Volume 257, number 2, 460–464 FEB 07815 November 1989

Imaging of cell membraneous and cytoskeletal structures with a scanning tunneling microscope

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Received 8 September 1989

The first observation of unstained cell membraneous structures by a scanning tunneling microscope is reported. An adhesive preparation method was used for imaging human medulloblastoma cells from the cell line TE 671 and oocytes from the clawed toad *Xenopus laevis*. The images show filaments, stacks of molecules and hilly structures. The possible identity of the filamentous structures is discussed, although the observed structures cannot yet be fully characterized. The work suggests possible future experiments on various biological structures in their natural environment.

Scanning tunneling microscope; Cell membrane; Cytoskeleton; Intermediate filament; Vinculin

1. INTRODUCTION

Scanning tunneling microscope (STM) is a powerful method for imaging structures of conductive surfaces [1]. Stable tunneling images have, however, even been obtained of thin layers of organic materials that were attached to conductive substrates [2–7]. Tunneling microscopy can be performed at room temperature, at ambient pressure and even in electrolyte solutions [8] and permits in principle the imaging of biomolecules in vivo. The conditions, however, under which imaging of organic molecules may be possible are not fully understood [8]. The STM can generally image only the structure of a surface. It is therefore necessary that the biological material be prepared on well-defined clean and atomically flat surfaces in order to be able to attribute the measured structures to the topology of the biological molecules and not to be misled by inhomogeneities of the substrate. In this work we used dry preparations of cell membranes since under these conditions a good connection between a cell membrane and a conductive substrate can be achieved. It is assumed that a membrane well-adsorbed to the supporting surface is stable and is not distorted by the drying procedure.

2. MATERIALS AND METHODS

The STM used in our experiments is identical to the one used by Smith et al. [2]. The tunneling needle was made from a tungsten wire (0.5 mm) by an AC-etching procedure (12 V in 5 M KOH). Two different cell preparations were used: cultured neural cells and oocytes from toads.

Cells from the human medulloblastoma cell line (TE 671) established by McAllister et al. [9] were cultured in Dulbecco's minimal essential medium with 10% fetal calf serum, 5% CO_2, and were kept at 37°C. To prepare the cells for the STM, small pieces of freshly cleaved, high-orientated pyrolytic graphite (HOPG) (Union Carbide Corp., Parma, OH, USA) were placed in plastic culture dishes prior to adding a suspension of TE 671 cells (10^6 cells/ml) in the culture medium. The dishes containing the cells were kept in an incubator for at least 2 h. During this period, the cells settled to the bottom of the dishes and to the HOPG surface. The quality of settling was checked under an electron microscope: a melamine foil prepared on a coverslip was placed in a dish instead of the HOPG, using the method of Westphal et al. [10]. After the cells had settled, the HOPG pieces were removed from the culture dish, briefly rinsed with distilled water, blotted at the edges and then dried in air.

The second preparation used was oocytes of the clawed toad *Xenopus laevis*. The oocytes were microsurgically extracted from the ovaries of cold anesthetized female toads and prepared as described in [11]. Briefly, the procedure included treatment with Collagenase Ia, Sigma, 1 mg/ml (1 h at 27°C) and mechanical dissection of the vitelline membrane. This procedure renders the cell membrane of the oocyte completely nude so that, e.g. a gigaseal can easily be achieved in a patch clamp experiment. Nude oocytes settle on any hydrophilic or even slightly hydrophobic support material and become irreversibly attached to it. We used small pieces of HOPG and allowed the oocyte to settle for about 20 min. The oocyte was then sucked away with a pasteur pipette, leaving a piece of membrane attached to the HOPG surface. The HOPG piece was then briefly rinsed with distilled water and then dried.

