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IMAGING OF THE CYTOPLASMIC LEAFLET OF THE PLASMA MEMBRANE BY ATOMIC FORCE MICROSCOPY

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Abstract

The cytoplasmic face of ventral cell membranes of Madin-Darby canine kidney (MDCK) cells grown on glass coverslips was imaged by atomic force microscopy (AFM) in air and under aqueous medium, in "contact" mode. Micrometer range scans on air-dried samples revealed a heterogeneous structure with some filaments, likely corresponding to actin filaments that abut the inner leaflet of the membrane, and a few semi-organized lattice structures that might correspond to clathrin lattices. Experiments in phosphate-buffered saline confirmed the heterogeneity of the inner membrane surface with the presence of large (> 100 nm) globular structures emerging from the surface. Using sub-micrometer scan ranges, protruding particles, that occupy most of the membrane surface, were imaged in liquid medium and in air. These particles, 8 to 40 nm x-y size, were still present following ethanol dehydration which extracts a large fraction of membrane lipids, indicating their proteic nature. Due, at least partly, to the presence of some peripheral proteins, high magnification images of the inner membrane surface were heterogeneous with regard to particle distribution. These data compare with those previously reported for the external membrane leaflet at the surface of living MDCK cells. They show that details of the cytosolic membrane surface can be resolved by AFM. Finally, the images support the view of a plasma membrane organization where proteins come into close proximity.

Key Words: MDCK cells, isolated membranes, fluorescence, membrane structure.

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Introduction

Upon sub-micrometer imaging of cells in aqueous medium by atomic force microscopy (AFM), the membrane surface appears as constituted mostly of protruding globular particles down to 8-10 nm x-y size (Butt *et al.*, 1990; Häberle *et al.*, 1992; Hörber *et al.*, 1992; Le Grimellec *et al.*, 1994). Both their altered lateral distribution upon lectin addition (Häberle *et al.*, 1989; Hörber *et al.*, 1992) and their degradation by pronase (Le Grimellec *et al.*, 1994), indicate that these particles correspond to proteins present at the membrane surface. For Madin-Darby Canine Kidney (MDCK) cells, an epithelial cell line derived from the kidney, imaging such particles required partial degradation of the glycocalyx, a complex structure composed of highly branched sugars that covers the cell surface. Otherwise, fuzzy images were generally obtained, even in the micrometer range.

So far, there is no AFM image of the structure of the inner membrane leaflet of the plasma membrane which faces the cytosol. Indeed, a large proportion of membrane proteins is accounted for by transmembrane proteins which may or may not interact with peripheral proteins (Houslay and Stanley, 1982). This suggests that images from the inner membrane surface must share common features with images from the outer surface. For the plasma membrane, however, sugars linked to lipids and to proteins are only present on the outer membrane leaflet (Op den Kamp, 1981). Incidentally, this suggests that the structure of the inner membrane leaflet, which faces the cytosol, can be imaged without the treatments required for imaging the outer surface. Several methods have been described which allow the exposure of the inner leaflet of the plasma membrane of cultured cells (the ventral cell membrane, which is firmly bound to the support), for experimental manipulation (Avnur and Geiger, 1981; Larkin *et al.*, 1986; Shannon Moore *et al.*, 1987).

In the present experiments, ventral cell membranes from MDCK cells were imaged by AFM both under liquid medium and in air. As predicted, imaging of membrane preparations even at sub-micrometer range did not

require any enzymatic treatment. Partly due to the presence of extra-membranous structures such as cytoskeletal elements still attached to the membrane, the inner surface of the ventral membrane appeared heterogeneous. At high magnification, the membrane, still heterogeneous, was constituted of globular particles that occupy most of the surface. The size of these particles, down to 8 nm, was comparable to that determined from the outer membrane surface and their proteic nature was supported by the fact they were still observed following ethanol dehydration. These data support the proposal (Houslay and Stanley, 1982) that proteins come into close proximity in plasma membranes.

Materials and Methods

Materials

Culture media were from Gibco (Paisley, Scotland). Polycarbonate filters, 0.4 μm pore diameter, 13 mm diameter were obtained from Nucleopore Corp. (Pleasanton, CA). Anti-Clathrin goat antiserum and Anti-Goat IgG TRITC Conjugate were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Cell culture

MDCK cells were obtained from Flow Laboratories (Irvine, CA). They were used between passages 75 and 84. Cells were grown to confluence at 37°C, in a 5% CO_2/95% air atmosphere, in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum directly on glass coverslips (14 mm diameter). They were used 1-2 days after confluence was reached. Under these conditions, they form a continuous monolayer of cells which firmly adheres to the support, and covers it entirely.

Preparation of isolated ventral cell membranes

MDCK cells on glass coverslips were rinsed three times briefly with ice-cold buffer A {50 mM [2(N-morpholino) ethanesulfonic acid], 5 mM $MgCl_2$, 3 mM ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), pH 6.0}, then incubated at room temperature for three minutes with buffer A containing 1 mM $ZnCl_2$ (Avnur and Geiger, 1981). Excess buffer was removed by blotting with a Whatman No. 4 filter paper for a few seconds. Then, a polycarbonate filter with 0.4 μm pore diameter was carefully placed on the top of the sample. After 10 seconds, the polycarbonate filter was peeled away from the culture, leaving essentially sheets of ventral cell membranes still attached to the coverslip. Membranes were washed three times with ice-cold buffer B {20 mM [tris(hydroxymethyl)methylaminopropane sulfonic acid], 1 mM benzamidine, 1 mM 1,10-phenanthroline, 10 μM leupeptin, pH 9.0), incubated on ice for 20 minutes

