

Fusion of Liposomes with the Plasma Membrane of Epithelial Cells: Fate of Incorporated Lipids as Followed by Freeze Fracture and Autoradiography of Plastic Sections

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Abstract. The fusion of liposomes with the plasma membrane of influenza virus-infected monolayers of an epithelial cell line, Madin-Darby canine kidney cells (van Meer et al., 1985. *Biochemistry*. 24:3593–3602), has been analyzed by morphological techniques. The distribution of liposomal lipids over the apical and basolateral plasma membrane domains after fusion was assessed by autoradiography of liposomal [³H]dipalmitoylphosphatidylcholine after rapid freezing or chemical fixation and further processing by freeze substitution and low temperature embedding. Before fusion, radioactivity was solely detected on the apical cell surface, indicating the absence of redistribution artifacts and demonstrating the reliability of lipid autoradiography on both a light and electron microscopical level. After induction of fusion by a low pH treat-

ment, the basolateral plasma membrane domain became progressively labeled, indicative of rapid lateral diffusion of [³H]dipalmitoylphosphatidylcholine in the plasma membrane. Analysis of individual fusion events by freeze fracture after rapid freezing confirmed the rapid diffusion of the liposomal lipids into the plasma membrane, as intramembrane particle-free lipid patches were never observed. After the induction of liposome-cell fusion, well-defined intramembrane particles were present on the otherwise smooth liposomal fracture faces and on the fracture faces of the plasma membrane. Morphological evidence thus was obtained in favor of a local point fusion mechanism with an intramembrane particle as a specific structural fusion intermediate.

As yet, the mechanisms of biological membrane fusion remain ill defined (Düzgünes, 1985; Sowers, 1987; Ohki et al., 1988). The system best characterized at the molecular level is that of the fusion of enveloped viruses with their target membranes. In particular, the fusogenic properties of the hemagglutinin (HA)¹ spike glycoprotein of influenza viruses have been described in great detail (White et al., 1983; Wiley and Skehel, 1987; Doms et al., 1988). In the present study, we have used these properties to create a fusion event that is fast and efficient as shown by both biochemical and biophysical techniques (van Meer and Simons, 1983; van Meer et al., 1985). Liposomes were fused with the apical plasma membrane of epithelial (Madin-Darby canine kidney; MDCK) cells that had been infected with influenza virus. Specific morphological features of the fusion event were studied using freeze-fracture EM, and the fate of incorporated lipids was followed by lipid autoradiography.

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1. *Abbreviations used in this paper:* HA, hemagglutinin; IMP, intramembrane particle; [³H]DPCC, L- α -dipalmitoyl-[choline-methyl-³H] phosphatidylcholine.

Lateral diffusion of liposomal lipids after fusion with the apical plasma membrane has been monitored before by the use of fluorescently labeled phospholipid analogues (van Meer and Simons, 1983, 1986; van Meer et al., 1985, 1986). Although this approach allowed direct observation of the lipidic dye did not permit general conclusions concerning the behavior of natural phospholipids to be made. This handicap can be overcome, in principle, by the use of radioactive phospholipids and the analysis of their distribution by autoradiography. However, conventional autoradiographic techniques have serious shortcomings when applied to lipids because they lead to lipid extraction and lipid redistribution (Poste et al., 1978). Recently developed low temperature techniques, i.e., the combination of freeze substitution and low temperature embedding, could be expected to overcome these limitations (Weibull et al., 1984; Verkleij et al., 1985).

Little is known about the mechanism and the intermediates of the actual joining of the two lipid bilayers during membrane fusion. Because the membrane architecture can be visualized using freeze fracturing (see for example Verkleij and Ververgaert, 1975), this technique has been used extensively to investigate membrane fusion events. These studies

are complicated by the high sensitivity of fusion events to the chemical pretreatments used in conventional preparation techniques (Chandler and Heuser, 1980; Plattner, 1981; Chandler, 1984), and by the short lifetime of fusion intermediates (Heuser et al., 1979; Siegel, 1986b).

In early freeze-fracture studies, intramembrane particle (IMP) clearance from fusion-competent membrane areas was observed. This led to the assumption that membrane proteins would have to be removed from the membrane areas participating in the fusion process (e.g., Orci and Perrelet, 1978). However, recently, by using pure cryofixation without chemical pretreatments, membrane fusion has been consistently observed as a local-point event that does not involve either IMP clearance or the formation of naked membrane diaphragms (Heuser et al., 1979; Ornberg and Reese, 1981; Plattner, 1981).

Pure cryofixation avoids preparation artifacts but does not overcome the difficulty of catching such short-lived phenomena as membrane fusion events (Knoll et al., 1987). Even if fusion intermediates could be captured, their frequency of occurrence is often low and their identification is easily hampered by the intrinsic heterogeneity of biological membranes (Pinto da Silva and Kachar, 1980). No structural intermediate of fusing biological membranes has been described unequivocally to date.

Liposomes can be induced to fuse with influenza virus-infected cells by lowering the pH (van Meer and Simons, 1983; van Meer et al., 1985). The high density of cell-associated liposomes at neutral pH and the possibility to trigger membrane fusion by lowering the pH provides both a high density and a good synchrony of fusion events and thus maximizes the chance of catching fusion intermediates. In addition, any change in freeze-fracture morphology of the normally smooth fracture faces of the liposomal membrane can be clearly seen and directly correlated with the fusion process. Altogether, the liposome-cell fusion system provides a promising means with which biological fusion can be studied, as compared with many other biological fusion systems.

In this study, we demonstrate that freeze substitution and low temperature embedding are suited to prevent extraction and redistribution of a radioactive phosphatidylcholine and therefore give the opportunity to follow the fate of incorporated lipids by autoradiography. In addition, we are able to show a specific structural feature during liposome-cell fusion, using freeze fracture after rapid freezing, that is an intramembrane particle presumably at the junction between the two fusing membranes. Finally, both methods successfully show that fusion has occurred in this system and that it is followed by rapid lateral diffusion of incorporated lipids in the plasma membrane.

Materials and Methods

Materials

Egg phosphatidylcholine, egg phosphatidylethanolamine, cholesterol, and buffer salts were obtained from Sigma Chemical Co. (St. Louis, MO). The ganglioside G_{D1a} was purchased from Supelco, Inc. (Bellefonte, PA). Octyl- β -D-glucoside was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Octadecyl rhodamine B chloride (R 18) was obtained from Molecular Probes, Inc. (Eugene, OR), and 1- α -dipalmitoyl-[choline-methyl- 3H]phosphatidylcholine ($[^3H]DPPC$; 51 Ci/mmol) was from New England Nuclear

(Dreieich, FRG). Trypsin-L-(tosylamide-2-phenyl)-ethyl-chloromethylketone and soybean trypsin inhibitor were obtained from Millipore Corp. (Freehold, NJ), as were the nitrocellulose acetate filters. Cell culture media and reagents were purchased from Gibco Bioculture (Glasgow, UK). FCS and 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid were from Boehringer (Mannheim, FRG). Ilford L4 emulsion was obtained from Ilford (Amsterdam, The Netherlands). Chemicals and solvents were of analytical grade and were obtained from E. Merck (Darmstadt, FRG).

Cells

MDCK strain II cells were passaged and grown on plastic as described before (van Meer et al., 1985). For some of the freeze-fracture experiments, the cells were grown as 2-d-old confluent monolayers on plastic coverslips (Thermanox, Lux Scientific Corp.) in 40-mm-diam Petri dishes.

For the other freeze-fracture experiments and all autoradiography experiments, the cells were grown on nitrocellulose filters, with a pore size of 0.22 μ m as described (Hansson et al., 1986). Experiments were performed on day 3 after seeding. The results obtained in freeze-fracture experiments with cells grown on either plastic coverslips or nitrocellulose filters did not differ significantly.