3. RESULTS

To ensure that the tungsten tip could achieve atomic resolution, we tested it at the beginning of each experiment by imaging an area of the HOPG surface at which no cell or membrane fragment could be seen in the light microscope. In nearly all of such tests, the atomically

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Published by Elsevier Science Publishers B.V. (Biomedical Division)

flat surface showing the typical corrugation of the HOPG was imaged (scanning area, 60 Å × 60 Å; fig.2c, inset). At the lower magnificiation of figs 2 and 3 (scanning area 600 Å × 600 Å), imaging of the HOPG surface always yielded a plane surface. Occasional straight step lines corresponding to steps on the graphite were observed. The same result, i.e. that the tip resolves only a plane HOPG surface, was often obtained in the initial experiments in which cells or cell membrane pieces were dried on an HOPG surface without having allowed them to settle.

Fig.1 shows two electron microscope images of TE 671 cells on a plane melamine foil [10]. The cell in fig.1a did not settle. A gap can be seen between the

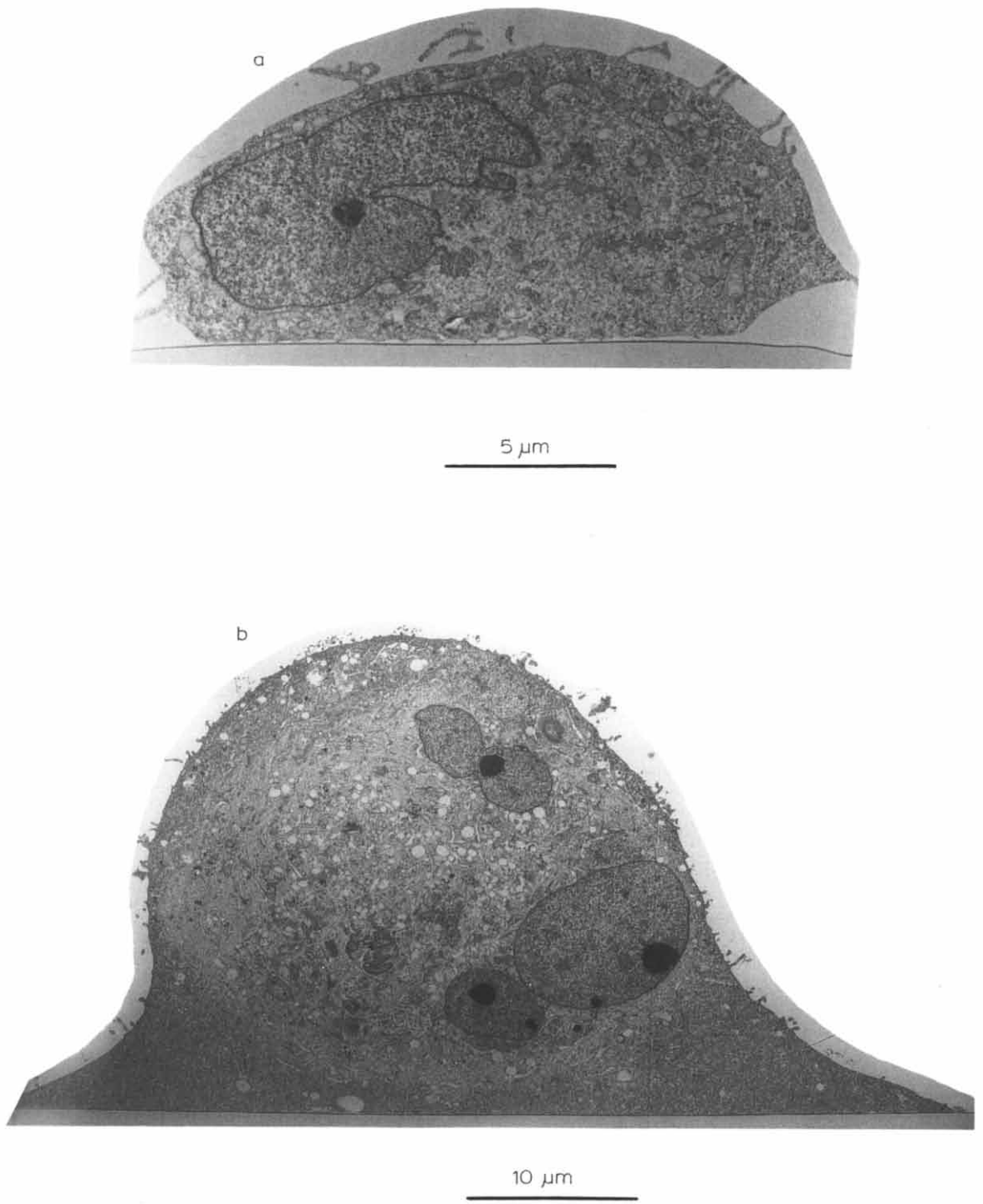

Fig.1. Electron microscopic picture of TE 671 cells placed on a melamine foil. (a) The cell was fixated after a few minutes of settling. Note the gap between the cell membrane and the support. (b) The cell was allowed to become attached to the support while kept for 2 h at 37°C. The cell membrane pictured is in direct contact with the support.