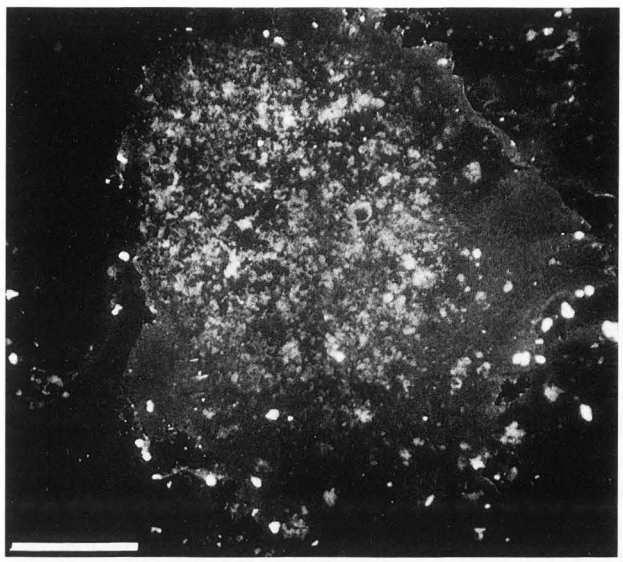
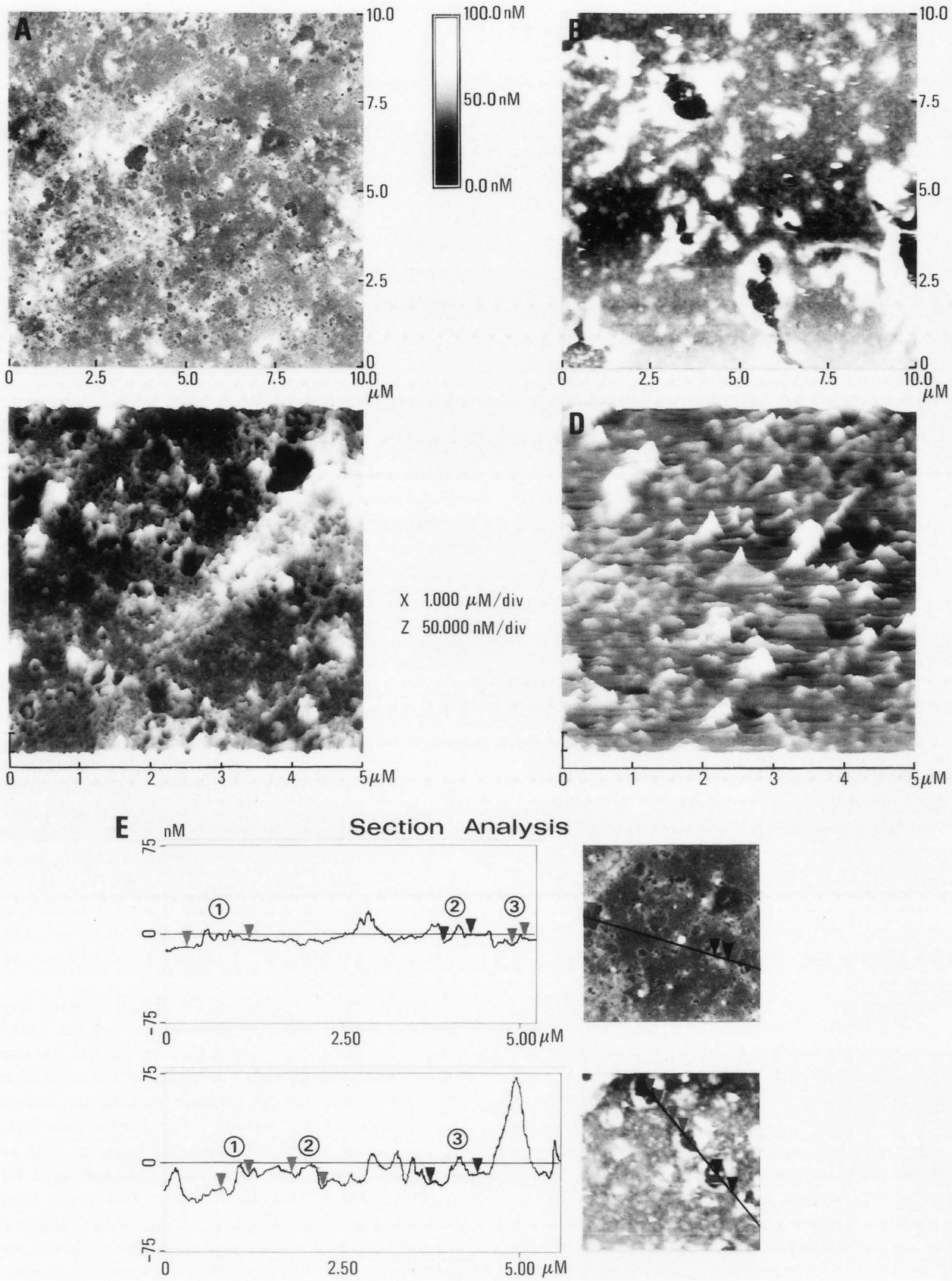


Figure 1. Immunofluorescence staining of clathrin binding sites of ventral cell membranes isolated and labelled with anti-clathrin antiserum, and then with anti-Goat IgG as described in **Materials and Methods**. No significant fluorescence signal was observed for controls, run in parallel, without anticlathrin antiserum. Bar = 20 μm.

Figure 2 (on the facing page). Low-magnification AFM images of ventral cell membrane preparations. Images **2A** and **2C** (zoom of 2A) were from an air-dried sample. Images **2B** and **2D** correspond to membrane preparations examined in aqueous medium. A and B: top view; C and D: quick surface plot. **2E**: section analysis of dried (top) and aqueous medium (bottom) membrane samples. Vertical distances between the pairs of arrows were 5.70 (1), 6.35 (2), and 5.23 (3) nm, respectively, in dried samples (top), and 11.83 (1), 11.36 (2), and 8.45 (3) nm, respectively, for the membrane examined in buffer (bottom).