All incubations at neutral pH were performed in binding medium: bicarbonate-free MEM with Earle's salts supplemented with 10 mM Hepes and 0.2% BSA, pH 7.4. Incubations at low pH were performed in fusion medium: bicarbonate-free MEM supplemented with 20 mM succinate and 0.2% BSA, pH 5.0.

Liposomes

Octyl- β -D-glucoside liposomes were prepared as described before (van Meer et al., 1985). For the autoradiography studies, liposomes were prepared from the following lipid mixture: 57.5 nmol egg phosphatidylcholine, 62.5 nmol egg phosphatidylethanolamine, 125 nmol cholesterol, 12.5 nmol G_{D1a} , 2.5 nmol R18, 5 nmol (250 μ Ci) $[^3H]DPPC$, and 4.5 mg octyl- β -D-glucoside in 500 μ l of Dulbecco's PBS without Ca^{2+} and Mg^{2+} . Liposomes were added to cells in a 1:4 dilution (in binding medium).

For freeze-fracture studies, R18-containing octyl- β -D-glucoside liposomes were prepared in the same way but without $[^3H]DPPC$.

Fusion of Liposomes with the Apical Plasma Membrane of MDCK Cells

The fusion procedure was essentially that described in van Meer et al. (1985) and van Meer and Simons (1986). In short, monolayers of MDCK cells were infected with 20 pfu of an avian influenza A virus (influenza N virus: A/chick/Germany/49, Hav2 Neq1). At 4.5 (plastic) or 5 h (filters) after infection, when the cells expressed the viral HA on their surface, the apical surface was treated with trypsin at 0°C to bring the HA into its fusogenic form. In the experiments on plastic and the initial experiments on filters, 500 μ l of liposomal suspension (31 nmol phospholipid per 2×10^6 cells on plastic or per 2.3×10^6 cells on filters) were added to the apical cell surface and the cells were incubated for 30 min on ice.

The neuraminidase inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid was present at a concentration of 10 mM to prevent the release of bound liposomes by the action of the viral neuraminidase (van Meer and Simons, 1983). Fusion of the liposomes with the apical plasma membrane was induced by replacing the apical medium by prewarmed fusion medium and an incubation at 37°C for 60 s. The cells were washed with binding medium and cooled on ice in a Petri dish containing 2 ml of binding medium (pH 7.4). After incubation for various times at neutral pH, the cells were fixed chemically or subjected to rapid freezing (see below). Lipid analysis performed on cell monolayers on filters showed that after the low pH treatment, 2.4% of the added liposomes, 0.75 nmol of liposomal phospholipid per filter, were cell associated, on the average 1 dpm of $[^3H]DPPC$ per cell. Assuming a diameter of 200 nm of the liposomes (van Meer et al., 1985), on the average one cell associated with 500 liposomes. A significant amount of the cell-associated liposomes had fused with the apical plasma membrane, because fluorescence microscopy (cf. van Meer and Simons, 1986) showed that liposomal R18 had diffused into the lateral plasma membrane domain. Assuming a 40–55% efficiency of fusion (van Meer and Simons, 1986), an apical surface area of a monolayer of MDCK II cells of 9.6 cm^2 (von Bonsdorff et al., 1985) and a surface area of 90 \AA^2 for a cholesterol-phospholipid pair, it can be calculated that 8–12% of additional lipids had been fused into the apical plasma membrane (calculation according to van Meer and Simons, 1986).

In the experiments with cells on filters for freeze fracture, binding and fusion were performed in a slightly different way: after trypsinization of the apical surface, the filters were removed from the holders and 3-mm-diam pieces were punched out. These pieces were immersed in a 50- μ l droplet of liposomes (3.1 nmol phospholipid/50 μ l) on parafilm and incubated for 30 min on ice. Fusion was induced by dipping a piece of filter into a beaker with fusion medium (60 s at 37°C). Filters were rapid frozen before, during, and after the low pH treatment (see later).

Fixation of Cells for Microscopy

Rapid Freezing. 3-mm disks were punched out of either the plastic or the filter, which served as substrates for the cell monolayers. This was done after liposome binding or at various time points after fusion. The disks (the filters were always supported by a plastic disk of the same size) were covered with a 0.05-mm-thick copper cover (treatment of copper covers used for autoradiography experiments, see below). Damage to the cells was prevented by the insertion of a copper spacer, 0.015 mm thick. Alternatively, cells were grown on plastic substrates with a 0.015-mm-deep milled depression, thus making the spacer unnecessary. Some filter disks (not for autoradiography) were punched out before the incubation with the liposomes, which could then be performed economically in only 50- μ l droplets. This allowed for very precise timing of a large number of specimens.

The assembled sandwich was frozen by a jet of propane at its melting temperature that was directed onto the copper cover of the sandwich (Pscheid et al., 1981; Knoll et al., 1982; Plattner and Knoll, 1984). For some specimens (frozen during the low pH treatment) the incubation temperature (37°C) was maintained until the moment of freezing by holding the specimen in a stream of warm air just in front of the propane nozzle. After being frozen, the sandwiches were stored in liquid nitrogen.

Chemical Fixation. Plastic substrates or filters were placed in Petri dishes containing 1% glutaraldehyde in 100 mM Pipes, pH 7.4, for 30 min on ice or at room temperature (the latter for all autoradiographic experiments). After three 20-min washes in Pipes, cells were impregnated with 30% glycerol in 100 mM Pipes, pH 7.4, for 30 min at room temperature. Samples were frozen either as a sandwich, assembled as described above, or as free filters, both by vigorously dipping in liquid propane at its melting point. Storage again was in liquid nitrogen.

Freeze Fracture. The sandwiches were inserted under liquid nitrogen into a mobile clamping table (normally used for gold holders; Balzers, Liechtenstein) with either the copper part or the plastic part uppermost. In the freeze-fracture apparatus (Balzers 300 or 360 M) the exposed sandwich parts were broken away using a cold knife, exposing preferentially either the protoplasmic or exoplasmic fracture face (nomenclature according to Branton et al. 1975) of the apical plasma membrane. Replicas (usual platinum/coal evaporation at nominally -110°C and 4×10^{-7} mbar) were obtained by floating either the copper cover on dilute chromic acid according to Costello et al. (1982) or the filter on sulfuric acid. Both procedures dissolve the supporting material and result mostly in intact replicas; replicas on plastic come off more easily and can be floated on water.

Freeze Substitution and Low Temperature Embedding

Sandwiches were split under liquid nitrogen to remove the copper cover. To avoid splitting the apical membrane by this procedure (freeze fracture), these copper covers had been coated by a film of lipids (crude phosphatidylcholine) and dried before assembling and freezing the sandwich. Plastic disks or filters with the cells attached were immersed into a mixture of 3% glutaraldehyde, 1% osmium tetroxide, and 0.5% uranyl acetate in methanol according to Müller et al. (1980) at -80°C and kept at that temperature for 24 h. After the temperature was slowly raised to -50°C , the specimens were washed with pure methanol of the same temperature and gradually infiltrated with Lowicryl HM20 (HM20/MeOH 1:1, 2:1, 100% HM20 for 1 h each). After an additional change of 100% Lowicryl, the specimens were stored overnight at -35°C in a deep freezer (or at -50°C in the Reichert unit, see below). After another change of Lowicryl, UV polymerization was initiated at -35°C , essentially according to the method of Humbel and Müller (1984). After 24 h, the specimens were brought to room temperature and further hardened for 48 h under UV illumination. To control specimen temperature either a Balzers spray freeze unit equipped with a brass block (with drilled holes for insertion of Eppendorf vials) or a Reichert Auto CS unit was used. Low temperature polymerization was carried out in a deep freezer containing UV lamps.