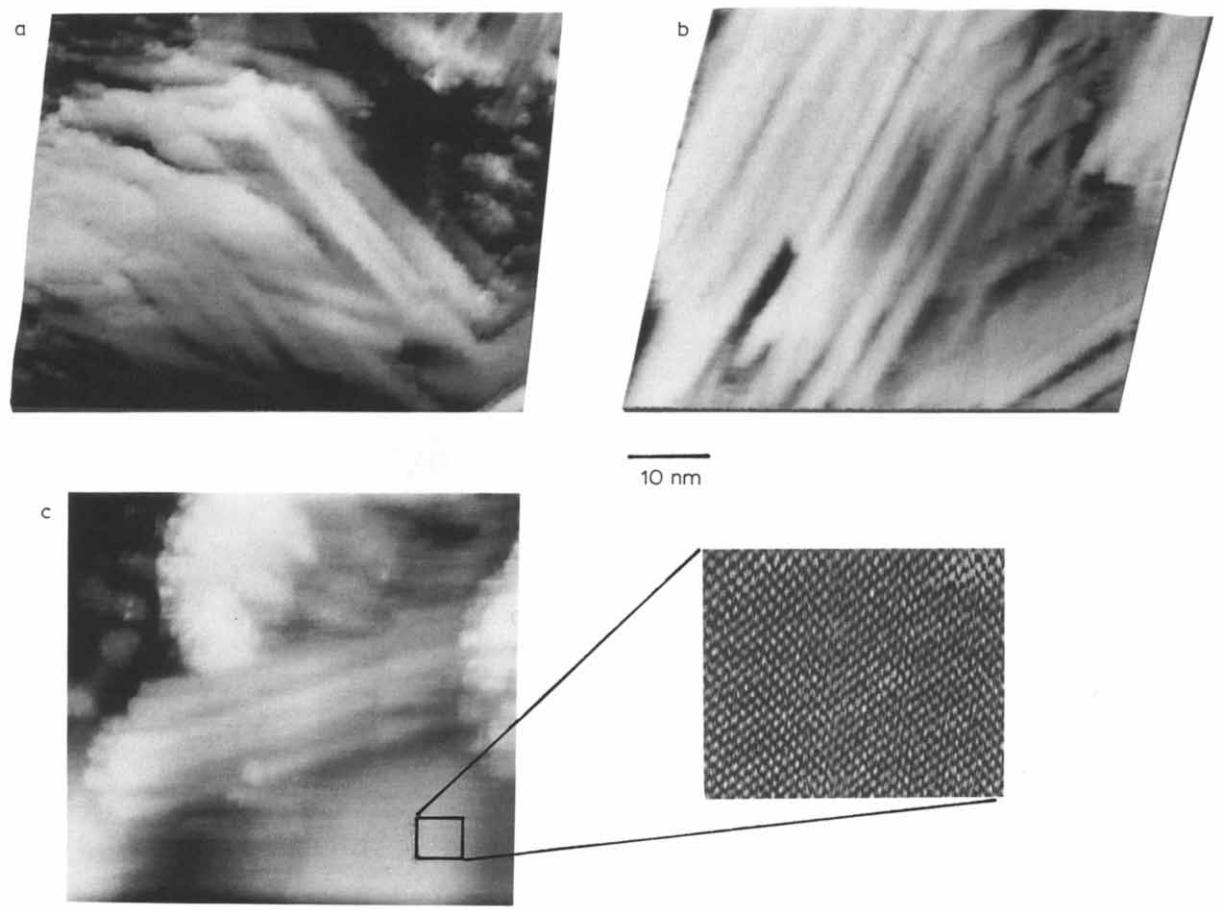

Fig.2. (a) View of the oocyte membrane structure. Hilly as well as filamentous structures are resolved by the STM. (b) An area in which only filamentous structures are visible. (c) Edge of the adhesive plaque. The inset shows the typical HOPG corrugation at a magnification five times higher than for the rest.

membrane and the foil. In fig.1b, there is no visible space between membrane and substrate; the cell settled and formed an adhesive connection.

Imaging areas where a cell membrane was in direct contact with the HOPG (as demonstrated in fig.1b), usually produced pictures quite different from those of a plane HOPG surface. Using low tunneling current (<250 pA) and relatively high tunneling voltage (0.5–1 V), a hilly landscape with a peak altitude of more than 100 Å was imaged reproducibly by scanning

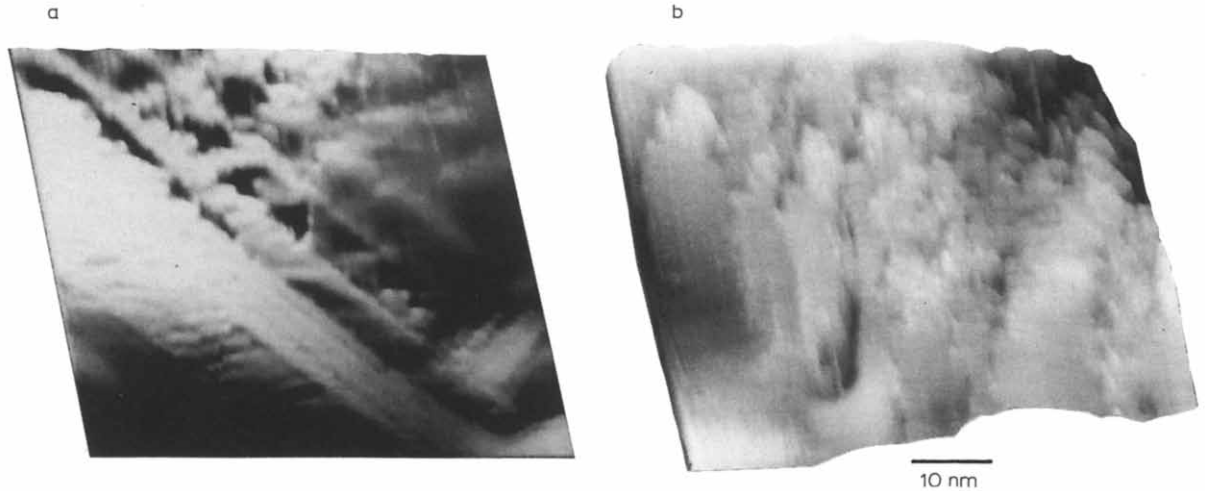

Fig.3. (a) STM image; view of the membrane structure of a medulloblastoma cell. (b) Edge of the adhesive plaque. The lower corner of the picture represents the plane HOPG surface.

slowly (line frequency less than 10 Hz) in the constant current mode. The structures found were not dependent on the scanning directions. By moving the imaged window over the surface of the sample, the observed structures shifted systematically without being distorted. Fig.2a shows a typical arrangement of the main structural elements generally found in the oocyte membrane preparation. Filamentous as well as small hilly structures can be seen. The filaments (about 30 Å in diameter) are arranged in bundles about 120 Å in diameter. The small hills are rather irregular in size, 50 Å on the average. Fig.2b shows an area of the oocyte membrane where the filaments are tightly packed so that the hilly structures are not visible. Fig.2c represents the edge of an adhesive area of an oocyte. There are hilly and filamentous structures which in the lower right-hand corner of the picture, end in a smooth plane area. At that location, a typical HOPG corrugation (inset) was revealed at a five times higher magnification with a difference in height of more than 100 Å between the filaments and the HOPG surface.