(Shannon Moore *et al.*, 1987), and fixed at room temperature in phosphate-buffered saline (PBS, pH 7.4) containing 2% paraformaldehyde. To make certain that ventral membranes were still attached to the support, coverslips, taken at random, were processed for localization of clathrin by indirect immunofluorescence (Shannon Moore *et al.*, 1987). Briefly, fixed membranes were washed three times in PBS, then incubated overnight at 4°C, in PBS containing 0.1% (w/v) bovine serum albumin (PBS/BSA) and anti-clathrin antiserum diluted 1:30. After three washes in PBS/BSA, samples were incubated for 30 minutes at room temperature in PBS/BSA containing anti-Goat IgG conjugated to TRITC at a dilution of 1:40. Controls without anti-clathrin were run in parallel. Samples were examined under an


A
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inverted microscope equipped for epifluorescence as previously described (Le Grimellec *et al.*, 1988). Air-dried samples were obtained by washing the membrane preparations three times with distilled water, followed by air drying at room temperature. Ethanol (EtOH) dehydration was performed using the usual procedure for the preparation of samples for electron microscopy (EM) (15 minutes EtOH 70%, 5 minutes EtOH 80%, 5 minutes EtOH 90%, 5 minutes EtOH 95%, 2 x 10 minutes EtOH 100%).

Atomic force microscopy

A Nanoscope III AFM (Digital Instruments, Santa Barbara, CA) was used for the experiments. The microscope was equipped with a "D" type scanner (12 μm). Samples were glued with super-glue-3 (Loctite) to magnetic stainless steel punches and mounted in either the standard support or in the fluid cell, without using the O ring. V-shaped cantilevers with a spring constant of 0.06 N/m (Digital Instruments) were used. Samples were examined either in air or under PBS in the "contact" mode. Samples imaged in liquid were also transferred under liquid onto the AFM scanner so that they never dried out. To control the force the sample was subjected to, the tip was engaged onto a 10 nm by 10 nm area in the center of the field. Typically, the estimated imaging forces were below 0.1 nN for scans in liquid medium and below 5 nN for scans in air. Scan frequency was 1-4 Hz. All AFM images were acquired in constant force mode. They were processed only by flattening to remove background slope.

Results

Control of ventral cell membranes preparations

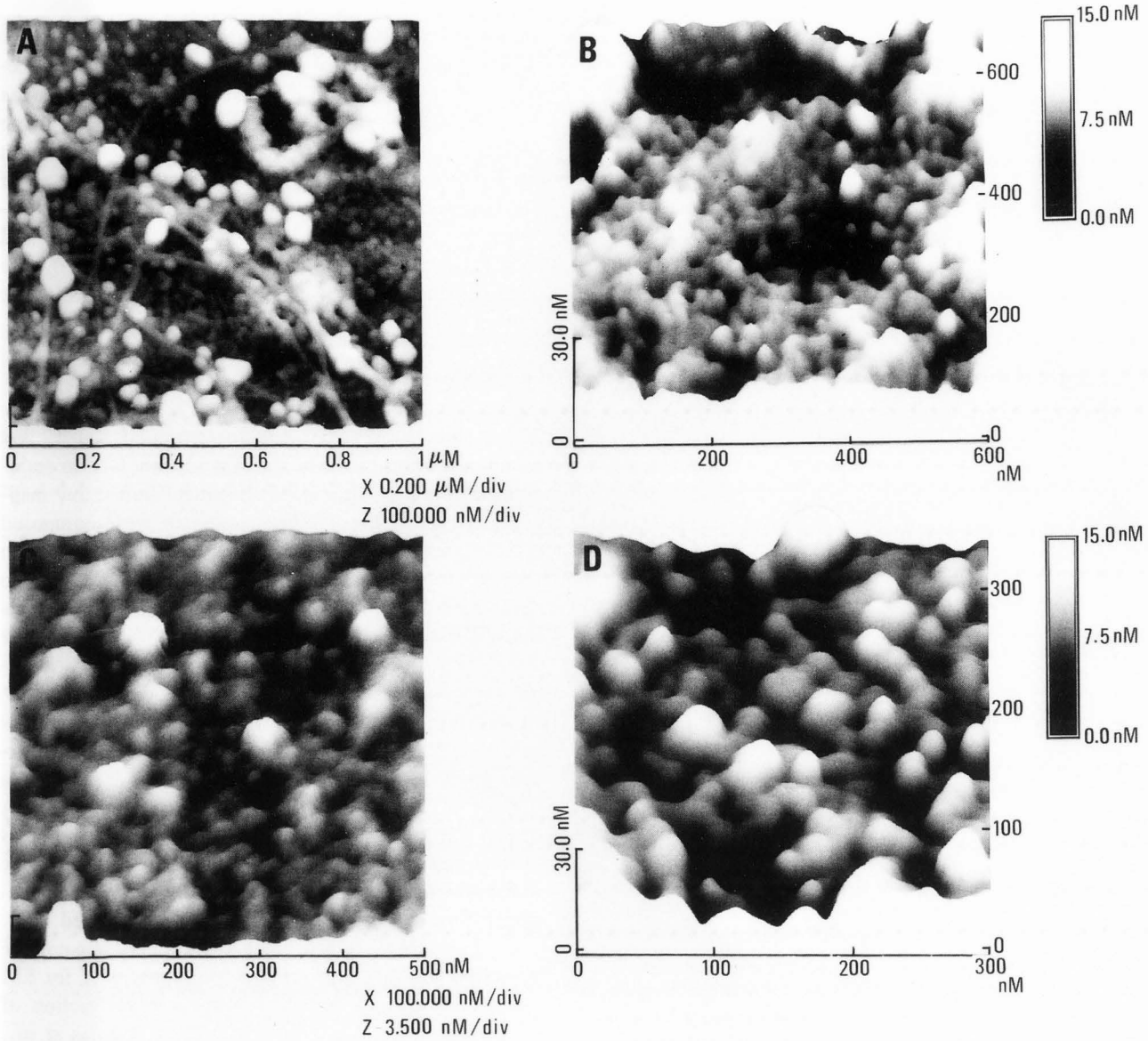
Examination by phase-contrast microscopy of coverslips after the treatment for membrane preparation revealed large zones where cells were destroyed. To ascertain the presence of ventral cell membranes still attached to the support in these zones, we used indirect immunofluorescence staining of clathrin, a protein present on the inner surface of the membrane where it assembles to form coated pits (Heuser, 1980; Shannon Moore *et al.*, 1987). Aggregates of fluorescent material, corresponding to antibody-labeled clathrin, were observed on fragments and sheets of plasma membrane that remained attached to the coverslips following the treatment (Fig. 1). This indicated that the pH 9.0 treatment we used for washing membranes (see **Materials and Methods**) did not remove all peripheral proteins. In our hands, hypotonic shock and unroofing of cells with polycarbonate filters gave larger and better preserved membrane sheets than sonication. Attachment of MDCK cells to the glass coverslips was generally good, leading to a satisfactory yield of ventral cell membrane.

