Imaging of the Membrane Surface of MDCK Cells by Atomic Force Microscopy

C. Le Grimellec,* E. Lesniewska,‡ C. Cachia,‡ J. P. Schreiber,‡ F. de Fornel,§ and J. P. Goudonnet§

*Institut National de la Santé et de la Recherche Médicale U251, Faculté de Médecine Xavier-Bichat, 75018 Paris; ‡Laboratoire de Biophysique, Faculté de Pharmacie, Université de Bourgogne, 21033 Dijon; and §Laboratoire de Physique du Solide, U.R.A. 785 CNRS Faculté des Sciences Mirande, Université de Bourgogne, 21004 Dijon, France

ABSTRACT The membrane surface of polarized renal epithelial cells (MDCK cells) grown as a monolayer was imaged with the atomic force microscope. The surface topography of dried cells determined by this approach was consistent with electron microscopy images previously reported. Fixed and living cells in aqueous medium gave more fuzzy images, likely because of the presence of the cell glycocalix. Treatment of living cells with neuraminidase, an enzyme that partly degrades the glycocalix, allowed sub-micrometer imaging. Protruding particles, 10 to 60 nm *xy* size, occupy most of the membrane surface. Protease treatment markedly reduced the size of these particles, indicating that they corresponded to proteins. Tip structure effects were probably involved in the exaggerated size of imaged membrane proteins. Although further improvements in the imaging conditions, including tip sharpness, are required, atomic force microscope already offers the unique possibility to image proteins at the membrane surface of living cells.

INTRODUCTION

The atomic force microscope (AFM) introduced by Binnig et al. (1986) is a scanning probe microscope that offers the possibility of imaging soft surfaces in aqueous environment with lateral resolution down to 1–50 nm (Hoh and Hansma, 1992). AFM has been used recently for investigating surface changes and displacements (Butt et al., 1990; Häberle et al., 1991; Kasas et al., 1993; Fritz et al., 1993), cell surface viscosity (Radmacher et al., 1992), cytoskeleton dynamic (Henderson et al., 1992; Chang et al., 1993), and virus exocytosis on isolated cells (Hörber et al., 1992; Häberle et al., 1992). Except for red blood cells, where structures down to 8–10 nm were detected (Butt et al., 1990; Häberle et al., 1991), details of the membrane surface of isolated cells in aqueous medium are lacking, cells being imaged in most of the experiments using scanning ranges of several micrometers.

Madin Darby canine kidney (MDCK) cells, a cell line derived from dog kidney, form an epithelial monolayer with structural characteristics and biochemical and biophysical properties close to those of epithelia in situ (Rindler et al., 1979). This cell line is one of the most popular models in studies on cell polarization processes (Louvard, 1980; Simons and Fuller, 1985; Rodriguez-Boulan and Powell, 1992). So far, AFM has not been used for imaging the surface of such highly differentiated cells grown as a monolayer.

With the AFM, we studied the apical membrane surface of dried, fixed, and living MDCK cells grown to confluence. Morphological characteristics of MDCK cells including structures such as microvilli were easily imaged from dried cells. AFM further allowed us to visualize particles, 8–70 nm *xy* size at the membrane surface using submicrometer scans. Fixed and living cells in aqueous medium gave more fuzzy images. This was likely due to the presence of the cell glycocalix. Partial disruption of the glycocalix allowed to image particles 10–60 nm in diameter, identified as proteins, at the membrane surface of living MDCK cells.

Received for publication 19 January 1994 and in final form 7 April 1994.

Address reprint requests to C. Le Grimellec, INSERM U251, L.E.M., Fac. Med. X-Bichat, 16 rue Henri Huchard, 75018 Paris, France. Tel.: 011-33-44-85-62-80; Fax: 011-33-42-28-15-64.

© 1994 by the Biophysical Society

0006-3495/94/07/36/06 $2.00

MATERIALS AND METHODS

Materials

Culture media were from Gibco (Paisley, Scotland). Neuraminidase from Vibrio Cholerae and pronase were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Cell culture

MDCK cells were obtained from Flow Labs (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, either in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum or in serum-free medium (Le Grimellec et al., 1988) directly on glass cover slips (14-mm diameter) or on green mica. They were used 1–2 days after the confluence was reached. Under these conditions, they form a continuous monolayer of cells that firmly adheres to the support, covering it entirely.