Autoradiography

The procedure used was essentially the flat substrate method according to Bachmann and Salpeter (1967). In short, sections were cut perpendicularly to the plane of the monolayer either 0.5 μ m thick (semithin) or 0.1 μ m thick (pale gold, ultrathin) and transferred to collodion-coated glass slides. Ultrathin sections were covered after staining (1% KMnO_4 for 10 min) with 5 nm carbon. In later experiments, ultrathin sections were covered with an additional layer of 50 nm Butvar B98 (according to Futaesaku and Mizuhira, 1986) to prevent the destruction of ultrastructural detail as a result of the deleterious interaction of the developer with the ultrathin section. Semithin sections were not stained and covered only with carbon. A monolayer of Ilford L4 silver bromide crystals was applied by slow withdrawal (80 mm/min) of the slides from the liquid emulsion ($\sim 20\%$ in distilled water at 25°C) using an electric motor. After drying overnight, the slides were stored in light-tight boxes at 4°C . Exposure was varied from 4 to 10 d; the autoradiograms were developed in Kodak D19 (for 2 min at 20°C). The ultrathin sections with the accompanying films were floated on distilled water and picked up on EM grids for analysis with a Phillips 420 at 80 kV. Semithin sections were analyzed with a Leitz Orthoplan microscope equipped with a $63\times$ (1.4 NA) objective in phase-contrast mode or with incident illumination.

Results

Autoradiography of a natural phospholipid (DPPC) was used as independent proof of liposome-cell fusion and to follow the fate of incorporated lipids in the plasma membrane. Cells were either frozen after chemical fixation and cryoprotection or rapid frozen without any pretreatments, before and at various time points after the induction of fusion. To avoid extraction and redistribution artifacts, the frozen cells were processed by freeze substitution and low temperature embedding. These procedures have been shown to prevent the extraction of phospholipids (Weibull et al., 1984; Verkleij et al., 1985). At neutral pH liposomes are bound to, but have not fused with, the apical plasma membrane. This defined situation with precisely located radioactive label allows for a critical evaluation of a possible redistribution artifact. Fig. 1 *a* shows that there is no such artifact. The distribution of silver grains, analyzed by light microscopy, is absolutely restricted to the apical cell surface under these conditions. This exclusive localization of silver grains at neutral pH was confirmed by electron microscopical analysis (data not shown).

Distribution of [^3H]DPPC after the Fusion of Radioactive Liposomes with the Apical Plasma Membrane

At neutral pH, liposomes bind to, but do not fuse with, the apical plasma membrane. Under these conditions, silver grains are found at the apical cell surface exclusively (Fig. 1 *a*). The low pH treatment (1 min at 37°C , pH 5.0) results in fusion of liposomes with the apical plasma membrane. The introduced [^3H]DPPC freely diffuses within the plane of the bilayer and the basolateral domain becomes labeled in a time-dependent way (Fig. 1, *b-e*, incubated on ice for 0, 5, and 10 min after the low pH treatment respectively; Table I). Endocytosis of radioactive liposomes as a reason for the translocation of the radiation source can be excluded because the endocytic activity of the cells is negligible, both at 37°C during the low pH treatment (cf. Davoust et al., 1987) and at 0°C during the subsequent neutral pH incubation (Table I). In contrast, if the low pH treatment is followed by a neutral pH incubation at 37°C for 10 min, label is found throughout the cell (with exception of the nucleus; data not shown).

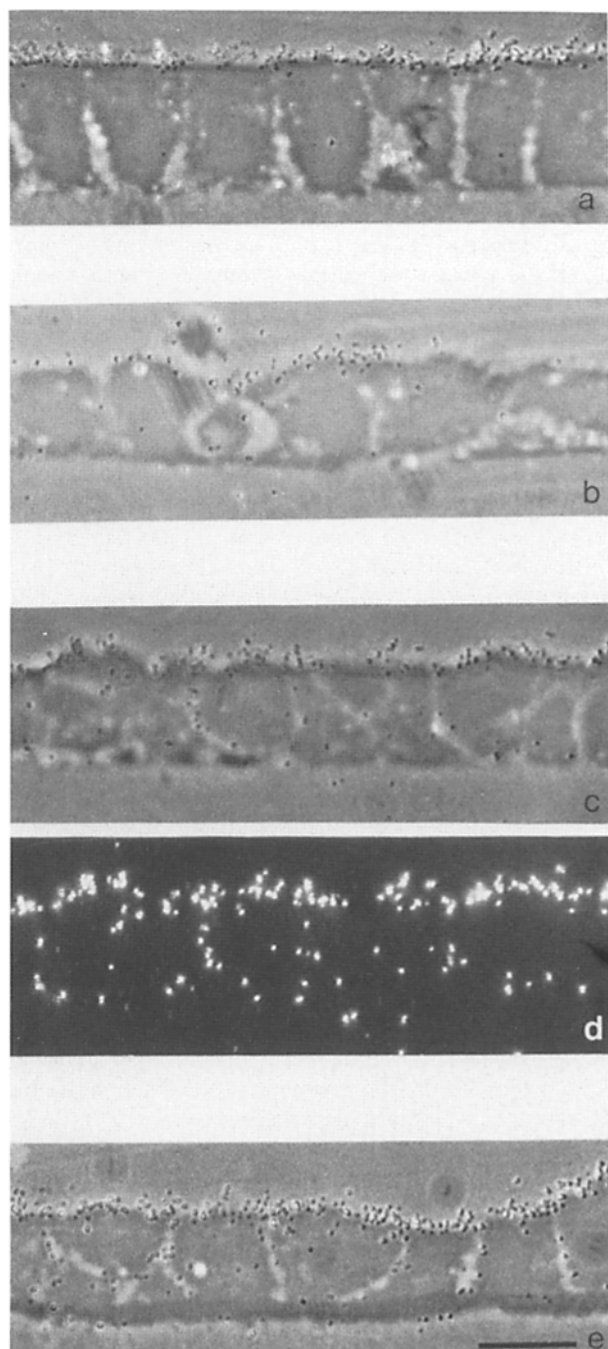


Figure 1. Light microscopical autoradiography of liposomal [^3H]DPPC before and after fusion of liposomes with the apical plasma membrane of influenza virus-infected MDCK cells. All samples were chemically fixed, cryoprotected, and further processed as indicated in Materials and Methods. No difference with regard to the distribution of label was found between chemically fixed and rapid-frozen samples. (a) Radioactive liposomes bound to the apical surface of a confluent monolayer of MDCK cells on filter at neutral pH (30 min on ice). (b) Liposomes have been induced to fuse with the apical plasma membrane by a low pH treatment (1 min, pH 5, at 37°C). (c and d) The low pH treatment was followed by an additional incubation at pH 7.4 for 5 min on ice. (c) In the transmitted light the silver grains appear as black dots, (d) incident light is reflected by the silver grains. In addition to the apical cell surface, basolateral domains are labeled. (e) Sample incubated for 10 min at pH 7.4 on ice after the low pH treatment. Bar, 10 μm .

Cells fixed immediately after the low pH treatment show a label distribution largely restricted to the apical cell surface (Figs. 1 b and 2; Table I). The low pH treatment is apparently too short to allow for considerable long range diffusion of [^3H]DPPC, subsequent passage of the tight junction, and labeling of the basolateral domain. Interestingly, a grain distribution largely restricted to the apical cell surface is found for all samples fixed immediately after the low pH treatment, irrespective of the mode of fixation (glutaraldehyde fixed, glycerol impregnated and frozen, or immediately rapid frozen, Figs. 1 b and 2, respectively). Because chemical fixation and glycerol impregnation were performed for longer time periods (total procedure took 2 h at room temperature; see Materials and Methods for details), the glutaraldehyde action clearly prevented gross diffusion of [^3H]DPPC into the basolateral domain (see Discussion).

If the low pH treatment is followed by a longer incubation at neutral pH, more [^3H]DPPC passes the tight junction and the basolateral domain is progressively labeled (Fig. 1, c-e; Table I).