Figure 3 (on the facing page). High-magnification AFM images of air dried ventral cell membranes. Filaments and vesicular structures (**A**), as well as semi-organized structures composed of tightly packed particles (**B**) were frequently imaged at the surface of dried samples. (**C**) and (**D**) are images of the membrane surface of different samples, in zones without filaments and semi-organized structures. Apparent size of the globular particles was determined from section analysis (**E**). Horizontal distances between the pairs of arrows were 12.7 (1), 13.7 (2), and 27.3 (3) nm, respectively, for the section of Figure 3C (top) and were 15.8 (1), 14.1 (2), and 14.6 (3) nm, respectively, for the section of Figure 3D (bottom).

AFM imaging of ventral cell membranes

Low magnification imaging. Once dried, membrane fragments were easily visualized using the optical microscope provided with the Nanoscope III. This allowed us to position the AFM tip where the membranes were located. As shown in Figure 2A, scanning of large zones (10 μm x 10 μm, scanner "D") of air-dried membrane sheets obtained from MDCK cells gave images that resembled those previously reported by EM for ventral cell membrane preparations (Larkin *et al.*, 1986; Samuelsson *et al.*, 1993), with holes of various sizes in the membrane sheet revealing the substrate. These holes were present in raw data, i.e., before the image flattening process. At this low magnification, the surface of the dried samples appeared heterogeneous, being overlaid, in places, by filaments and by semi-organized structures which might correspond to the clathrin lattice organization (Fig. 2C). Estimates of the apparent thickness of the membrane, from the analysis of different sections from different images, through zones including holes, gave minimal values close to 6 nm (Fig. 2E). A similar result was obtained from scans across the edge of membranes, in unfolded zones (data not shown), indicating that in most cases a single membrane layer was covering the support.

In contrast with air-dried samples, membrane fragments maintained in aqueous medium were not visible using the Nanoscope III-coupled optical microscope. This constituted the major difficulty encountered when imaging ventral cell membranes in aqueous medium (PBS): using the "D" scanner (12 μm), which was found to be less sensitive to liquid leaks than the 130 μm scanner ("J" scanner), repeated approaches were frequently required to find the membrane fragments. Scanning of large areas (10 μm x 10 μm) with satisfactory resolution was then easily accomplished, without any prior treatment with glycocalyx-degrading enzymes which contrasted with the situation encountered when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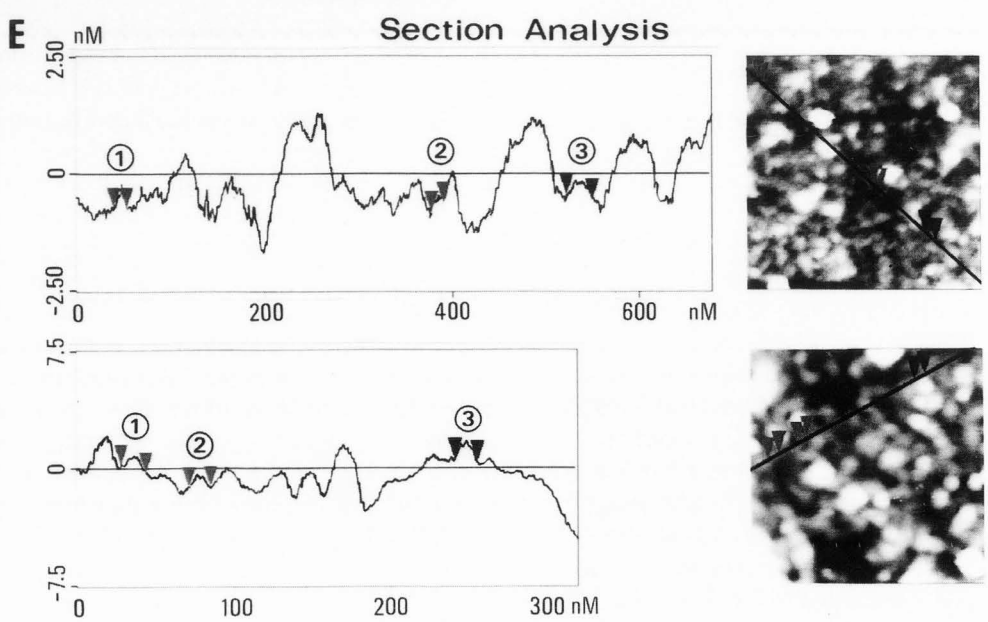
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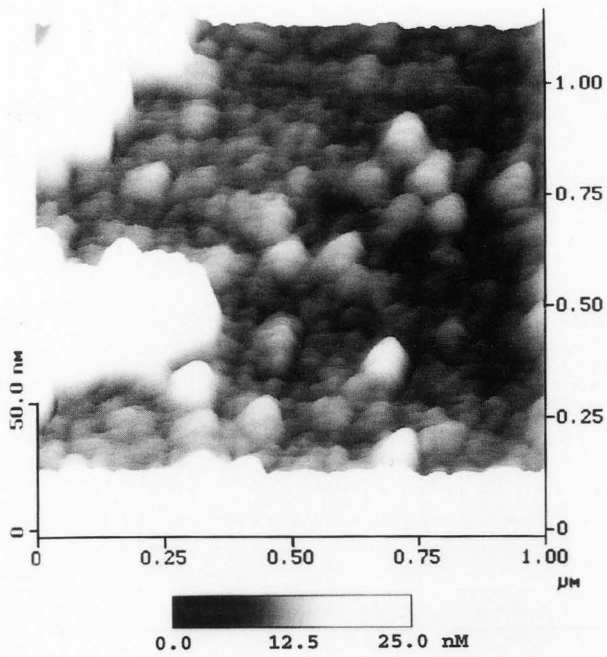

Figure 4. High-magnification AFM image of inner membrane surface after ethanol dehydration.