Atomic force microscopy

A Nanoscope III AFM (Digital Instruments, Santa Barbara, CA) was used for the experiments. The microscope was equipped with a "J" (130 μm)-, an "E" (12 μm)-, or an "A" (800 nm)-type scanner. 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 spring constant of 0.06–0.38 N/m (Digital Instruments) were used. To control the force to which the sample was subjected, the tip was engaged onto a 10 × 10 nm scan area in the center of the field. Typically, imaging forces were then adjusted to less than 5 nN. The scan frequency was 1–2.5 Hz. All AFM images were acquired in constant force mode (i.e., "height mode"). They were processed only by flattening to remove background slope.

Sample preparation

Living cells

Before examination in the "fluid cell," living MDCK cells were washed 3 times with serum-free medium devoid of pH indicator and imaged in the same medium. Alternatively, living cells were washed with PBS (NaCl 125 mM, Na_2HPO_4 20 mM, NaH_2PO_4 5 mM, KCl 5 mM, pH 7.4) containing 0.5 mM $CaCl_2$ and 2 mM glutamine (supplemented PBS: S-PBS) and imaged in this solution, which preserves their functions (Le Grimellec et al., 1988). When required, cells imaged with the "J" scanner were treated with 30 mU/ml neuraminidase in S-PBS for 1 h on ice or for 15 min at room temperature, washed 3 times and further imaged in the same buffer. For pronase treatment, cells were incubated in S-PBS containing BSA (1 mg/ml) and 0.25% (w/v) pronase for 15 min at room temperature and washed 3 times with S-PBS before imaging in the same buffer. Cellular viability was estimated by ability to exclude the vital dye, trypan blue (Blais et al., 1993).

Fixed cells

Cells were fixed in 3% paraformaldehyde/S-PBS solution for 30 min at room temperature, washed 3 times with PBS, and imaged in the same solution. In some experiments, cells were treated with pronase as described above, before fixation.

Dried cells

Fixed cells were dehydrated with ethyl alcohol/water solutions of increasing ethanol concentration (from 70 to 100%) as described for EM samples preparation (Bergeron et al., 1994). They were examined in air.

RESULTS AND DISCUSSION

Imaging of living cells at low magnification ("J" scanner) confirmed the monolayer organization of the cultured MDCK cells as well as the highly corrugated and heterogeneous character of their membrane surface (Fig. 1 *A*). Intercellular junctions generally appeared as a fence-like structure forming the characteristic "honeycomb pattern" observed when using fluorescent probes that label the plasma membrane (Dragsten et al., 1981; Le Grimellec et al., 1988). In most cells, depression of the membrane surfaces were observed in zones generally close to an intercellular junction. Comparable images were obtained for paraformaldehyde-fixed cells in liquid medium (not shown). These depression zones were still visible for dried cells examined in air (Fig. 1 *B*). For living cells, fuzzy images were obtained when decreasing the scan size down to a few micrometers, using either the "J" or the "E" scanner, in an attempt to improve the resolution. The use of cantilevers with spring constants between 0.06 and 0.38 N/m and typical forces applied to the samples from less than 0.5 to 5 nN did not modify the image pattern. The fuzzy aspect of cells imaged in liquid medium in the micrometer scale range is common in AFM and is more or less pronounced according to the cell type studied (see, for instance, Fig. 3 in Kasas et al., 1993; Fig. 3 in Fritz et al., 1993; Fig. 2 in Chang et al., 1993). The quality of images was improved in fixed cells but was really satisfactory only for dried cells (Fig. 1 *C*). In accordance with electron microscopy and scanning electron microscopy images (Imhof et al., 1983; Behrens et al., 1985; Bergeron et al., 1994), AFM allowed us to visualize, under these conditions, finger-like structures, the microvilli, present at the membrane surface of MDCK cells. These microvilli were abundant in the region of contact between cells (Louvard, 1980), which could explain the fence-like AFM image obtained at lower magnification. Also in accordance with EM, the presence of pits, which might be involved in endocytic processes, was frequently observed at the basis of microvilli.