Together, the data indicate a strictly pH-dependent fusion of liposomes with the apical plasma membrane of influenza virus-infected MDCK cells, followed by a rapid lateral diffusion of incorporated [^3H]DPPC.

Intermediates during the Fusion of Liposomes with the Plasma Membrane

After liposome binding, the fusion of liposomes with the apical plasma membrane was triggered by lowering the pH of the incubation medium. The cell monolayer with associated liposomes was rapid frozen within 30 s after the start of the

Table I. Increase of Basolateral Label after Fusion of [^3H]DPPC-containing Liposomes with the Apical Plasma Membrane of Influenza Virus-infected MDCK Cells

| Time after fusion (0°C)* | Basolateral label as percent of total grains † | Number of samples/Total number of cells |
|--|---|---|
| min | | |
| bound | 0.4 ± 0.6 | 2/20 |
| 0 | 8.5 ± 2.5 | 5/38 |
| 5 | 22.1 ± 2.1 | 3/32 |
| 10 | 31.3 ± 2.8 | 4/46 |

* A confluent monolayer of MDCK cells was incubated on ice for 30 min at pH 7.4 with the labeled liposomes added to the apical cell surface (bound); the endocytic activity of the cells on ice is negligible. Fusion was induced by a low pH treatment (1 min, pH 5, at 37°C), after which pH and temperature were readjusted. Samples were taken at three time points after the low pH treatment; the basolateral plasma membrane domain is progressively labeled.

† Grain densities were measured on light microscopical autoradiograms either in an area from 2.5 μm above to 2.5 μm below the apical cell surface (as a measure of the label associated with the apical part of the plasma membrane) and in the area of the rest of the cell from 2.5 μm below the apical surface to 2.5 μm below the basal membrane (as a measure of the label present in the basolateral plasma membrane domain). The grain densities were corrected for background (on the average 0.3 grains/100 μm^2 , accounting for <3% of the absolute number of total grains (apical + basolateral). The corrected absolute number of total grains (apical + basolateral) were summed on each micrograph and the part attributed to the basolateral plasma membrane domain was determined (δ_{BL} as indicated).

‡ The relatively low amount of basolateral label, even at later time points, is at least partially due to an incomplete fusion of the liposomes with the apical plasma membrane; ~50% of the liposomes bound at neutral pH do not fuse with the apical plasma membrane upon lowering of the pH but stay bound to the apical cell surface (van Meer and Simons, 1986).

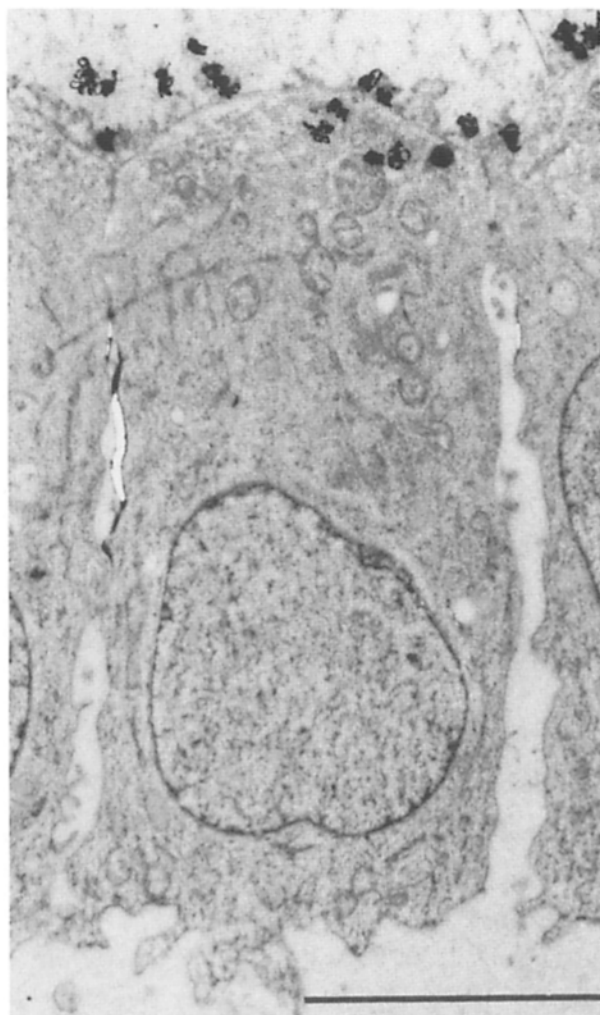


Figure 2. Electron microscopical autoradiography of liposomal [^3H]DPPC after fusion of liposomes with the apical plasma membrane of influenza virus-infected MDCK cells. After liposome-cell binding at neutral pH (30 min on ice), liposomes have been induced to fuse with the apical plasma membrane of a confluent monolayer of MDCK cells on filter by a low pH treatment (1 min, pH 5, at 37°C). Immediately afterwards, the cells were rapid frozen and then further processed as described in Materials and Methods. Label is largely restricted to the apical cell surface. Bar, 5 μm .

low pH incubation, whilst fusion processes were still occurring (van Meer and Simons, 1983). Freeze fracture exposes extended areas of both fracture faces of the apical plasma membrane (Fig. 3).

Analysis of many hundreds of cells rapid frozen during the low pH treatment never showed smooth IMP-free regions in the plasma membrane, as they might be expected to result either from IMP clearance before fusion, diaphragm formation during fusion, or from the insertion of liposomal-pure lipid after fusion (see Discussion). Instead, pronounced protrusions of the plasma membrane are observed bearing a central particle or pit (Fig. 3). Liposomes probably just fusing with microvilli are also visualized; their membranes seem to be continuous (Fig. 4). Liposomes often exhibit particles, both on the convex and the concave fracture face. These par-

ticles are never observed in liposome samples, at either pH 7.4 or 5. As will be argued later, these structures may be interpreted as fusion intermediates.

All experiments were performed at 4.5–5 h after infecting the cells with influenza virus (see Materials and Methods). At this time point, the very first virions start budding from the apical plasma membrane (according to a plaque titration, van Meer, G., and K. Simons, unpublished observations). Virus budding also involves membrane fusion, and the morphological features of the plasma membrane (Fig. 3) could, in principle, be connected to virus budding rather than to liposome-cell fusion (as opposed to the morphological features of the liposomal membrane, as shown in Fig. 4, which can only be due to liposome-cell interaction). Therefore, in control experiments, virus-infected cells were analyzed, and indeed the apical plasma membrane occasionally contained extended areas of protrusions bearing a central particle or pit (Fig. 5). These structures could be easily identified as being virus-budding intermediates. However, the morphology of the virus-budding intermediates, ordered clusters of very small protrusions of the plasma membrane with narrow spacings (Fig. 5), clearly deviates from the morphology observed in Fig. 3, large protrusions and wider spacings. Consequently, Fig. 3 is thought to represent liposome-cell fusion rather than virus budding.

Altogether, the data indicate the absence of smooth IMP-free lipid patches during liposome-cell fusion, and the presence of fusion-correlated particles on both fracture faces of the fusing liposomes and the apical plasma membrane.

Discussion

The fusion of liposomes with the plasma membrane of influenza virus-infected cells (van Meer and Simons, 1983; van Meer et al., 1985) was analyzed morphologically using low temperature methods. This approach made it possible to monitor the fate of incorporated liposomal lipids by autoradiography of liposomal [^3H]DPPC, and to identify fusion intermediates.

Autoradiography

Autoradiography of phospholipids is hampered by extraction and redistribution artifacts, especially during sample dehydration and embedding (Stein and Stein, 1971). Apart from the fact that only a limited number of phospholipid species can be fixed chemically, the chemical pretreatments can often give rise to artifacts including membrane blebbing and the formation of multilamellar membrane structures (Saffitz et al., 1981).

