Stratum Corneum Lipid Liposomes: Calcium-Induced Transformation Into Lamellar Sheets

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The epidermal water barrier in mammalian stratum corneum is formed of broad lamellar sheets of lipids consisting principally of ceramides (40%), cholesterol (25%), cholesteryl sulfate (10%), and free fatty acids (25%). Such lipid mixtures have been shown to form lipid bilayers in the form of small, unilamellar liposomes when sonicated at 80°C in water containing Tris buffer and 100 mM NaCl. In the present study it is shown that such liposomes are slowly transformed into large unilamellar liposomes and then into broad lamellar sheets after the addition of stoichiometric amounts of calcium chloride. The presence of free fatty acids was a necessary condition for this calcium-induced fusion. These observations may provide a useful analogy for the transformation of flattened liposomes into broad lamellar sheets that occurs during transition of epidermal granular cells into corneocytes. *J Invest Dermatol 88:212–214, 1987*

Mammalian stratum corneum contains multiple intercellular lipid bilayers that constitute the epidermal water barrier [1]. The stratum corneum lamellae are made up of ceramides (40%), free fatty acids (25%), cholesterol (25%), cholesteryl sulfate (10%), and, unlike all other biologic membranes, contain no phospholipids [2]. In a previous study, small unilamellar vesicles were prepared from a variety of such mixtures [3], thus establishing the bilayer-forming capability of stratum corneum lipids. In the present study we report the transformation of small unilamellar liposomes prepared from stratum corneum lipids into broad multilamellar sheets in the presence of Ca^{++}. This may provide a useful analogy for the formation of the intercellular lamellae that constitute the epidermal water barrier.

MATERIALS AND METHODS

Lipids Ceramides were isolated by preparative thin-layer chromatography from total lipid extracts of full-thickness pig epidermis as described previously [4]. Palmitic acid (reagent grade) was obtained from Fisher Scientific Co. (Springfield, New Jersey), and cholesterol was obtained from Sigma Chemical Co. (St. Louis, Missouri). Cholesteryl sulfate was prepared by reaction of cholesterol with excess chlorosulfonic acid in pyridine and purified chromatographically.

Preparation of Liposomes Individual lipids were dissolved in chloroform:methanol, 2:1, and appropriate volumes were combined to obtain several mixtures, as shown in Table I. Mixture A was chosen as a close approximation of the composition of stratum corneum lipids, without the small proportions of triglycerides, cholesteryl esters, and minor polar components. Liposomes were prepared from these mixtures as described previously [3]. Ca^{++} was then added as a dilute solution of $CaCl_2$ (5 mM) to the liposome suspension at 37°C with stirring. The mole ratio of Ca^{++} to the acidic lipids (free fatty acids and/or cholesteryl sulfate) in these mixtures was 1:2

The suspensions containing Ca^{++} were incubated at 37°C until analyzed by freeze-fracture. For this, the lipid dispersions were centrifuged at 100,000 *g* at 4°C for 1 h to form a pellet. Samples of the wet pellet were quenched from 4°C in liquid propane, fractured, and analyzed by electron microscopy as described previously [3]. Portions of the wet pellet were fixed in osmium-ferrocyanide for 30 min at 4°C. The fixed pellets were preembedded in agar, dehydrated in graded acetones, embedded in Epon, and sectioned. Silver-gray sections were stained with uranyl acetate and lead citrate and examined in a Hitachi H-600 electron microscope operating at 75 kV.

RESULTS

All of the mixtures shown in Table I formed small unilamellar vesicles upon sonication, as shown in Fig 1 (*1A, 1B, 1C*). The freeze-fracture micrographs show these liposomes to be in the size range of 20–150 nm. Within 1 h after adding Ca^{++}, the liposomes seem to have undergone fusion to form large unilamellar vesicles in mixtures A and B, as seen in Fig 1 (*2A, 2B*). Figure 1 (*2B*) also shows the presence of some lamellar sheets. Mixture C did not show any effect of Ca^{++}, as seen in Fig 1 (*2C*). Figure 1 (*3A, 3B, 3C*) shows the freeze-fracture micrographs of mixtures A, B, and C, containing amounts of Ca^{++} equivalent to the fatty acids present, after incubation at 37°C for 2 weeks. Mixture A produced large liposomes ranging in size from 50 to 800 nm, along with lamellar sheets, as in Fig 1 (*3A*). Mixture B produced mostly lamellar sheets with few large liposomes [Fig 1 (*3B*)], while mixture C remained unaffected, although the pres-

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Table I. Composition of Lipid Mixtures Used for Making Liposomes (wt%)

Mixture	Ceramides	Cholesterol	Palmitic Acid	Cholesteryl Sulfate
A	40.0	25.0	25.0	10.0
B	44.4	27.8	27.8	—
C	44.4	27.8	—	27.8

ence of a few large liposomes indicated fusion of some of the liposomes [Fig 1 (*3C*)]. Figure 2 shows a thin-section electron micrograph of mixture A that had been incubated with Ca^{++} for 2 weeks.