When scanning 800 × 800 nm areas ("A" scanner), globular structures, heterogeneous in size, can be detected emerging from the membrane surface of dried cells (Fig. 2 *A*). The structures seen in this image resemble those observed on blood cells examined in liquid medium (Butt et al., 1990; Häberle et al., 1991). The *xy* size of these protrusions (range 8–70 nm) varied little between the experiments. As shown, these particles occupied the major part of the cell surface. For paraformaldehyde-fixed MDCK cells imaged in PBS, we could zoom in certain regions and scan 800 × 800 nm areas (Fig. 2 *B*). Protruding structures of *xy* size comparable to that estimated from dried cells, but of more irregular shape, were also visualized. Part of the particles seemed to be arranged along lines whose directions differed from that of scanning, as previously reported for red blood cells (Häberle et al., 1991; Hörber et al., 1992). Again, the cell surface appeared highly corrugated, with corrugation amplitudes mostly of 1–5 nm. Similar images were obtained from different cell culture batches, examined at different times. Preatreatment with pronase, a protease commonly used to digest proteins at the membrane surface of cells under conditions that keep the membrane impermeable to macromolecules (Karin and Mintz, 1981), drastically altered membrane surface images by reducing the *xy* size, the height, and the number of the protruding particles that eventually became undetectable (Fig. 2 *C*). This indicated that these particles were of proteic nature and likely corresponded to proteins (peripheral and/or integral, perhaps) exposed at the membrane surface.

In living cells, the fuzzy character of images at intermediate magnification mentioned above was associated with the impossibility of obtaining satisfying force curves and images from 800 × 800 nm scans. The surface of most cells is covered by a glycocalix constituted of highly branched sugar residues linked to proteins that can extend well above the membrane surface (Underhill and Toole, 1982; Lee et al., 1993). The glycocalix is often damaged during the fixation/drying processes, and faulty fixation of pericellular substances during the preparative procedure might account for the failure to visualize glycocalix on the surface of most cells in conventionally fixed tissues (Behnke and Zelander, 1970; Hunziker et al., 1983). We hypothesize that the fuzzy appearance at low magnification and the problems encountered when trying to scan sub-micrometer areas were linked to the presence of this glycocalix with the sugar chains moving under the AFM tip during scanning. Examination of the glycocalix organization in toad bladder epithelial cells using quick-freeze, deep-etch techniques eventually coupled with freeze-fracture on glutaraldehyde-fixed samples revealed an anastomosing mesh composed of filaments, 8–12 nm in diameter, that attach directly to intramembrane particles