An alternative approach for the preservation of phospholipids makes use of low temperature techniques and renders chemical pretreatments unnecessary. This approach guarantees an almost complete retention of all phospholipid species (Weibull et al., 1984; Verkleij et al., 1985) although neutral lipids are largely extracted (Verkleij, A. J., unpublished results). Even if gross extraction of phospholipids is prevented by using freeze substitution and low temperature embedding, the hazard of redistribution remains.

In this study, the exclusive localization of bound liposomes at the apical plasma membrane before the pH drop offers a sensitive system with which the possible redistribution of phospholipids can be tested. The results show that no redis-

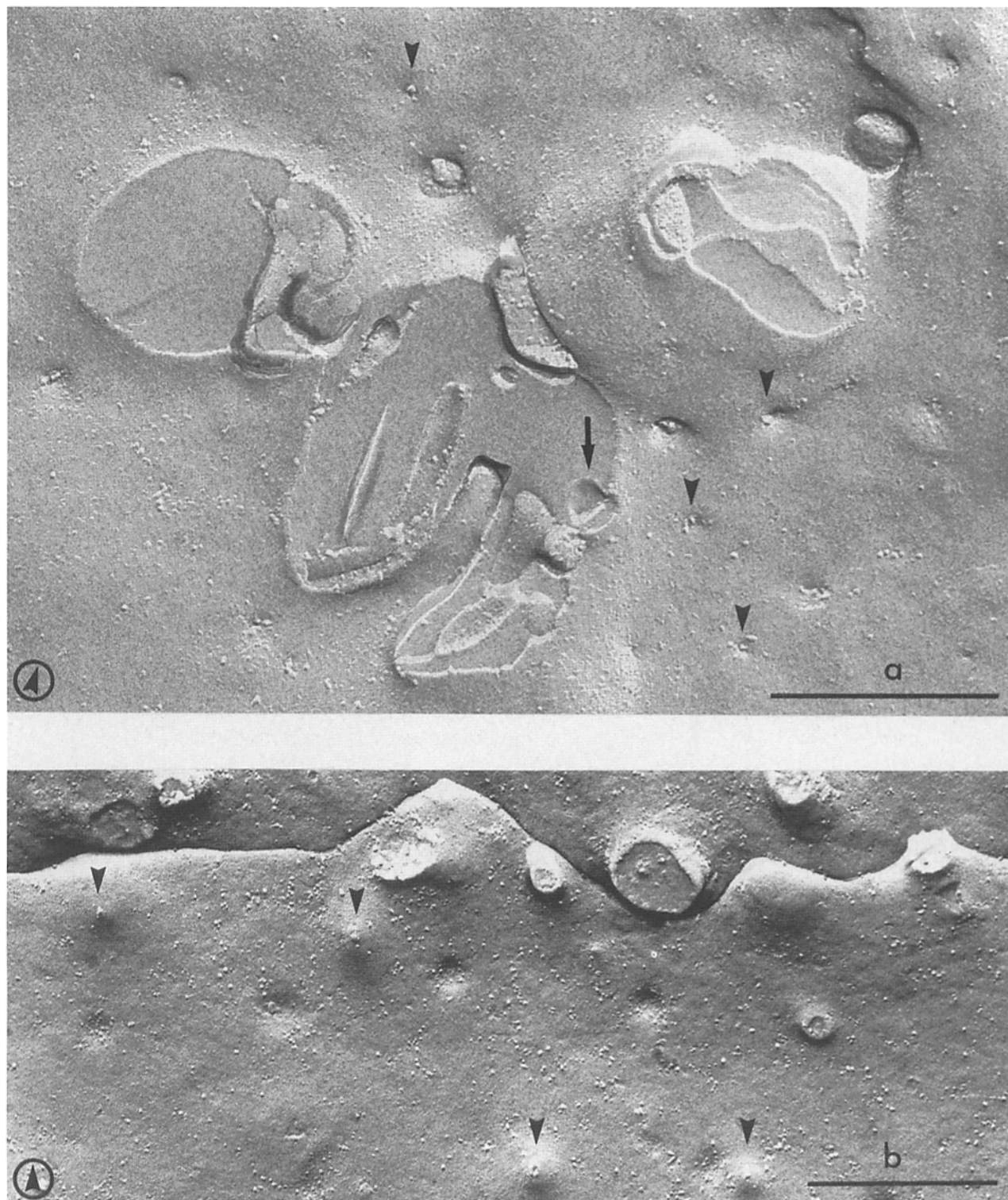


Figure 3. Freeze fracture of the apical plasma membrane during fusion with associated liposomes. Cells with associated liposomes were rapid frozen after a 30-s incubation at pH 5, 37°C. Depressions in the exoplasmic fracture face (a) and protrusions in the protoplasmic fracture face (b) with a central particle or pit were marked by arrowheads. In the case of a deviation of the fracture plane to the outside of the cell, a small liposome fusing with the plasma membrane becomes visible (arrow in a). Direction of Pt/C shadowing indicated by encircled arrowhead. Bars, 0.5 μ m.

