Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 9–10, January 1997

Commentary

Cellular and molecular mechanics by atomic force microscopy: Capturing the exocytotic fusion pore *in vivo*?

J. M. Fernandez

Department of Physiology and Biophysics, Mayo Clinic, Rochester, MN 55905

The atomic force microscope (AFM) is a remarkably simple instrument that can measure forces down to piconewtons and can resolve force changes caused by the displacement of its probe by a fraction of a nanometer. Furthermore, these measurements can be done under water, allowing for the study of biological material under conditions that resemble those *in vivo*. The AFM functions like a miniature phonograph. A sharp tip, mounted on a cantilever, interacts with the sample, causing minute deflections that can be calibrated as a force. The AFM, in its force-measuring mode, has been applied to the study of molecular mechanics. For example, the laboratory of Hermann Gaub in Munich succeeded in the remarkable feat of measuring the adhesion force between single avidin–biotin pairs (≈160 pN; ref. 1). These measurements were independently confirmed by molecular dynamic simulations of the avidin–biotin interaction that revealed the atomic trajectory of a rupture and predicted the associated forces (2). Hence, the techniques of molecular mechanics now rival the capabilities of patch-clamp techniques (3) in describing the activity of single proteins. The patch-clamp technique seals a lipid membrane, containing ion channel proteins, onto a glass pipette electrode. Ionic currents flowing through a single ion channel protein are then easily measured, reporting on the conformational states of the transmembrane protein. In contrast to the patch-clamp technique, useful mainly for the characterization of ion channels, molecular mechanics studies can be done on single proteins or more complex structures that do not carry an associated electrical activity.

The most familiar use of the AFM is in creating topographic images of a sample. This is done by scanning the AFM tip over the sample and recording the z axis displacement required to maintain a constant contact force. The nanometer resolution of the AFM imaging technique has been used to examine the structural features of proteins, producing data that is comparable to that obtained by x-ray crystallography (4). However, the biggest promise of the AFM technique is in the measurement of the activity of single protein structures *in vivo* during signaling events or during other types of cellular activities. Indeed, we may be getting close. A recent example demonstrated that it is possible to observe conformational changes in the structure of a single nuclear pore in *Xenopus* oocyte nuclear envelopes (5). However, these measurements were done in fixed tissue. Recent work from the laboratory of Bhanu Jena may be the first to show a macromolecular structure undergoing conformational changes during cellular activity (6).

Jena and colleagues used an AFM to image the exposed apical region of isolated pancreatic acini. They found a set of puzzling structures: wide pits punctuated by smaller depressions that averaged about 150 nm. Significantly, the depressions widened to about 200 nm during amylase secretion and returned to their control size upon cessation of the secretory response. The authors proposed that the depressions corresponded to the sites where the exocytotic fusion pores form during amylase secretion. The exocytotic fusion pore, the structure that connects the lumen of a secretory vesicle with the extracellular environment, was first observed in mast cells and in *Limulus* amebocytes using a combination of rapid freezing techniques and freeze-fracture electron microscopy (7, 8). Similar to the depressions observed with the AFM, the electron micrographs of degranulating mast cells revealed plasma membrane dimples that reached through the cortical cytoskeleton and fused with the granule membrane to form fusion pores (ref. 7; Fig. 1). The mast cell dimples measured about 100 nm at their mouths and are, thus, similar to the size of the depressions measured in the acinar cells. However, in mast cells, the dimples were observed only upon stimulation, whereas the depressions in pancreatic acinar cells seen by use of the AFM seem to be permanent features of the surface of these cells.

Another significant finding was that the depressions enlarged transiently, indicating that amylase is released mostly by fusion pores that only open transiently. This contrasts with the widely accepted belief that exocytosis always occurs by irreversibly merging the granule membrane with the plasma membrane. However, their finding agrees well with electrophysiological measurements of the activity of fusion pores.

The development of patch-clamp-capacitance techniques permitted, for the first time, direct observation of the activity of single fusion pores in isolated living cells undergoing exocytosis (10–12). These measurements revealed that the exocytotic fusion pore, after opening, expanded rapidly and then either closed again completely (transient fusion, sometimes called "flicker") or expanded irreversibly (irreversible fusion). These experiments also showed that a secretory vesicle could undergo multiple transient fusion events. Furthermore, the release of secretory products during the various modes of fusion was investigated by measuring the oxidation of electroactive substances (e.g., serotonin) with a carbon fiber microelectrode placed near the cell (13). This approach demonstrated that a significant amount of secretion takes place during transient fusion events, establishing secretion by transient fusion as a well documented and common form of exocytosis (14).