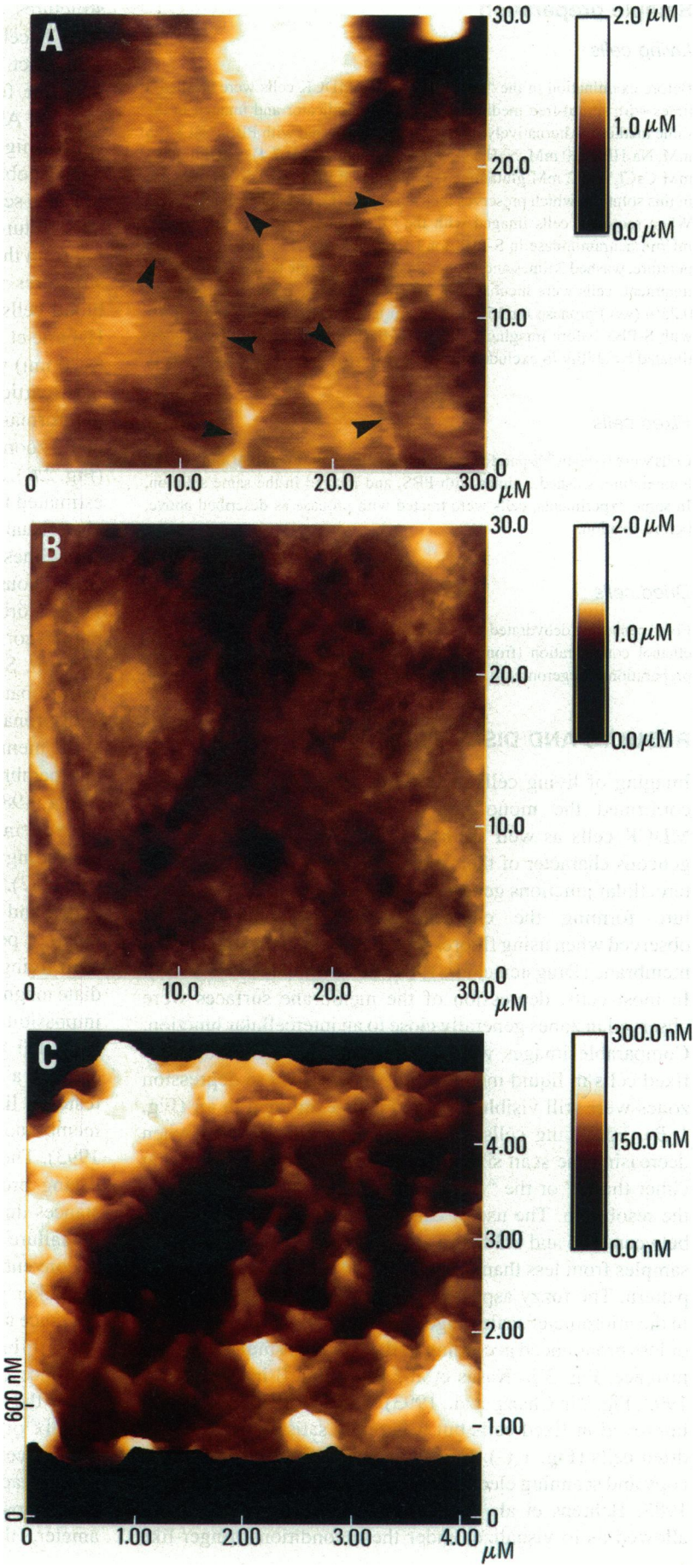

FIGURE 1 Low-magnification AFM images of MDCK cells. AFM image of a monolayer of living (*A*) and dried (*B*) MDCK cells grown to confluence (field size 30 × 30 μm). Living cells were imaged in serum-free medium. (*Arrow heads*) Contact regions between adjacent cells. (*C*) AFM image (4.2 × 4.2 μm) of a dried monolayer showing part of the contact region between two adjacent cells at the left upper corner. Note the presence of numerous microvilli at the membrane surface. The images were processed only by flattening to remove background slope. To control the force to which the sample was subjected, the tip was engaged onto a 10 × 10 nm scan area in the center of the field. Typically, imaging forces were then adjusted to 0.5–5 nN.