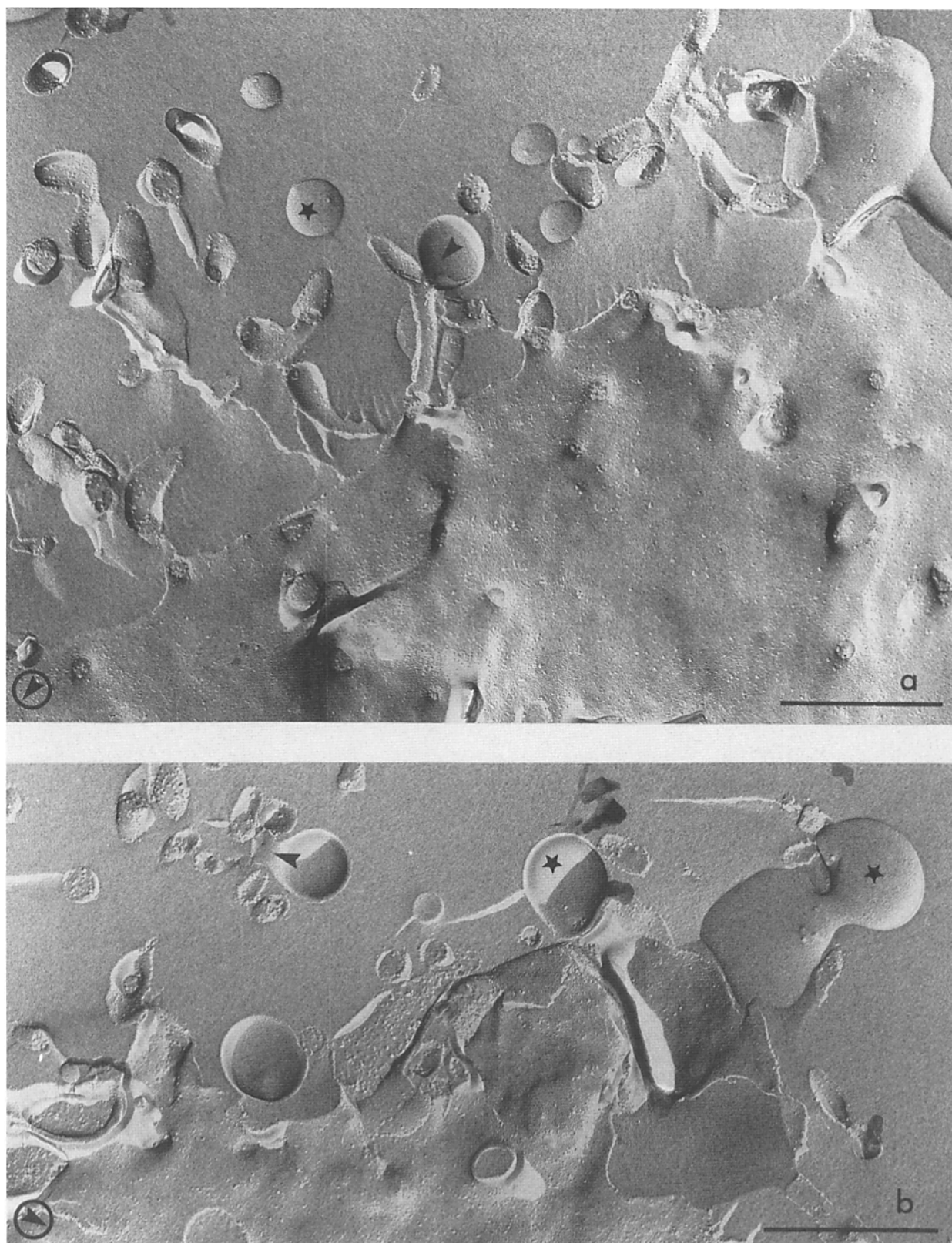


Figure 4. Freeze fracture of liposomes fusing with the apical plasma membrane of influenza virus-infected MDCK cells. Cells with associated liposomes were rapid frozen after a 30-s incubation at pH 5, 37°C. In both *a* and *b*, the exoplasmic fracture face of the apical plasma membrane is exposed (*bottom*), the fracture plane locally leaves the membrane and continues into the incubation medium next to the cells (*top*). Liposomes probably just fusing with microvilli (*arrowheads*) become visible. Some liposomes (*stars*) reveal single particles, presumably in areas in contact with the plasma membrane. Direction of Pt/C shadowing indicated by encircled arrowhead. Bars, 0.5 μ m.

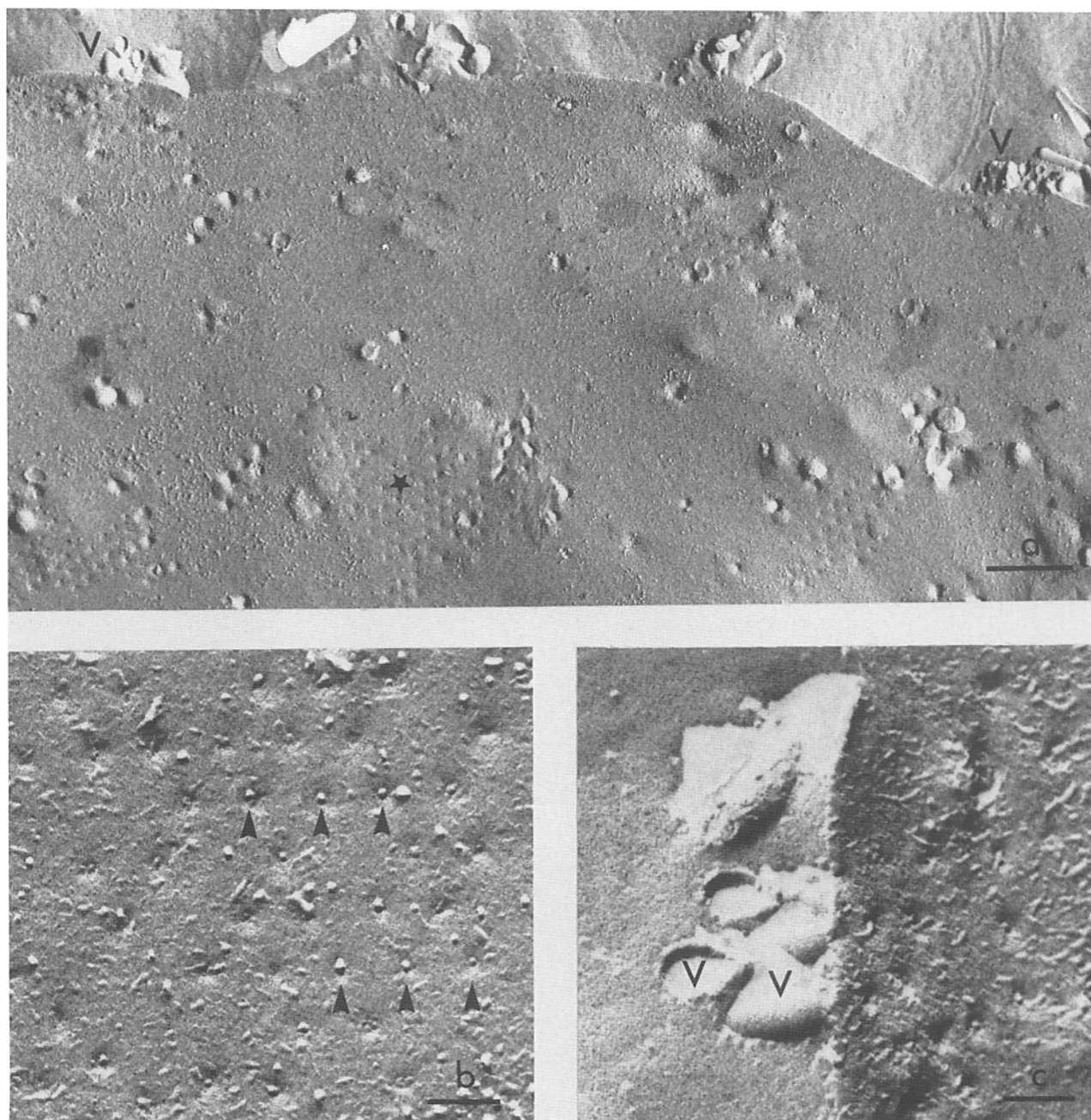


Figure 5. Freeze fracture of apical plasma membrane during influenza virus budding. Infected cells without liposomes were rapid frozen from room temperature (pH 7.4) (a) The exoplasmic fracture face contains ordered clusters of depressions (*star*) with central particles or pits (at higher magnification in *b*, marked by arrowheads). Where the fracture plane leaves the plasma membrane aggregates of budding virions (*V*) as effectors become visible (at higher magnification in *c*). Bars: (a) 0.5 μm ; (b and c) 0.1 μm .

tribution of [^3H]DPPC occurred between these closely associated membranes. This provided a solid basis for the data obtained for the diffusion of liposomal lipids into the plasma membrane after the induction of fusion.

Time Course and Extent of Lipid Diffusion

Cells fixed immediately after the low pH treatment showed only a minor labeling of the basolateral domains (Figs. 1 *b* and 2; Table I). If the low pH treatment was followed by an additional incubation at neutral pH (on ice) long range lipid

diffusion resulted in a progressive labeling of the basolateral domain. Assuming a diffusion coefficient of 0.35 $\mu\text{m}^2/\text{s}$ for the lateral diffusion of phospholipids at 5°C (Jacobson et al., 1981), an average 1.5–5 min is needed for a phospholipid molecule to reach the lateral membrane at midheight by an unrestricted random walk after its incorporation in the apical plasma membrane by liposome–cell fusion (mean displacement 11–20 μm , estimate based on the morphometric data of von Bonsdorff et al., 1985; for calculation see Beck, 1987). This is in fair agreement with the data on lipid diffusion obtained by lipid autoradiography (Table I).

Taken together, the time course of lateral diffusion points to an extremely rapid integration of liposomal lipids into the plasma membrane continuum. Indeed, in freeze-fracture experiments, the IMP-free lipid patches described by Papahadjopoulos et al. (1974); see also conclusions drawn by Poste and Papahadjopoulos, 1978, and Orci and Perrelet, 1978) were not observed when cells were either rapid frozen or chemically fixed at room temperature (Figs. 3 and 4; for further discussion see later).

