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Neurone decapping characterization by atomic force microscopy: a topological systematic analysis

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WE tested a new approach to cell decapping on rat cerebellar neurones, and observed its effects on cell topography by atomic force microscopy (AFM). The results clearly demonstrate the effectiveness of our decapping approach, and also the ability of AFM to reveal fine details of the decapped cells. Specifically, varying the conditions and duration of the decapping process modifies the extent of the decapping. Such a method can be used to investigate the cytoplasm with surface sensitive techniques.

Key words: Rat cerebellar granule cells; Neurones; Decapping; Atomic force microscopy (AFM); Surface sensitive techniques

Introduction

In recent years, new analytical techniques have investigated the local chemistry of biological samples.[1–5] Their application domain has significantly expanded including the observation of toxic and physiological elements in neurones.[1,4–6] Techniques such as photoemission-based synchrotron spectromicroscopy[1–6] are somewhat limited by the fact that they are only capable of investigating the neurone surface, i.e., the membrane and membrane proteins. AFM has been recently applied to the study of cultured neurones.[7,8] In particular, in the work of Cricenti *et al*[8] granule cells and their axons were imaged in a region of several tens of microns with a lateral resolution of a few tens of nanometres. The AFM micrographs were acquired from a monolayer of fixed and dried cells. Details of cell processes such as neurites and of cell bodies were easily revealed.

In the present article, we show AFM images of uncoated cultures of rat primary neurones (granule cells), grown on glass cover slips, and decapped in order to gain access to the cytoplasm and the inner portion of the cell. This makes it possible to analyse the cytoplasm with surface-sensitive techniques such as atomic-force microscopy,[9] synchrotron spectromicroscopy and electron microscopy.

Materials and Methods

Cerebellar granule cells from 7-day-old rats were seeded in 35 mm diameter Petri dishes at a density of 1.5×10^6 cells per well, containing four round glass coverslips, pretreated with a 10 $\mu g\,ml^{-1}$ poly-L-lysine solution. The cells were obtained by enzymatic and mechanical dissociation of the cerebellar tissue and plated in Basal Medium (Eagle's salt), containing 10% fetal calf serum, and allowed to grow in an incubator at 37°C in a 5% CO_2 humidified atmosphere.[10] After 8 days *in vitro*, the granule neurone network was fully developed. We removed the glass coverslips from the culture medium, and treated the samples, from four different cultures, according to four different conditions. Control samples (condition 1) were simply fixed with 4% glutaraldehyde in PBS (phosphate buffered saline solution). After 20 min in the fixing solution, the cell cultures were rinsed twice in DD H_2O. A second

set of samples (condition 2) was prefixed for 30 s with 0.4% glutaraldehyde in PBS. They were then soaked with CS buffer [100 mM PIPES (piperazine-*N*,*N*′-bis(2-ethanesulphonic acid); laboratory reagent grade from Sigma), 5 mM EGTA (ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Sigma), 2 mM $MgCl_2$ (Carlo Erba), pH 6.8],[11] and covered with prewetted membrane filter (Millipore HAWP 0.45 μm pore size, but GSWP 0.25 μm pore size can be used as well). The buffer induces the cell membrane to adhere to the membrane filter. After 30 s, the membrane filter was quickly removed, peeling off the cell membrane. At this point, the decapped cell cultures were rapidly fixed with 4% glutaraldehyde in PBS and washed. Samples prepared under condition 3 were prefixed for 30 s with 0.4% glutaraldehyde in PBS, exposed to CS buffer and allowed to adhere to the membrane filter for 2 min, peeled off, fixed and washed. The final set of samples (condition 4) was not prefixed, but was exposed to CS buffer and allowed to adhere to the membrane filter for 5 min, peeled off, fixed and washed.

The effectiveness of the decapping procedure was tested by immunofluorescence. Some of the samples prepared under the different conditions reported above were stained with polyclonal anti-tubulin antibodies. Several areas of the cell cultures were illuminated to reveal immunofluorescence of TRITC-labelled secondary antibodies. Samples for immunofluorescence were decapped using PBS instead of CS buffer.