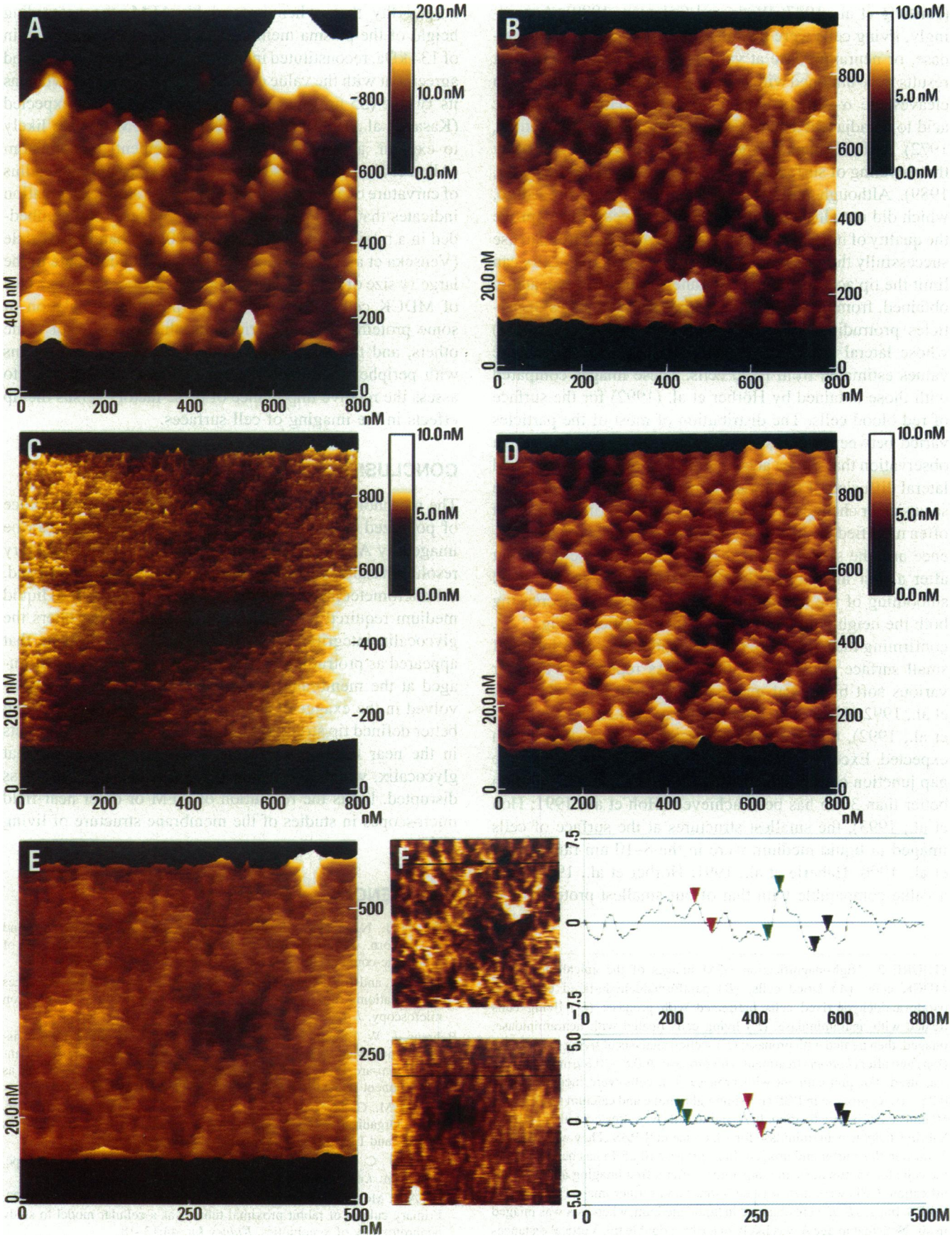

FIGURE 2 Legend on next page.