Samples chemically fixed immediately after the low pH treatment reveal a grain distribution almost restricted to the apical cell surface. Although the total procedure of glutaraldehyde fixation and glycerol impregnation took 2 h at room temperature, [^3H]DPPC is not able to pass the tight junction and label the basolateral domain. The action of glutaraldehyde with respect to the chemical cross-linking of phospholipid molecules is confined to aminophospholipids and a direct influence of glutaraldehyde fixation on the lateral diffusion of phosphatidylcholines seems highly unlikely (Jost et al., 1973). Instead glutaraldehyde treatment may well result in the chemical cross-linking of membrane proteins and aminophospholipids, hindering the lateral diffusion of lipid molecules and possibly preventing their passage from the apical to the basolateral domain. Alternatively, one might speculate, based on the protein model of tight junction structure (van Meer and Simons, 1986), that lipid passage could be blocked by the glutaraldehyde fixation and denaturation of certain tight junction-associated proteins.

Fusion Intermediates Visualized by Freeze Fracture after Rapid Freezing

Rapid freezing is considered to be the only reliable fixation method for the analysis of fast processes like membrane fusion (Plattner, 1981; Knoll et al., 1987). In agreement with earlier studies using pure cryofixation (Heuser et al., 1979; Chandler and Heuser, 1980; Ornberg and Reese, 1981; Plattner, 1981), particle clearance or similar structural features preceding membrane fusion were not observed (Figs. 3 and 4). Smooth lipid patches in the plasma membrane of cells fusing with liposomes have been described by Papahadjopoulos et al. (1974) and were interpreted as evidence for fusion (Poste and Papahadjopoulos, 1978). We observed similar phenomena only after chemical pretreatments on ice (and under these conditions also in control cells in the absence of liposomes; results not shown), but not after rapid freezing or after chemical pretreatments at room temperature. This is in line with the observations of Kachar et al. (1980), who pointed out that routine fixation at low temperatures can lead to intramembrane particle displacement. We interpret the absence of smooth lipid patches in our study as evidence for the rapid lateral diffusion of incorporated liposomal lipids in the plasma membrane.

Samples rapid frozen during the low pH treatment displayed specific morphological features on the fracture faces of both the liposomal membrane and the plasma membrane (Figs. 3 and 4). These distinct features, i.e., well-defined particles and pits, are correlated to the interaction of liposomes with the apical plasma membrane induced by low pH as they were only observed in samples containing both liposomes and cells after lowering the pH and never in liposomes or cells incubated separately at low or neutral pH (Fig. 3 is

thought to represent liposome-cell fusion as opposed to the virus budding intermediates shown in Fig. 5; see Results).

The interpretation of the (liposome-cell) fusion-correlated particles and pits is complicated for two reasons, however. First, the particles and pits could represent prefusion structures. A local-point contact between two membranes can give rise to particles and pits without the necessity of actual membrane fusion ("intermembrane attachment sites," Miller, 1980). In the case of influenza virus-liposome interaction, deformations of the convex fracture face of the liposomal membrane have been observed at neutral pH, often with a central, quite well-defined particle. This central particle was interpreted as a local-point adhesion site (Burger et al., 1988). In the current study, the possibility of local-point adhesion sites is at least partially ruled out, because both particles and pits were found on the protoplasmic fracture face of the plasma membrane (Fig. 3 *b*) and particles were present on both fracture faces of the liposomal membrane (Fig. 4); local-point adhesion sites would give rise to particles only on the protoplasmic or convex fracture face and pits on the exoplasmic or concave fracture face (see Verkleij, 1984).

Even if the possibility of a prefusion structure could be ruled out, the real nature of the particles and pits remains obscure because no technique is currently available to determine whether a fusion-correlated particle or pit represents a lipid, a protein, or a lipid-protein complex. However, if the fusion-correlated particles observed on the fracture faces of the plasma membrane, on the one hand, and the fusing liposome, on the other hand, would be representative of the HA glycoprotein and the NH_2 -terminal fusion sequence of the HA subunit-two (which is thought to induce membrane fusion by insertion into the target membrane; Doms et al., 1988), respectively, (protein) particles would be expected on the exoplasmic fracture face of the plasma membrane and the concave fracture face of the liposome exclusively. Particles on the protoplasmic or convex fracture faces, as shown in Figs. 3 *b* and 4, are not expected, as the HA glycoprotein is almost entirely exposed at the extracellular surface (cf. Burger et al., 1988; in the intact influenza virion intramembrane particles are present on the concave fracture face exclusively).

Based on the presence of fusion-correlated particles on both fracture faces and the similarity, in size and shape, of the particles and pits with those found in pure lipid systems (Verkleij, 1984), we tend to conclude, although direct proof is lacking, that the particles are lipidic and represent a specific structural intermediate of HA-induced liposome-cell fusion. Different molecular organizations have been proposed for the particles observed in pure lipid systems, and whether the observed lipidic particles represent real fusion intermediates, i.e., inverted lipid micelles at the joining stage of two fusing membranes (Verkleij, 1984), or rather represent postfusion structures with a minute aqueous channel already formed (interlamellar attachment sites, Siegel, 1986*a*) is still a matter of debate (cf. Verkleij, 1984; Siegel, 1986*a,b*).

Conclusions and Outlook

On a structural level, all evidence obtained in this study supports the suggestion that influenza virus HA-induced mem-

brane fusion involves a local-point adhesion without IMP clearance, followed by a local-point fusion with a lipidic particle as a specific structural fusion intermediate. After HA-induced liposome-cell fusion, liposomal lipids are very rapidly integrated in the plasma membrane.

In our opinion, the combination of freeze substitution with lipid autoradiography will prove to be a reliable localization technique with general applicability to all phospholipids. The possibility to stop redistribution of radiolabeled phospholipids after specific time periods offers the opportunity to obtain kinetic data on the lateral diffusion of natural phospholipids in a biomembrane, which to date has not been possible. It should also be a valuable tool to study lipid traffic and sorting, and offers the opportunity to determine the intracellular fate of lipophilic drugs.