Atomic force microscopy: Our AFM microscope is described in detail elsewhere.[12] In short, it consists of a unit made of two separable cylindrical supports. The lower unit contains the sample holder mounted on top of a piezoelectric scanner. A laser deflection circuit is mounted on the top cylinder. The AFM microscope is mounted, using a vibration isolation system, inside a stainless steel chamber. After loading the sample, the AFM chamber is closed to perform the experiments in a controlled constant and reproducible environment. Constant force images have been obtained with the microscope working in the repulsive mode with a force smaller than 1 nN from zero cantilever deflection. Gold-coated Si_3N_4 microlevers (Park Scientific Instruments) with a diamone integrated pyramidal tip and a spring constant of 0.023 N m^{-1} were used. An optical microscope (up to 120-fold magnification) together with a millimetre x-y stage enabled us to select areas of the sample to observe, and to avoid in particular large aggregates of cells.

The only data-processing used for the images discussed here was rigid plane subtraction. No degradation of the specimen was observed due to the interaction with the microscope and the data were reproducible over several days.

Results

Figure 1 shows a 6 × 6 μm^2 AFM micrograph typical of the present study. A cell body of a neurone of the control sample (prepared under condition 1, i.e. not decapped) is clearly visible, and its surface corrugation profile is shown on the right-hand side of the figure. Figure 2 shows a similar set of data, image and surface corrugation profile, for a decapped cell, from the culture of sample allowed to adhere to the membrane filter for 5 min. Figure 3 presents a survey of AFM results. Each curve reported in this figure is representative of the typical corrugation profile of the cell bodies in the corresponding sample. A line plot for each cell body has been extracted from an AFM image, on a 4 μm line, chosen across a cell body; 5–8 cell body line plots were analysed and averaged for each sample. In Figure 3 we see that the cell in the control sample has a height of ~1.6 μm; in the sample allowed to adhere to the membrane filter for 5 min (condition 4) the cell height is ~0.6 μm; for conditions 3 and 2 the cell height is ~1.1 and ~1.5 μm, respectively.

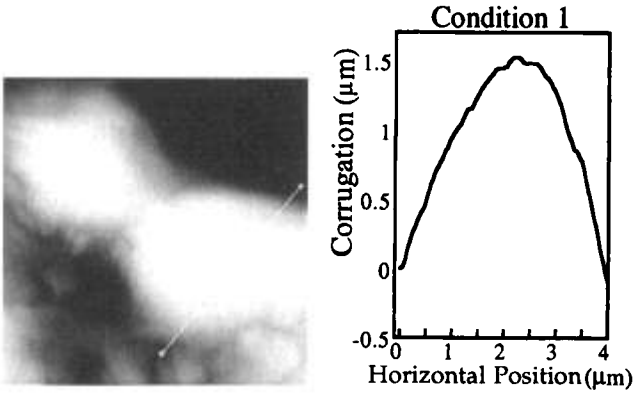

FIG. 1. **(Left)** AFM micrograph showing an undecapped neurone (6 × 6 μm^2), from the control sample. **(Right)** The corrugation profile of the same cell acquired along the line on the cell body, visible in the left-hand image.

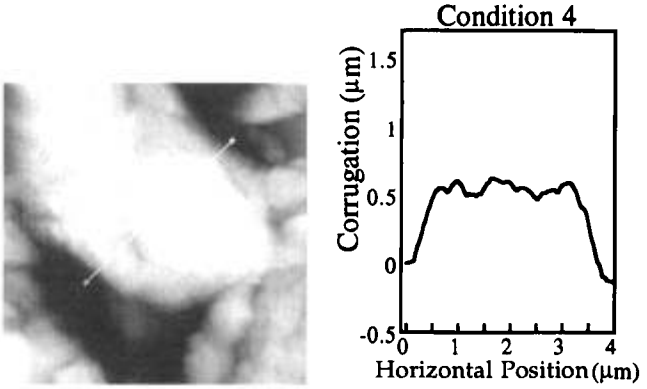

FIG. 2. **(Left)** AFM micrograph showing a decapped neurone (6 × 5 μm^2), from the sample allowed to adhere to the membrane filter for 5 min. **(Right)** The corrugation profile of the same cell, acquired on the line on the cell body shown in the left-hand image.

