978.
5. Bloj B, Zilversmit DB: Biochemistry 16:3943–3948, 1977.
6. Backer JM, Dawidowicz EA: Biochim Biophys Acta 551:260–270, 1979.
7. Sen PC, Ray TK: Arch Biochem Biophys 198:548–555, 1979.
8. Forte JG, Machen TE, Obrink KJ: Ann Rev Physiol 42:111–126, 1980.
9. Ray TK, Fromm D: J Surg Res: 31:496–505, 1981.
10. Sen PC, Ray TK: Biochem J 182:637–640, 1979.
11. Sen PC, Ray TK: Arch Biochem Biophys 202:8–17, 1980.
12. Ray TK: FEBS Lett 92:49–52, 1978.
13. Forte JG, Ray TK, Poulter JL: J Appl Physiol 32:714–717, 1972.
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265–275, 1951.
15. Sanui H: Anal Biochem 60:489–504, 1974.
16. Lee HC, Forte JG: Biochim Biophys Acta 508:339–356, 1978.
17. Sen PC, Meng-Ai Z, Ray TK: Arch Biochem Biophys 205:340–351, 1980.
18. Bligh EG, Dyer WJ: Can J Biochem Physiol 37:911–917, 1959.
19. Sen PC, Ray TK: Biochim Biophys Acta 618:300–307, 1980.
20. King EJ, Wotton IDP: "Microanalysis in Medical Biochemistry," 3rd Ed. London: Churchill, 1956, p 42.
21. Bartlett GR: J Biol Chem 234:466–468, 1959.
22. Palade GE: J Exp Med 95:285–298, 1952.
23. Luft JH: J Biophys Biochem Cytol 9:409–414, 1961.
24. Watson ML: J Biophys Biochem Cytol 4:475–478, 1958.
25. Reynolds ES: J Cell Biol 17:208–212, 1963.
26. Haschemayer RH, Meyers RJ: In Hayat MA (ed): "Principles and Techniques of Electron Microscopy," Vol 2. New York: Van Nostrand Reinhold, 1972, pp 99–147.
27. Saccomani G, Chang HH, Spisni A, Helander HF, Spitzer HI, Sachs G: J Supramol Struct 11:429–444, 1979.
28. Sen PC, Ray TK: Biochem J 195:515–518, 1981.
29. Forte JG, Ganser AL, Ray TK: In Kasbekar DK (ed): "Mechanisms of Physiological H^+ Secretory Process." New York: Marcel Decker, 1976, pp 302–330.
30. Ray TK: Can J Physiol Pharmacol 58:1189–1191, 1980.
31. Ray TK, Tague LL: Acta Physiol Scand Special Suppl 1978:283–292.
32. Ray TK, Nandi J, Pidhorodeckyj N, Meng-Ai Z: Proc Natl Acad Sci USA 79:1448–1452, 1982.
33. Dourmashkin RR, Dougherty RM, Harris RJC: Nature 194:1116–1119, 1962.
34. Greven H, Robenek H: J Submicrosc Cytol 14:123–130, 1982.
35. Bittman R, Rottem S: Biochem Biophys Res Commun 71:318–324, 1976.
36. Miller MW, Shamoo AE (eds): "Membrane Toxicity." New York: Plenum Press, 1977.
37. Elias PM, Goerke J, Friend DS, Brown BE: J Cell Biol 78:577–596, 1978.
38. Devente J, Zaagsma J: Arch Biochem Biophys 209:249–255, 1981.
39. Malinowska DG, Kolez HR, Hersey SJ, Sachs G: Proc Natl Acad Sci USA 78:5908–5912, 1981.
40. Orci L, Singh A, Amherdt M, Brown D, Perrelet A: Nature 293:646–647, 1981.
41. Forte JG, Poulter JL, Dykstra R, Rivas J, Lee HE: Biochim Biophys Acta 644:257–265, 1981.