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imaging the outer membrane surface of living MDCK cells (Le Grimellec *et al.*, 1994). Again, membrane edges or presence of holes in the membrane sheets (Fig. 2B) allowed us to unambiguously identify the material examined and to select smaller zones for scanning. Heterogeneity was still observed upon imaging of large membrane areas in PBS (Fig. 2B), although details of the surface were more clearly resolved using a smaller scan size (5 μm x 5 μm) (Fig. 2D). Protruding structures, some of them tubular with a diameter of about 100 nm, others arranged in clusters, were imaged at the membrane surface. Minimal values of the apparent thickness of the membrane estimated as above were about 8 nm (Fig. 2E).

High magnification imaging. As a consequence of the membrane surface heterogeneity, submicrometer scanning of ventral cell membranes gave rise to various types of images. Considering first the air-dried samples, zones with long filaments and structures likely corresponding to vesicles overlaying the membrane surface were imaged (Fig. 3A). Filaments were about 15 nm in diameter which, taking into account the broadening by convolution with the finite tip shape (Allen *et al.*, 1992; Vesenka *et al.*, 1992), suggest they corresponded to actin filaments. Small spherical structures were found along the filaments in accordance with EM images (Larkin *et al.*, 1986; Samuelsson *et al.*, 1993). Their diameter was 30-40 nm. Vesicular structures, between

Figure 5 (on the facing page). High-magnification AFM images of ventral cell membranes in aqueous medium. (**A**) and (**B**) correspond to two different areas from the same ventral cell membrane. (**C**) and (**D**) were obtained from membrane preparations that differ from the one imaged in (A) and (B). (**E**): section analysis of (C) (top) and (D) (bottom). Horizontal distances between the pairs of arrows were 19.9 (1), 12.9 (2), and 17.6 (3) nm, respectively, for the top section, and were 12.9 (1), 8.6 (2), and 7.8 (3) nm, respectively, for the bottom section.

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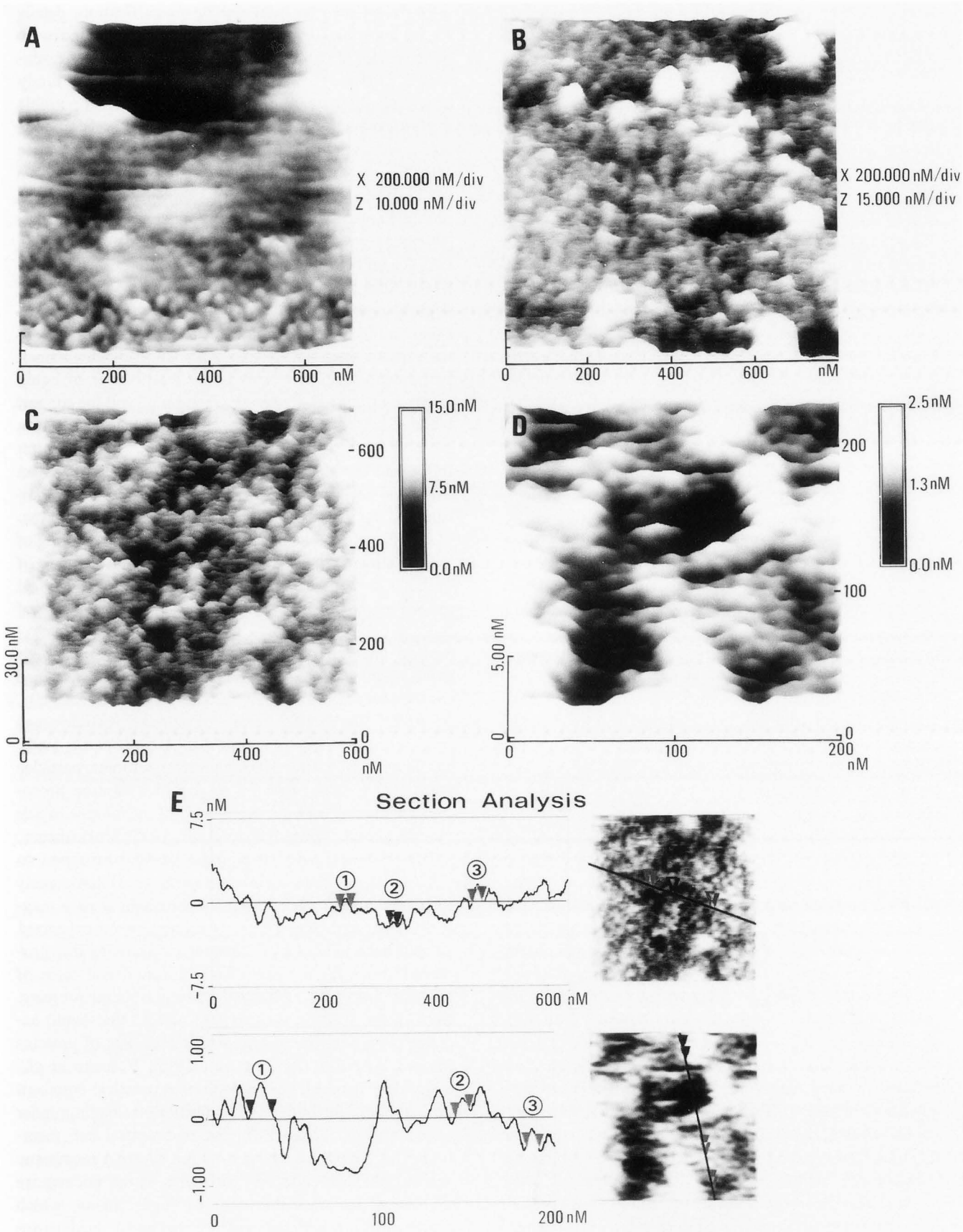
50 nm and 90 nm x-y size, of irregular shape, that might correspond to forming vesicles involved in endocytosis were also imaged. Zones identified at low magnification as semi-organized structures were composed of very abundant and tightly packed particles that often formed crown-like structures (Fig. 3B). In addition to these characteristic structural features, the inner membrane surface was highly corrugated on a nanometer scale. When small areas, apparently devoid of peripheral material (filaments, vesicles and "semi-organized" zones) were scanned, globular structures, heterogeneous in size, were detected emerging from the membrane surface (Figs. 3C and 3D). Similar to the outer membrane surface of dried MDCK cells (Le Grimellec *et al.*, 1994), these globular particles occupied the major part of the inner membrane surface. Their minimal x-y size was 13-14 nm (Fig. 3E). Interestingly, linear arrangements formed of particles were often observed (Fig. 3D). Particles were still imaged following ethanol dehydration (Fig. 4), a procedure commonly used for EM sample preparation, which extracts a large fraction of membrane lipids (Verkleij *et al.*, 1985; Fornas *et al.*, 1993; Mollenhauer, 1993). This indicated that these particles likely correspond to membrane proteins.