DISCUSSION

The results presented here indicate that Ca^{++} causes the liposomes of mixtures A and B (containing free fatty acids) to form broad lamellar sheets. In both these mixtures the fusion leads to the formation of large liposomes prior to the formation of extended sheets. The first step, wherein the small unilamellar liposomes aggregate and fuse to form large unilamellar liposomes is perhaps a spontaneous effect of the divalent Ca^{++}, as seen by the disappearance of small unilamellar liposomes in mixtures A and B within 1 h after adding Ca^{++}. Also, these preparations became cloudy with the addition of the very first drop of Ca^{++}. However, this initial aggregation seems to be extremely difficult in mixture C, which does not have any free fatty acid. This mixture remained clear even after several days of incubation with Ca^{++} although some sediment began to appear after about 2 weeks. Earlier studies have shown Ca^{++}-induced fusion of acidic phospholipid-containing liposomes to be almost instantaneous [5,6], probably because in those studies the lipid mixtures used were in the liquid crystalline form at the temperature of Ca^{++} addition. On standing for several days with stoichiometric amounts of Ca^{++}, mixture A forms more and more lamellar sheets coexisting with many large liposomes, while mixture B is mostly made up of lamellar sheets. Addition of Ca^{++} at 80°C instead of 37°C produced the same results.

One major concern in this study is the possibility of calcium-induced phase separation. The crystalline phases of fatty acids, their calcium salts, and cholesterol, each form lamellar fracture faces [7] when analyzed by freeze-fracture. We are not presently able to establish whether the lamellar sheets seen in our lipid systems are made up of all of the lipids in the mixture or of only