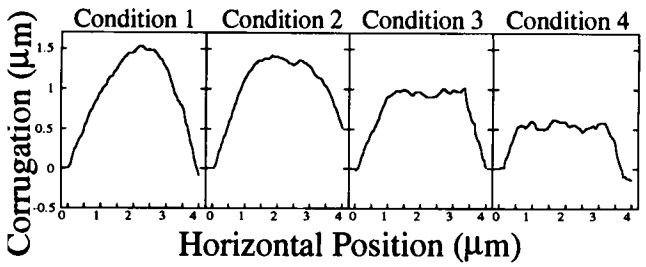

FIG. 3. Cell bodies surface corrugation curves of: (1) the undecapped control sample; (2) the sample allowed to adhere to the membrane filter for 30 s (prefixed); (3) the sample allowed to adhere to the membrane filter for 2 min (prefixed); and (4) the sample allowed to adhere to membrane filter for 5 min.

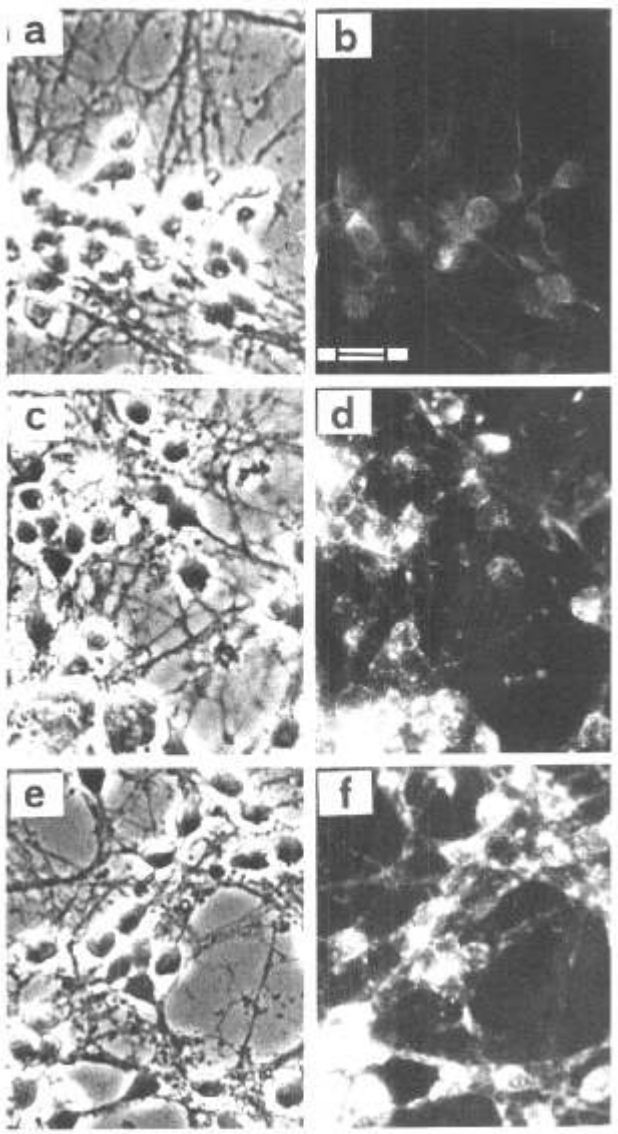

FIG. 4. Phase contrast micrographs (**a**, **c** and **e**) and fluorescence micrographs (**b**, **d** and **f**) of the same fields of rat cerebellar granule neurone cultures: (a, b) undecapped (condition 1); (c, d) decapped for 30 s; (e, f) decapped for 5 min. Note the increase in the fluorescence intensity from (b) to (f). Bar = 10 μm. Note that the cell morphology is not appreciably altered by decapping as visible in the phase contrast micrographs.