The presence of particles and the heterogeneity of the surface were also observed when imaging the ventral cell membranes under PBS. Working under liquid medium, imaging of filaments such as those present in Figure 3A was a very rare event, but heterogeneity of the surface organization was still evident in images such as Figure 5A, where a zone, probably corresponding to the "semi-organized" structures ("clathrin lattices") detected on dried samples, coexists with a smoother area still bearing particles. In Figure 5B, in addition to structures that protrude more than 15 nm above the surface, particles are imaged that appear to be arranged along lines with a direction different from that of the scanning. Figure 5C was obtained from a membrane preparation that differed from that presented in Figure 5A (and with a different tip). Most of our images were obtained using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Z 10.000 nM/div
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scan sizes between 800 nm x 800 nm and 500 nm x 500 nm. Further reduction in the scan size did not generally improve the image resolution. At some occasions, on "flat" zones, 200 nm x 200 nm scans provided reasonably good quality images still showing the presence of membrane particles, but with less height (Fig. 5D). Section analysis suggested that the minimal x-y size of the particles was 8-13 nm, depending on the zone chosen (Fig. 5E). In contrast to intact cells (Le Grimellec *et al.*, 1994), pronase treatment could not be used on hydrated samples to confirm the proteic nature of particles established for dry samples, because of the detachment of the membrane fragments from their support during the assay. It is noteworthy that most of the images of the ventral membrane surface resembled those obtained from the outer membrane surface of living MDCK cells treated with neuraminidase (see Fig. 2 in Le Grimellec *et al.*, 1994).

Discussion

The present data indicate that the cytosolic surface of the ventral cell membrane can be easily imaged by AFM both in air and under aqueous medium. At low magnification, the well established heterogeneity of the ventral cell membrane surface was readily apparent, especially on dried samples. High magnification imaging revealed the existence of small particles heterogeneous in size, similar to the proteins present at the outer membrane surface, that occupy the major part of the surface. The observation that these particles were still present after ethanol dehydration indicated that they most likely corresponded to proteins. The exceptional vertical resolution of the AFM thus provides a new view of the plasma membrane structure where proteins come into close proximity.

Ventral membrane preparations have been used by various laboratories to study the assembly of clathrin-coated pits as well as the relationships between the plasma membrane and the cytoskeleton. In accordance with EM micrographs on carbon-platinum replicas of such preparations, AFM examination of dried samples at intermediate magnification, i.e., with scanning fields in the μm range, shows the presence of cytoskeletal elements, likely corresponding to actin filaments, decorated by vesicles and that abut the plasma membrane (Larkin *et al.*, 1986; Samuelsson *et al.*, 1993). Semi-organized lattice structures emerging from the surface were frequently observed. Taking into account our indirect immunofluorescence images, it seems reasonable to assume that these correspond to clathrin lattices. The difference between our AFM images and those obtained after quick-freeze deep etch technique (Heuser, 1980) probably reflects the difference in sample preparation. Interestingly, at such an intermediate magnification, details of the cytosolic surface of the ventral membrane were better resolved on dried samples than on samples examined in buffer. In particular, thin filaments were rarely observed when scanning was done in buffer. A possible explanation is that, during scanning, the lateral force exerted by the tip on filaments floating at a long distance from the site of their interaction with the membrane was sufficient to break this interaction. Estimates of the minimal apparent thickness of the membrane in the flattest zones gave minimal values close to 6 nm for dried samples and close to 8 nm for samples in buffer, respectively. For dried samples, this value was close to (but slightly higher than) that previously reported. Differences in the degree of attachment of the membranes to the support seem a plausible explanation for these small variations. Similarly, it is likely that a layer of liquid remained trapped between the membrane and the support when samples were examined in buffer. Added to a possible swelling of the membrane in aqueous medium, this could account for the difference recorded between dried and non-dried samples. These measurements indicate that the images were from a single membrane layer.

In air and under liquid, sub-micrometer scanning of the membrane cytosolic surface revealed the presence of globular structures, heterogeneous in size, that occupied the major part of the surface. The minimal x-y size of these particles was approximately 8 nm for samples examined under liquid medium and about 13 nm for air dried samples. In accordance with the images obtained for the outer surface of red blood cells and MDCK cells, in which the proteic nature of similar particles was established by different approaches (Hörber *et al.*, 1992; Le Grimellec *et al.*, 1994), these membrane particles were still present after the ethanol dehydration procedure, which extracts a large part of membrane lipids (Verkleij *et al.*, 1985; Fornas *et al.*, 1993; Mollenhauer, 1993), indicating that they most likely correspond to proteins of the inner membrane surface. Heterogeneity of the membrane surface remained present at such magnifications, with zones corresponding to semi-organized lattice characterized by "clusters" of particles that protruded above the surface. This indicated that some of the globular particles imaged were peripheral proteins. Besides the well-recognized "tip effect" that could account for a possible enlargement of the size of proteins imaged by AFM (Allen *et al.*, 1992; Vesenka *et al.*, 1992), our data call for an additional practical comment on membrane imaging. High resolution imaging under liquid was much easier for isolated ventral cell membranes of MDCK cells than for the external membrane surface of intact cells. In particular, it did not require the use of enzymes that degrade sugar chains, which agrees with biochemical evidence that sugar residues are

present only at the outer surface of the membrane (Op den Kamp, 1981) and support the view that the glycocalyx may constitute a major obstacle to high resolution imaging of the membrane surface of intact cells (Le Grimellec *et al.*, 1994).