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References

- Bachmann, L., and M. M. Salpeter. 1967. Absolute sensitivity of electron microscope autoradiography. *J. Cell Biol.* 33:299-305.
- Beck, K. 1987. Mechanical concepts of membrane dynamics: diffusion and phase separation in two dimensions. In *Cytomechanics*. J. Bereiker-Hahn, O. R. Anderson, and W. E. Reif, editors. Springer-Verlag, Berlin. 79-99.
- Branton, D., S. Bullivant, N. B. Gilula, M. J. Karnovsky, H. Moor, K. Mühlethaler, D. H. Northcote, L. Packer, B. Satir, P. Satir, V. Speth, L. A. Staehelin, R. L. Steere, and S. Weinstein. 1975. Freeze-etching nomenclature. *Science (Wash. DC)*. 190:54-56.
- Burger, K. N. J., G. Knoll, and A. J. Verkleij. 1988. Influenza virus-model membrane interaction. A morphological approach using modern cryotechniques. *Biochim. Biophys. Acta*. 939:89-101.
- Chandler, D. E. 1984. Comparison of quick-frozen and chemically fixed sea-urchin eggs: structural evidence that cortical granule exocytosis is preceded by a local increase in membrane mobility. *J. Cell Sci.* 72:23-36.
- Chandler, D. E., and J. Heuser. 1980. Arrest of membrane fusion events in mast cells by quick-freezing. *J. Cell Biol.* 86:666-674.
- Costello, J. J., R. Fetter, and M. Höchli. 1982. Simple procedures for evaluating the cryofixation of biological samples. *J. Microsc.* 125:125-136.
- Davoust, J., J. Gruenberg, and K. E. Howell. 1987. Two threshold values of low pH block endocytosis at different stages. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3601-3610.
- Doms, R. W., J. White, F. Boulay, and A. Helenius. 1988. Influenza virus hemagglutinin and membrane fusion. In *Cellular Membrane Fusion: Fundamental Mechanisms and Applications of Membrane Fusion Techniques*. J. Wilschut and D. Hoekstra, editors. Marcel Dekker, Inc., New York. In Press.
- Duzgunes, N. 1985. Membrane fusion. *Subcell. Biochem.* 11:195-287.
- Futasesaku, Y., and V. Mizuhira. 1986. Negative-staining autoradiography: a new technique for ultracytometry utilizing an interposed film. *J. Histochem. Cytochem.* 34:1085-1094.
- Hansson, G. C., K. Simons, and G. van Meer. 1986. Two strains of the Madin-Darby canine kidney (MDCK) cell line have distinct glycosphingolipid compositions. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:483-489.
- Heuser, J. E., T. S. Reese, M. J. Dennis, Y. Jan, L. Jan, and L. Evans. 1979. Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. *J. Cell Biol.* 81:275-300.
- Humbel, B., and M. Müller. 1984. Freeze substitution and low-temperature embedding. In *Electron Microscopy, Proceedings of the Eighth European Congress on Electron Microscopy*. Vol. 3. A. Csányi, P. Röhlich, and P. Szabó, editors. Budapest. 1789-1798.
- Jacobson, K., Y. Hou, Z. Derzko, J. Wojcieszyn, and D. Organisiak. 1981. Lipid lateral diffusion in the surface membrane of cells and in multibilayers formed from plasma membrane lipids. *Biochemistry*. 20:5268-5275.
- Jost, P., U. J. Brooks, and O. H. Griffith. 1973. Fluidity of phospholipid bilayers and membranes after exposure to osmium tetroxide and glutaraldehyde. *J. Mol. Biol.* 76:313-318.
- Kachar, B., J. A. Serrano, and P. Pinto da Silva. 1980. Particle displacement in epithelial cell membranes of rat prostate and pancreas induced by routine low temperature fixation. *Cell Biol. Int. Rep.* 4:347-356.
- Knoll, G., G. Oebel, and H. Plattner. 1982. A simple sandwich-cryogen-jet procedure with high cooling rates for cryofixation of biological materials in the native state. *Protoplasma*. 111:161-176.
- Knoll, G., A. J. Verkleij, and H. Plattner. 1987. Cryofixation of dynamic processes in cells and organelles. In *Cryotechniques in Biological Electron Microscopy*. R. A. Steinbrecht and K. Zierold, editors. Springer-Verlag, Berlin. 258-271.
- Miller, R. G. 1980. Do 'lipidic articles' represent intermembrane attachment sites? *Nature (Lond.)*. 287:166-168.
- Müller, M., T. Marti, and S. Kriz. 1980. Improved structural preservation by freeze substitution. In *Electron Microscopy. Proceedings of the Seventh European Congress on Electron Microscopy*. Vol. 2. P. Brederoo and W. DePriester, editors. Seven European Congress Electron Microscopy Foundation, Leiden. 720-721.
- Ohki, S., D. Doyle, T. D. Flanagan, S. W. Hui, and E. Mayhew. 1988. Molecular Mechanisms of Membrane Fusion. Proceedings of an International Symposium on Molecular Mechanisms of Membrane Fusion, April 1987, Buffalo, New York. Plenum Publishing Corp., New York.
- Orci, L., and A. Perrelet. 1978. Ultrastructural aspects of exocytotic membrane fusion. In *Membrane Fusion*. G. Poste and G. L. Nicolson, editors. Elsevier/North Holland, Amsterdam. 630-651.
- Ornberg, R. L., and T. S. Reese. 1981. Beginning of exocytosis captured by rapid-freezing of *Limulus ameobocytes*. *J. Cell Biol.* 90:40-54.
- Papahadjopoulos, D., E. Mayhew, G. Poste, S. Smith, and W. J. Vail. 1974. Incorporation of lipid vesicles by mammalian cells provides a potential method for modifying cell behaviour. *Nature (Lond.)*. 252:163-166.
- Pinto da Silva, P., and B. Kachar. 1980. Quick freezing vs. chemical fixation: capture and identification of membrane fusion intermediates. *Cell Biol. Int. Rep.* 4:625-640.
- Plattner, H. 1981. Membrane behaviour during exocytosis. *Cell Biol. Int. Rep.* 5:435-459.
- Plattner, H., and G. Knoll. 1984. Cryofixation of biological materials for electron microscopy by the methods of spray-, sandwich-, cryogen-jet- and sandwich-cryogen-jet-freezing: a comparison of techniques. In *The Science of Biological Specimen Preparation for Microscopy and Microanalysis*. J. P. Revel, T. Barnard and G. H. Haggis, editors. AMF O'Hare, Chicago. 139-146.
- Poste, G., and D. Papahadjopoulos. 1978. The influence of vesicle membrane properties on the interaction of lipid vesicles with cultured cells. *Ann. NY Acad. Sci.* 308:164-184.
- Poste, G., C. W. Porter, and D. Papahadjopoulos. 1978. Identification of a potential artifact in the use of electron microscope autoradiography to localize saturated phospholipids in cells. *Biochim. Biophys. Acta*. 510:256-263.
- Pscheid, P., C. Schudt, and H. Plattner. 1981. Cryofixation of monolayer cell cultures for freeze-fracturing without chemical pretreatments. *J. Microsc.* 121:149-167.
- Saffitz, J. E., R. W. Gross, J. R. Williamson, and B. E. Sobel. 1981. Autoradiography of phosphatidyl choline. *J. Histochem. Cytochem.* 29:371-378.
- Siegel, D. P. 1986a. Inverted micellar intermediates and the transitions between lamellar, cubic, and inverted hexagonal lipid phases. I. Mechanism of the $L_{\alpha}H_{II}$ phase transitions. *Biophys. J.* 49:1155-1170.
- Siegel, D. P. 1986b. Inverted micellar intermediates and the transitions between lamellar, cubic, and inverted hexagonal lipid phases. II. Implications for membrane-membrane interactions and membrane fusion. *Biophys. J.* 49:1171-1183.
- Sowers, A. E. 1987. Cell Fusion. Plenum Publishing Corp., New York.
- Stein, O., and Y. Stein. 1971. Light and electron microscopic autoradiography of lipids: techniques and biological application. *Adv. Lipid Res.* 9:1-72.
- van Meer, G., and K. Simons. 1983. An efficient method for introducing defined lipids into the plasma membrane of mammalian cells. *J. Cell Biol.* 97:1365-1374.
- van Meer, G., and K. Simons. 1986. The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1455-1464.
- van Meer, G., J. Davoust, and K. Simons. 1985. Parameters affecting low-pH mediated fusion of liposomes with the plasma membrane of cells infected with influenza virus. *Biochemistry*. 24:3593-3602.
- van Meer, G., B. Gumbiner, and K. Simons. 1986. The tight junction does not allow lipid molecules to diffuse from one epithelial cell to the next. *Nature (Lond.)*. 322:639-641.
- Verkleij, A. J. 1984. Lipidic intramembranous particles. *Biochim. Biophys. Acta*. 779:43-63.
- Verkleij, A. J., and P. H. J. T. Ververgaert. 1975. Architecture of biological and artificial membranes, as visualized by freeze-etching. *Annu. Rev. Phys. Chem.* 26:101-122.
- Verkleij, A. J., B. Humbel, D. Studer, and M. Müller. 1985. 'Lipidic particle' systems as visualized by thin-section electron microscopy. *Biochim. Biophys. Acta*. 812:591-594.
- von Bonsdorff, C. H., S. D. Fuller, and K. Simons. 1985. Apical and

- basolateral endocytosis in Madin-Darby canine kidney (MDCK) cells grown on nitrocellulose filters. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2781-2792.
- Weibull, C., W. Villiger, and E. Carlemalm. 1984. Extraction of lipids during freeze-substitution of *Acholeplasma laidlawii*-cells for electron microscopy. *J. Microsc.* 134:213-216.
- White, J., M. Kielian, and A. Helenius. 1983. Membrane fusion proteins of enveloped animal viruses. *Q. Rev. Biophys.* 16:151-195.
- Wiley, D. C., and J. J. Skehel. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu. Rev. Biochem.* 56:365-394.