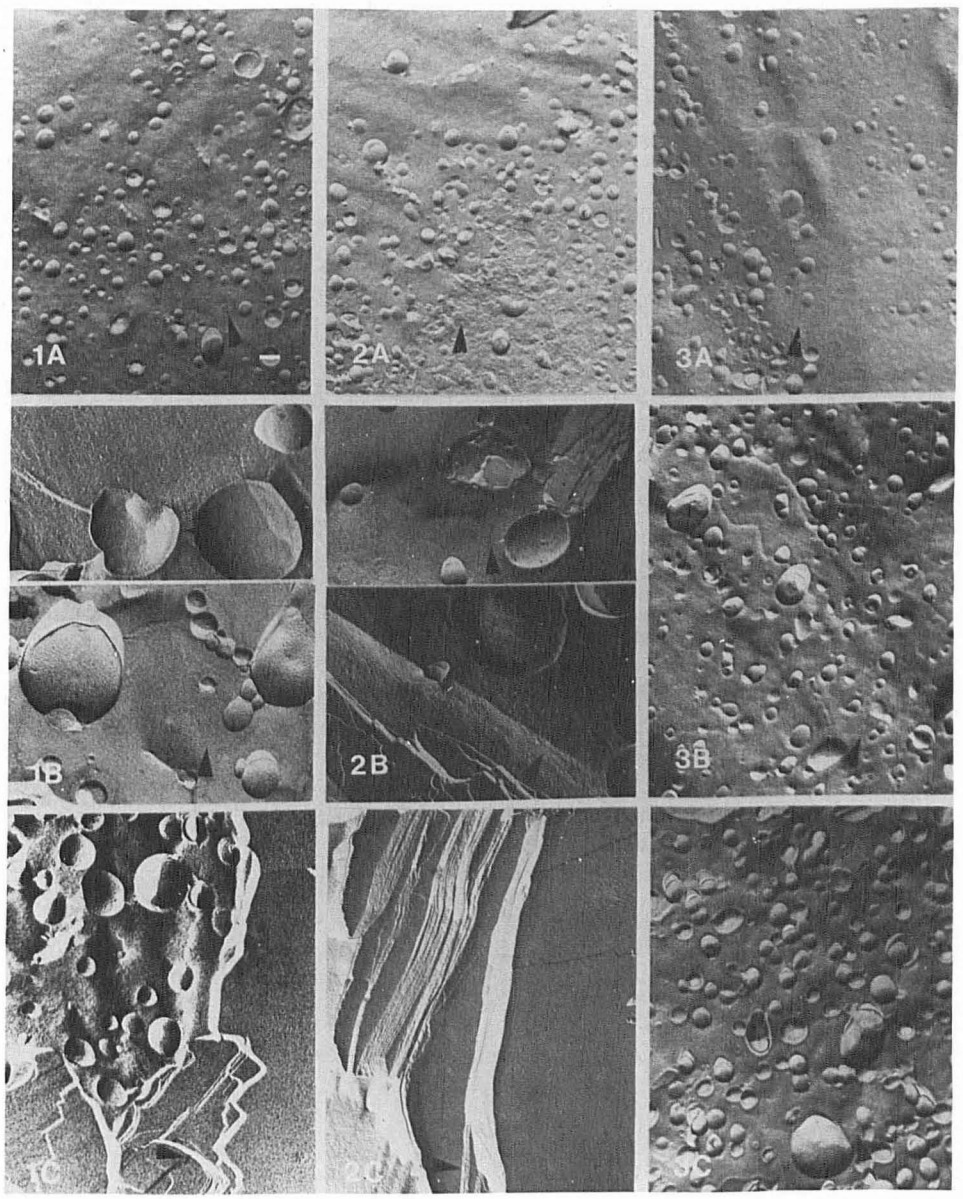

Figure 1. Freeze-fracture micrographs of mixture A (*1A*), mixture B (*1B*), and mixture C (*1C*) without any Ca^{++}; mixture A (*2A*), mixture B (*2B*), and mixture C (*2C*) 1 h after the addition of Ca^{++}; mixture A (*3A*), mixture B (*3B*), and mixture C (*3C*) 2 weeks after the addition of Ca^{++}. *Bar* in *1A* = 100 nm. *Arrowheads* indicate the direction of shadowing.

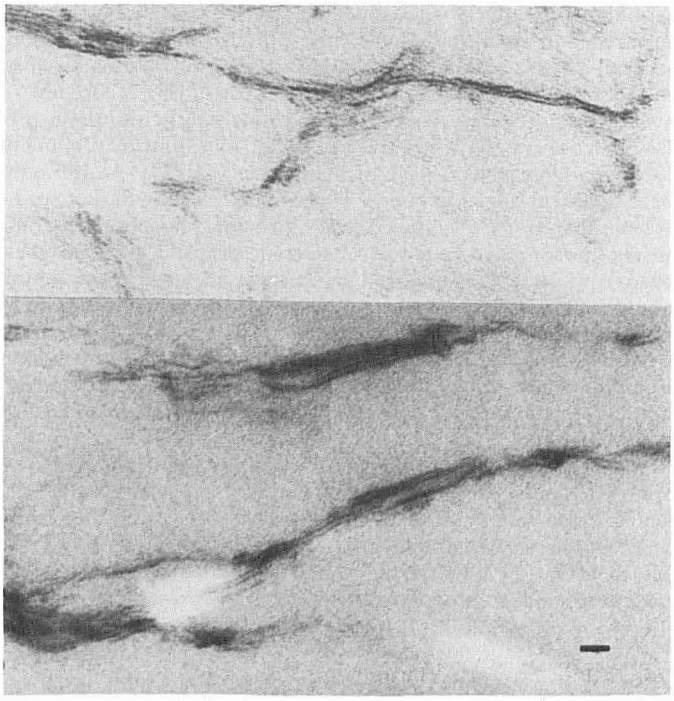

Figure 2. Thin-section electron micrograph of mixture A after incubation with a stoichiometric amount of Ca^{++} for 2 weeks at 37°C. The characteristic trilaminar structure of lipid lamellae is apparent, often in multiple layers. The lamellae are somewhat wavy and do not have the rigid planarity that would be expected of purely crystalline structures. *Bar* = 40 nm.

the crystalline Ca^{++} salts of free fatty acids separating out from the lipid mixtures. Calcium-induced fusion leading to the formation of a Ca–lipid complex has been identified by the appearance of phase-separated lipidic particles in some phospholipid systems by freeze-fracture and ^{31}P nuclear magnetic resonance (NMR) studies [6,8]. The smooth fracture faces of the large liposomes and the lamellar sheets do not indicate any such phase separation in the stratum corneum lipids. Moreover, the presence of large unilamellar liposomes in mixture B after the addition of Ca^{++} indicates that Ca^{++} has caused the fusion of the small vesicles by binding to the negatively charged carboxyl groups of the fatty acids in situ rather than by precipitating the fatty acids as Ca^{++} soaps, which would leave behind the ceramides and cholesterol. The latter would destroy the liposomal structures, because a mixture of ceramides and cholesterol is not capable of forming bilayered structures [3]. Furthermore, the multilaminated lines in thin-section electron micrograph (Fig 2) show the bilayer structure.

The results presented here demonstrate the ability of Ca^{++} to induce fusion of small unilamellar vesicles to form large unilamellar vesicles and to promote the formation of extended lamellar sheets like those seen in the intercellular space of the stratum corneum. The roles of acidic lipids, viz the fatty acids and cholesteryl sulfate, and the molecular mechanism of fusion, are not clear from this preliminary investigation, although absence of free fatty acids from the bilayer structures seems to slow down the aggregation process. Furthermore, an additional factor must be operative in vivo. In the upper granular layer, the lamellar granules discharge their contents in the intercellular space. The discharged material, appearing as short disks, consists of flattened unilamellar liposomes [9]. These disks rearrange to form lamellar sheets by a membrane fusion process. The high amounts of extracellular Ca^{++} that are present in the stratum granulosum–stratum corneum interface [10] might promote the edge-to-edge fusion of these flattened vesicles, acting through forces that in the present study promote the fusion of small liposomes and their subsequent transformation into broad sheets.

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