In the present images of the membrane, globular particles practically occupy all of the ventral surface. This differs from the early fluid mosaic membrane model where proteins were represented as globular structures surrounded by vast lipid domains (Singer and Nicholson, 1972), but is not unexpected: as previously pointed out, the protein to lipid ratio (w/w) of plasma membranes, generally exceeding unity, suggests that in these membranes, proteins are in close proximity (Houslay and Stanley, 1982). Moreover, our data compare to those obtained by high resolution scanning electron microscopy (HRSEM) of the surface of LLCPK1 cells, another renal epithelial cell line, where it was shown that the membrane is covered with small particles (Walther and Hentschel, 1989). It is noteworthy that, by AFM, a part of the particles on the ventral cell membrane was frequently found to be distributed along lines with a direction different from that of the scanning. Such an observation was previously made for proteins at the outer membrane surface of intact cells (Hörber *et al.*, 1992; Le Grimellec *et al.*, 1994). The fact that some particles are arranged in parallel lines was also reported in HRSEM experiments (Walther and Hentschel, 1989). Whatever the origin(s) of the phenomenon, which might be related to interactions between extracellular matrix, membrane proteins, and cytoskeleton elements (Edidin *et al.*, 1991; Sako and Kusumi, 1994), these observations agree with the proposal that part of the proteins might be not randomly distributed in the membrane plane (Yechiel and Edidin, 1987).

Conclusion

Atomic force microscopy of isolated ventral cell membranes provides structural images of the cytosolic leaflet of the plasma membrane in aqueous medium with details that so far were not described by other morphological techniques. As visualized by the AFM, particles, which likely correspond to proteins, occupy a major part of the membrane inner surface, in accordance with the images previously reported of the outer surface of cells. In addition to an improvement in the tip quality, which might result in a better resolution of the AFM images, the development of new near-field techniques (optical?) allowing the identification of the proteins imaged appears to be necessary for a better understanding of the membrane structure.

References

Allen MJ, Hud NV, Balooch M, Tench RJ, Siekhaus WJ, Balhorn R (1992). Tip-radius-induced artifacts in AFM images of protamine-complexed DNA fibers. Ultramicroscopy **42-44**: 1095-1100.

Avnur Z, Geiger B (1981). Substrate-attached membranes of cultured cells isolation and characterization of ventral cell membranes and the associated cytoskeleton. J Mol Biol **153**: 361-379.

Butt HJ, Wolff EK, Gould SAC, Dixon Northern B, Peterson CM, Hansma PK (1990). Imaging cells with the atomic force microscope. J Struct Biol **105**: 54-61.

Edidin M, Kuo SC, Sheetz MP (1991). Lateral movements of membrane glycoproteins restricted by dynamic cytoplasmic barriers. Science **254**: 1379-1382.

Fornas E, Renau-Piqueras J, Fortea A, Mayordomo F, Alborch E (1993). Cholesterol and 25-hydroxycholesterol retention in specimens of liver and aorta prepared for electron microscopy. Lipids **28**: 923-928.

Häberle W, Hörber JKH, Binnig G (1989). Force microscopy on living cells. J Vac Sci Technol **B9**: 1210-1212.

Häberle W, Hörber JKH, Ohnesorge F, Smith DP, Binnig G (1992) *In situ* investigations of single living cells infected by viruses. Ultramicroscopy **42-44**: 1161-1167.

Heuser J (1980). Three-dimensional visualization of coated vesicle formation in fibroblasts. J Cell Biol **84**: 560-583.

Hörber JKH, Häberle W, Ohnesorge F, Binnig G, Liebich HG, Czerny CP, Mahnel H, Mayr A (1992). Investigation of living cells in the nanometer regime with the scanning force microscope. Scanning Microsc **6**: 919-930.

Houslay MD, Stanley KK (1982). Dynamics of Biological Membranes. John Wiley and Sons, Chichester, U.K. pp. 24-29.

Larkin JM, Donzell WC, Anderson RGW (1986). Potassium-dependent assembly of coated pits: New coated pits form as planar clathrin lattices. J Cell Biol **103**: 2619-2627.

Le Grimellec C, Friedlander G, Giocondi M-C (1988). Asymmetry of plasma membrane lipid order in Madin-Darby Canine Kidney cells. Am J Physiol **255**: F22-F32.

Le Grimellec C, Lesniewska E, Cachia C, Schreiber J-P, de Fornel F, Goudonnet J-P (1994). Imaging of the membrane surface of MDCK cells by atomic force microscopy. Biophys J **67**: 36-41.

Mollenhauer HH (1993). Artifacts caused by dehydration and epoxy embedding in transmission electron microscopy. Microsc Res Tech **26**: 496-512.

Op den Kamp JAF (1981). The asymmetric

architecture of membranes. In: Membrane Structure. Finean JB, Michell RH (eds.). Elsevier/North-Holland, Amsterdam. pp. 83-126.

Sako Y, Kusumi A (1994). Compartmentalized structure of the plasma membrane for receptor movements as revealed by a nanometer-level motion analysis. J Cell Biol **125**: 1251-1264.

Samuelsson SJ, Luther PW, Pumplin DW, Bloch RJ (1993). Structures linking microfilament bundles to the membrane at focal contacts. J Cell Biol **122**: 485-496.

Shannon Moore M, Mahaffey DT, Brodsky FM, Anderson RGW (1987). Assembly of clathrin-coated pits onto purified plasma membranes. Science **236**: 558-563.