The TE 671 preparation gave slightly different pictures: the surfaces looked less corrugated. In fig.3a, a big filament runs through the lower half of the picture crossing a regular structure of stacks of molecules. The big filaments showed substructures similar to those of the oocyte preparations. Fig.3b again shows the edge of an adhesive plaque ending on the HOPG plane (left lower part of the picture).

4. DISCUSSION

The aim of the present study was to prove that imaging of biological molecular structures with an STM is possible. Previously this has been controversial [8]. In our opinion, the tight binding of the organic molecules to the substrate is a prerequisite for imaging. This finding opens a new field of application for STM in biology, particularly if STM is to be used with aqueous samples.

In our experiments, the tunneling tip has to penetrate a thick layer of organic material until the last molecular layer situated directly on the substrate can be imaged. Considering this situation, the most likely artifact that is difficult to recognize is friction between the tip and the molecules imaged, which results in a deformation of tip and sample while scanning [12]. In this case the imaged structures, which might be due to the substrate instead of the biological molecules, are distorted. There are many possible ways that distortions of the substrate might simulate structures of a sample, however, neither crossing filaments nor structures of an altitude as high as that found in our preparations can be due to this artifact.

The most striking structure found in both preparations were filaments. It is well known that nearly all types of cells may contain filaments belonging to the so-called cytoskeleton. Cytoskeletal filaments consisting of the protein actin would be expected in microvilli of oocytes [13] and in stress fibers attached to the adhesive plaques of cultured cells [14]. However, the filaments we found were not helically wound as expected of actin filaments, and the diameter (30 Å) was smaller than that of the actin filaments. Moreover, there are bundles of filaments (120 Å) arranged in parallel. Such a substructure atypical for actin filaments is known to occur in so-called intermediate filaments [15], highly stable structures insensitive to drying artifacts and often found in close contact with the cell membrane [16]. Another interpretation of the filaments deals with the fact that they can be imaged by the tunneling microscope: this is a strong indication that they are very close to the conducting HOPG surface, i.e. connected to the cell membrane. They might, for example, link the cell membrane to the cytoskeleton like the proteins vinculin or talin [17,18], whose tertiary structures are still unknown.

The hilly structures shown, e.g. in fig.1 have not been characterized. We assume that they belong to the cell membrane itself, formed by the protein and lipid components.

In the present investigation the preparations were dried in air, hence the possibility of drying artifacts must be considered. This problem could be avoided by STM imaging under water. In this case the tungsten needle would have to be insulated up to a few microns from the tip since the imaging of membrane structures requires a very small tunneling current (250 pA) which makes it quite sensitive to the capacitive current noise and to ionic offset current fluctuations.

By using underwater STM, it may make it possible in future experiments to identify certain molecular structures undergoing chemical reactions while the process of imaging is taking place. This could be done with antibodies and with specific toxins interacting with known components of the membrane or of the cytoskeleton.

Acknowledgements: We thank Professor Dr Reinhardt Rüdel and Professor Dr Bert Sakmann for their continuous interest in this work, Professor Dr John Connor, Mrs Gerda Hack and Mrs Sigrid Schäfer for the help with the cells, Dr H. Lorkovic for critical comments on the manuscript and Cristel Westphal and Dr Dieter Frösch for the electron microscopic pictures. We also would like to thank Professor Dr T.W. Hänsch for the generous disposal of the STM equipment.

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