(Hartwig et al., 1987; Wade and Coleman, 1989). Accordingly, living cells were treated with heparinase I, hyaluronidase, or neuraminidase at various concentrations. The best results were obtained with neuraminidase (30 mU/ml), which cleaves the α-ketosic linkage of N-acetylated neuraminic acid to an adjacent sugar residue (Gottschalk and Drzeniek, 1972). A similar neuraminidase treatment was used to study the recycling of surface glycoproteins in intact cells (Snider, 1989). Although not completely efficient, this procedure, which did not alter the cell viability, allowed us to improve the quality of images at intermediate magnification and to use successfully the "A" scanner, confirming that glycocalix can limit the tip access to the membrane surface. We repeatedly obtained, from different cell culture batches, images of particles protruding from the membrane surface (Fig. 2 *D*) whose lateral sizes (range 10–60 nm) compared with the values estimated from fixed cells. These images compared with those obtained by Hörber et al. (1992) for the surface of red blood cells. The distribution of most of the particles varied between successive scannings of the same area, an observation that can be accounted for by the well established lateral diffusion of membrane constituents. Reduction in the scan size or changes in the scan speed, after a first scan, most often modified the image quality but confirmed both the presence and the size range of the particles. Pronase treatment after a first imaging of the particles resulted in a marked smoothing of the membrane surface (Fig. 2 *E*), decreasing both the height and the *xy* size of these particles (Fig. 2 *F*), confirming that they were proteins and not a convolution of small surface structures of the tip. As reported before for various soft biological materials including DNA (Hansma et al., 1992; Allen et al., 1992; Murray et al., 1993; Venseka et al., 1992), the *xy* size of these proteins was larger than expected. Except for highly organized structures such as the gap junction cell-to-cell channels, where a lateral resolution better than 3 nm has been achieved (Hoh et al., 1991; Hoh et al., 1993), the smallest structures at the surface of cells imaged in liquid medium were in the 8–10 nm range (Butt et al., 1990; Häberle et al., 1991; Hörber et al., 1992), i.e., a value comparable with that of our smallest proteins. It is noteworthy that, when imaged by AFM, the protruding height of the plasma membrane calcium-ATPase, a protein of 134 kDa, reconstituted in liposomes (4–5 nm), was in good agreement with the value calculated for the crystals, whereas its *xy* size (35 nm) was about 3 times larger than expected (Kasas et al., 1992). Characteristics of AFM tips are likely to explain, at least partly, the enlargement phenomenom (Allen et al., 1992; Venseka et al., 1992). Assuming a radius of curvature of around 10 nm for the tips, a simple calculation indicates that, for instance, a 5 nm diameter protein embedded in a bilayer can be imaged as a 20 nm diameter particle (Venseka et al., 1992). Other factors might contribute to the large *xy* size of the particles imaged at the membrane surface of MDCK cells. They include the possible association of some proteins as multimeric units, the clustering of some others, and the association of integral membrane proteins with peripheral proteins. Future experiments will have to assess the relative importance of these factors versus the tip effects in the imaging of cell surfaces.

FIGURE 2 High-magnification AFM images of the apical surface of MDCK cells. (*A*) Dried cells; (*B*) paraformaldehyde-fixed cells; (*C*) paraformaldehyde-fixed cells pretreated with pronase; (*D*) living cells treated with neuraminidase; (*E*) living cells treated with neuraminidase, imaged, then treated with pronase; (*F*) section analysis of living cells before (*top*) and after (*bottom*) treatment with pronase. A 0.8 × 0.8 μm AFM head was used. For pretreatment with pronase (*C*), cells were incubated with 0.25% (w/v) pronase in PBS containing glutamine and calcium (S-PBS) for 60 min at 4°C before fixation. In *D*, living cells were treated with 30 mU/ml Vibrio Cholerae neuraminidase for 1 h on ice in S-PBS. They were washed 3 times in this buffer and imaged. In *E*, pronase (0.25%) has been added to the cells for 15 min at room temperature, after a first imaging at high magnification. Cells were then washed 3 times and further imaged as above in S-PBS. Images *B–E* were obtained in liquid medium, whereas *A* was imaged in air. Note that image *A* was likely made by a double tip. Vertical distances between the pairs of red, green, and black arrows (*F*) were 2.58, 4.45, and 1.79 nm, respectively, in controls (*top*) as compared to 1.45, 1.08, and 0.77 nm, respectively, in pronase-treated cells.

CONCLUSION

The morphological characteristics of the membrane surface of polarized epithelial cells grown as a monolayer can be imaged by AFM. Submicrometer imaging with satisfactory resolution can be performed on dried cells. On the other hand, submicrometer imaging of the surface of living cells in liquid medium required treatment with an enzyme that alters the glycocalix integrity. Under these conditions, proteins that appeared as protruding particles 10–60 nm *xy* size were imaged at the membrane surface. Tip effects were likely involved in the exaggerated size of the proteins. Sharper and better defined tip structures are likely to reduce these effects in the near future. Our results also strongly suggest that glycocalix, which covers the surface of most cells, unless disrupted, limits the resolution of AFM or other near-field microscopes in studies of the membrane structure of living cells.

REFERENCES

Allen, M. J., N. V. Hud, M. Balooch, R. J. Tench, W. J. Siekhaus, and R. Balhorn. 1992. Tip-radius-induced artifacts in AFM images of protamine-complexed DNA fibers. *Ultramicroscopy*. 42:1095–1100.