Figure 4 shows the time dependence of our decapping procedures. The left-hand panels (a, c and e) are phase contrast micrographs of undecapped neurones, neurones decapped for 30 s and those decapped for 5 min, respectively. The right-hand panels of Figure 4 show the same fields, illuminated to reveal immunofluorescence of TRITC-labelled secondary antibody. The increase in the fluorescence intensity from (b) to (f) indicates that membrane removal is time dependent during the decapping procedure.

Discussion

The main conclusion drawn from the results shown in Figure 3 is that an increase in the duration of adhesion to the membrane filter corresponds to an increase in the proportion of the cell removed during the decapping procedure. This result can be interpreted in terms of a time-dependent interaction between the cell membrane and the membrane filter, enhanced by the presence of the buffer. Such an interaction is probably due to the hydrophobic character of the fatty acid chains present in the cell membrane and in the membrane proteins, that would 'prefer' to adhere to membrane filter rather than to be immersed in the buffer. In the presence of a buffer, therefore, the adhesion of cell structures to filter should be accelerated. We used CS buffer or PBS, and they both gave positive results. In the case of AFM experiments, however, we preferred CS buffer because it is supposed to stabilize the cytoskeletal structures.[11] The use of membrane filter rather than any other kind of material is justified by its homogeneity. It has, in fact, a very smooth surface with small pores (only 0.45 μm wide).

The immunofluorescence results shown in Figure 4 further demonstrate the effectiveness of decapping and its time dependence. Such a test, in fact, will give positive results only after the removal of part of the membrane, which allows the antibodies to reach the cytoskeleton. This test rules out any other possible interpretation of the AFM results. No such time-dependent effect has been observed before. Previous studies[6,13] reported this decapping technique to be effective on glial cells and neurones, but a systematic study of the decapping effect and of the parameters that influence it was not performed.

We also note that gently prefixing the cells, with one tenth of the concentration of glutaraldehyde normally used to fix cells, was supposed to stabilize the chemical and consequently the mechanical structure of the cells, and to prevent decapping of a large area. Prefixing can also be expected to stabilize the cytoplasm, preventing extensive leaking after decapping. We finally note that our detection of the effects of cell decapping could

only be obtained by AFM, a technique which is extremely sensitive to the surface topography, and which does not require the sample to be a conductor, as in the case of scanning tunnelling microscopy or other electron microscopies. Therefore, we could leave the cells in a near natural state, with no need for labelling or coating.

As to the practical usefulness of our results, decapping can make it possible to reach the inner cell region and therefore to apply virtually every surface-sensitive technique to its study. This is important, since many such techniques have been developed in materials science and could be applied to cell studies if their surface sensitivity did not limit the standard domain of application to the outermost portion of the cell. The techniques that could profit from the decapping procedure, apart from AFM, include photoelectron spectromicroscopy[13] and electron microscopy in general.[11]

Conclusions

We successfully tested the cell decapping technique, already known for glial cells, on neurones, and found that it depends on the membrane filter adhesion time. Our results, therefore, demonstrate that it is possible to decap neurones removing only the desired portion of the cell. This decapping technique can extend the range of applications of many surface sensitive analytical techniques.

Note Added in Proof

While this manuscript was in preparation, we came across the manuscript by Le Grimellec *et al*, reporting the results of a decapping technique based on similar concepts, and applied on different cells from ours. The manuscript to be published in Scanning Microscopy is: C. Le Grimmellec, E. Lesniewska, M.-C. Giocondi *et al*, Imaging of the Cytoplasmatic Leaflet of the Plasma Membrane by Atomic Force Microscopy.

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