Singer SJ, Nicholson GL (1972). The fluid mosaic model of the structure of cell membranes. Science **175**: 720-731.

Verkleij AJ, Humbel B, Studer D, Müller M (1985). "Lipidic particles" systems as visualized by thin-section electron microscopy. Biochim Biophys Acta **812**: 591-594.

Vesenka J, Guthold M, Tang CL, Keller D, Delain E, Bustamante C (1992). Substrate preparation for reliable imaging of DNA molecules with the scanning force microscope. Ultramicroscopy **42-44**: 1243-1249.

Walther P, Hentschel J (1989). Improved representation of cell surface structure by freeze substitution and backscattered electron imaging. Scanning Microsc Suppl **3**: 201-211.

Yechiel E, Edidin M (1987). Micrometer-scale domains in fibroblast plasma membranes. J Cell Biol **105**: 755-760.

Discussion with Reviewers

Reviewer I: I am missing some experimental data about the roughness of the support used for the investigation of membranes.

Authors: The surface of the glass coverslips we used as support was much less corrugated than that of the membranes (Fig. 6). Accordingly, it is unlikely that roughness of the support had a significant influence on the three-dimensional images of the cytoplasmic membrane surface.

Reviewer I: How do the authors know that the bottom of holes really corresponds to the substrate and that the upper side of the membrane is not covered with other materials?

Authors: As we indicated in the text, estimates of the apparent thickness of the membrane from analysis of different sections through zones including holes or from scans across the edge of membranes in unfolded zones gave comparable results. This strongly suggests that the bottom of the holes really corresponds to the substrate.

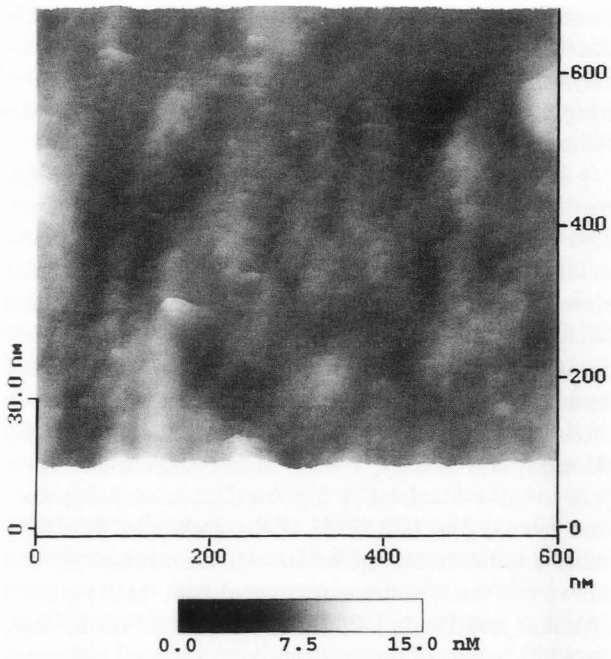

Figure 6. AFM imaging of the glass coverslip support.

Concerning the possibility that the upper side of the membrane was covered with other materials, we mentioned at different occasions that the incubation step at pH 9.0 did not remove all peripheral proteins, which explains why we could still get immunofluorescence staining of clathrin. The intensity of the fluorescence was, however, markedly reduced by the incubation at pH 9.0, indicating that most of the peripheral proteins were washed out by the treatment. The unavoidable presence of some contaminant material, that sticks to the preparation during washing, might explain that the depth of the bottom of some holes is not constant. In fact, the purpose of the sections was essentially to verify that we were scanning a single membrane layer, i.e., the ventral membrane. This was clearly the case.

A. Cricenti: What is the change in height on average between the untreated cells and after peeling both in air and in solution? Since a large part of the cell has been removed by peeling, I would also expect an increase in corrugation over the cells unless a very small volume of the cell has been left onto the support. If only a small part of the outer membrane has been removed how do you know that the images are from the inner part?

Authors: We know because, before peeling the confluent cells form a continuous monolayer, it is not possible to obtain the height of untreated cells by AFM. According to EM studies, the height of confluent MDCK

cells grown on solid support can vary between 2-3 μm and 6-8 μm depending in particular on the stage of confluence and the position of the section (near the tight-junctions or above the cell nucleus). For the same reason, the height of the dried control monolayer cannot be measured by AFM. However, it is known that air drying results in a severe shrinkage and decreases the cellular height. With the peeling method we used, large areas of the cell monolayer became "transparent" for conventional light microscopy. It was also often difficult to visualize the material remaining on the support by phase contrast microscopy. On the other hand, unbroken cells, or damaged cells were easily observed by the same technique. This suggested that in the light "transparent" zones, we looked either at the glass coverslip surface or at something very thin which adhered to the support, i.e., the ventral membrane surface, in accordance with previously published data (see for instance: Avnur and Geiger, 1991; Larkin *et al.*, 1986; Shannon Moore *et al.*, 1987). To ascertain the presence of ventral membranes in our preparations we used both immunofluorescence localization of clathrin, which when present on membranes, is on their inner leaflet, and lipophilic fluorescent probes (NBD-phosphatidylcholine, TMA-DPH) which label membranes. Both techniques confirmed that a large part of the light "transparent" areas was covered with membranes. The presence of clathrin, very abundant before the pH 9.0 washing step, confirmed the membranes were correctly oriented with their inner leaflet facing the medium. It is noteworthy that scanning of lightly or severely damaged cells that still contained intracellular material gave quite different images. For lightly damaged cells, big holes in the apical plasma membrane, revealing intricate intracellular tubular structures were imaged. Severely damaged cells that were not completely washed from intracellular structures gave characteristic highly corrugated images with abundant intracellular membranes, cytoskeleton elements, and from time to time the presence of the nucleus.

A. Cricenti: Did you observe any difference on the immunofluorescence of clathrin between the untreated cells and after peeling?
Authors: Yes. The fluorescence of cells only permeabilized by the treatment was diffuse and much more intense than that of isolated membranes.