Behnke, O., and T. Zelander. 1970. Preservation of intercellular substances by the cationic dye Alcian blue in preparative procedures for electron microscopy. *J. Ultrastruct. Res.* 31:424–438.

Behrens, J., W. Birchmeier, S. L. Goodman, and B. A. Imhof. 1985. Dissociation of Madin-Darby kidney epithelial cells by the monoclonal antibody anti-arc-1: mechanistic aspects and identification of the antigen as a component related to uvomorulin. *J. Cell. Biol.* 101:1307–1315.

Bergeron, M., G. Thiéry, F. Lenoir, M-C. Giocondi, and C. Le Grimellec. 1994. Organization of the endoplasmic reticulum in renal cell lines MDCK and LLC-PK1. *Cell Tisssue Res.* In press.

Binning, G., C. F. Quate, and C. Gerber. 1986. Atomic force microscope. *Phys. Rev. Lett.* 56:930–933.

Blais, A., J. Morvan-Baleynaud, G. Friedlander, and C. Le Grimellec. 1993. Primary culture of rabbit proximal tubules as a cellular model to study nephrotoxicity of xenobiotics. *Kidney Int.* 44:13–18.

Butt, H. J., E. K. Wolff, S. A. C. Gould, B. Dixon Northern, C. M. Peterson, and P. K. Hansma. 1990. Imaging cells with the atomic force microscope. *J. Ultrastruct. Biol.* 105:54–61.

Chang, L., T. Kious, M. Yorgancioglu, D. Keller, and J. Pfeiffer. 1993. Cytoskeleton of living, unstained cells imaged by scanning force microscopy. *Biophys. J.* 64:1282–1286.

Dragsten, P. R., R. Blumenthal, and J. S. Handler. 1981. Membrane asymmetry in epithelia: is the tight junction a barrier to diffusion in the plasma membrane? *Nature.* 294:718–722.

Fritz, M., M. Radmacher, and H. E. Gaub. 1993. In vitro activation of human platelets triggered and probed by atomic force microscopy. *Exp. Cell Res.* 205:187–190.

Gottschalk, A., and R. Drzeniek. 1972. Neuraminidase as a tool in structural analysis. *In* Glycoproteins. A. Gottschalk, editor. Elsevier, Amsterdam, 381–402.

Häberle, W., J. K. H. Hörber, and G. Binnig. 1991. Force microscopy on living cells. *J. Vac. Sci. Technol. B.* 9:1210–1213.

Häberle, W., J. K. H. Hörber, F. Ohnesorge, D. P. E. Smith, and G. Binnig. 1992. In situ investigations of single living cells infected by viruses. *Ultramicroscopy.* 42:44: 1161–1167.

Hansma, H. G., J. Vesenka, C. Siegerist, G. Kelderman, H. Morrett, R. L. Sinsheimer, V. Elings, C. Bustamante, and P. K. Hansma. 1992. Reproducible imaging and dissection of plasmid DNA under liquid with the atomic force microscope. *Science.* 256:1180–1184.

Hartwig, J. H., D. A. Ausiello, and D. Brown. 1987. Vasopressin-induced changes in the three-dimensional structure of toad bladder apical surface. *Am. J. Physiol.* 253:C707–C720.

Henderson, E., P. G. Haydon, and D. S. Sakaguchi. 1992. Actin filament dynamics in living glial cells imaged by atomic force microscopy. *Science.* 257:1944–1946.

Hoh, J. H., and P. K. Hansma. 1992. Atomic force microscopy for high-resolution in cell biology. *Trends Cell Biol.* 2:208–213.

Hoh, J. H., R. Lal, S. A. John, J. P. Revel, and M. F. Arnsdorf. 1991. Atomic force microscopy and dissection of gap junctions. *Science.* 253: 1405–1408.

Hoh, J. H., G. E. Sosinsky, J.-P. Revel, and P. K. Hansma. 1993. Structure of the extracellular surface of the gap junction by atomic force microscopy. *Biophys. J.* 65:149–163.

Hörber, J. K. H., W. Häberle, F. Ohnesorge, G. Binnig, H. G. Liebich, C. P. Czerny, H. Mahnel, and A. Mayr. 1992. Investigation of living cells in the nanometer regime with the scanning force microscope. *Scanning Microsc.* 6:919–930.

Hunziker, E. B., W. Herrmann, and R. K. Schenk. 1983. Ruthenium hexammine trichloride (RHT)-mediated interaction between plasmalemmal components and pericellular matrix proteoglycans is responsible for the preservation of chondrocytic plasma membranes in situ during cartilage fixation. *J. Histochem. Cytochem.* 31:717–727.

Imhof, B. A., P. Vollmers, S. L. Goodman, and W. Birchmeier. 1983. Cell-cell interaction and polarity of epithelial cells: specific perturbation using a monoclonal antibody. *Cell.* 35:667–675.

Karin, M., and B. Mintz. 1981. Receptor-mediated endocytosis of transferrin in developmentally totipotent mouse teratocarcinoma stem cells. *J. Biol. Chem.* 256:3245–3252.

Kasas, S., V. Gotzos, and M. R. Celio. 1993. Observation of living cells using the atomic force microscope. *Biophys. J.* 64:539–544.

Kasas, S., F. Hofmann, M. R. Celio, and E. Carafoli. 1992. Atomic force microscope imaging of the plasma membrane Ca^{2+} ATPase reconstituted in phospholipids. *Scanning.* 14:276–281.

Lee, G. M., F. Zhang, A. Ishihara, C. L. McNeil, and K. A. Jacobson. 1993. Unconfined lateral diffusion and an estimate of pericellular matrix viscosity revealed by measuring the mobility of gold-tagged lipids. *J. Cell. Biol.* 120:25–35.

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

Louvard, D. 1980. Apical membrane aminopeptidase appears at a site of cell-cell contact in cultured kidney epithelial cells. *Proc. Natl. Acad. Sci. USA.* 77:4132–4136.

Murray, M., H. G. Hansma, M. Bezanilla, T. Sano, D. F. Ogletree, W. Kolbe, C. L. Smith, C. R. Cantor, S. Spengler, P. K. Hansma, and M. Salmeron. 1993. Atomic force microscopy of biochemically tagged DNA. *Proc. Natl. Acad. Sci. USA.* 90:3811–3814.

Radmacher, M., R. W. Tillman, M. Fritz, and H. E. Gaub. 1992. From molecules to cells: imaging soft samples with the atomic force microscope. *Science.* 257:1900–1905.

Rindler, M. J., L. M. Chuman, L. Shaffer, and M. H. Saier, Jr. 1979. Retention of differentiated properties in an established dog kidney epithelial cell line (MDCK). *J. Cell. Biol.* 81:635–648.

Rodriguez-Boulan, E., and S. K. Powell. 1992. Polarity of epithelial and neuronal cells. *Annu. Rev. Cell. Biol.* 8:395–427.

Simons, K., and S. D. Fuller. 1985. Cell surface polarity in epithelia. *Ann. Rev. Cell. Biol.* 1:243–288.

Snider, M. D. 1989. Remodeling of glycoprotein oligosaccharides after endocytotis: a measure of transport into compartments of the secretory apparatus. *In* Methods in Cell Biol 32. A. M. Tartakoff editor, Academic Press, San Diego. 339–350.

Underhill, C. B., and B. P. Toole. 1982. Transformation-dependent loss of the hyaluronidate-containing coats of cultured cells. *J. Cell. Physiol.* 110: 123–128.

Venseka, J., M. Guthold, C. L. Tang, D. Keller, E. Delaine, and C. Bustamante. 1992. Substrate preparation for reliable imaging of DNA molecules with the scanning force microscope. *Ultramicroscopy.* 42:1243–1249.

Wade, J. B., and R. A. Coleman. 1989. Direct visualization of the interrelationship between intramembrane and extracellular structures. *Proc. Natl. Acad. Sci. USA.* 86:2723–2727.