In spite of the agreement of the AFM observations with the electron microscope and electrophysiological descriptions of fusion pore structures, many uncertainties remain. The most important limitation of the AFM, when applied to biological tissues, is that its resolution depends strongly on the elasticity of the sample (15). Since the AFM imaging technique demands the constant application of forces typically in excess of 1 nN, the AFM tip will indent into a soft sample by an amount dependent on the sample's elastic modulus. The indentation of the AFM tip into the sample causes the reduction in resolution. The atomic resolution of the instrument drops to less than 0.1 μm for samples with an elastic modulus within 10–100 kPa, which is typical of cells (Elliot Elson, personal communication) and gelatin in water (15). Some cellular structures, such as the condensed secretory granule matrices found in mast cells, have

0027-8424/97/949-2$2.00/0
PNAS is available online at **http://www.pnas.org**.

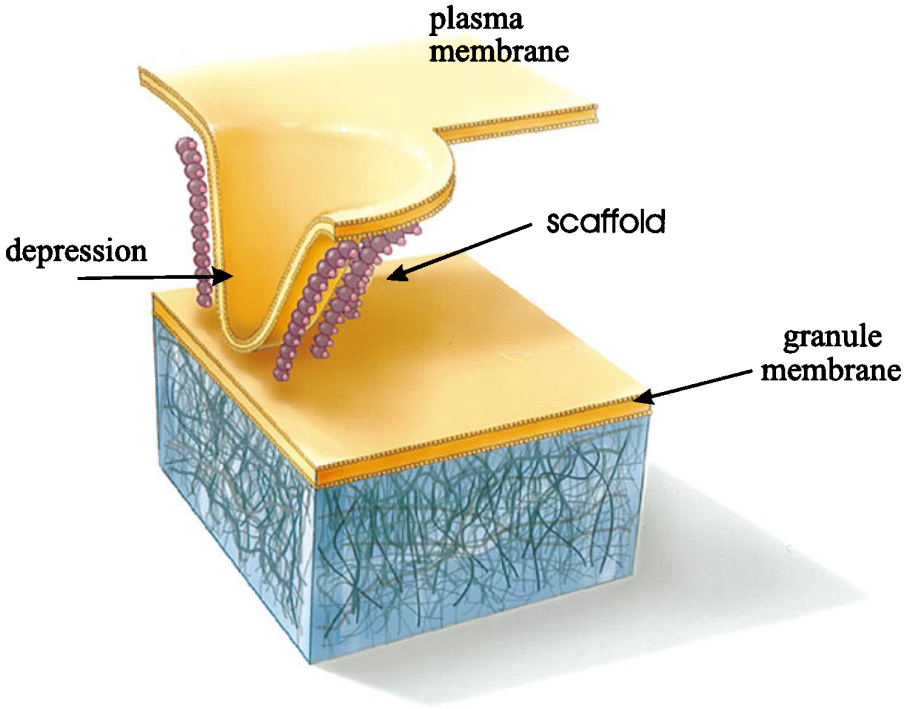

FIG. 1. Artistic rendition of the structures that form a fusion pore according to the scaffold hypothesis. The figure shows a depression of the plasma membrane that is caused by a scaffold of proteins. At the tip of the depression, the highly curved lipid membrane is thought to fuse, spontaneously, with the granule membrane, creating a lipid-lined fusion pore. It is possible that the AFM images of fusion pores presented by Schneider *et al.* (6) correspond to the structures associated with the mouth of the depression shown in the figure. This figure was modified from Monck and Fernandez (9).

been shown to have a much higher elastic modulus ($\approx 10^6$ Pa; ref. 16). Hence, rather than simply mapping the surface topography of a cell, it is likely that the surface structures imaged by AFM are convolved by a complex function that depends on the local elasticity of the sample. Thus, it is unlikely that the AFM images of the apical membrane showed the bilayer surface directly. It is possible that part of what is being imaged is the hard shell of the underlying cytoskeleton or other surface structures with a high elastic modulus. It is also significant that the elasticity of the cell surface has been shown to depend on the secretory status of the cell (17). All these considerations suggest that the AFM images, demonstrated by Jena's group (6), may be more complex than simple topographic representation of fusion pores. Given these considerations, it would seem like a good idea to confirm the AFM observations of the fusion pore using different imaging techniques, such as scanning electron microscopy, freeze-fracture techniques, or even high resolution video microscopy (18). Another feasible approach may be to combine patch-clamp-capacitance measurements of exocytosis together with AFM examination of the activity of the fusion pores (Bhanu Jena, personal communication). However, regardless of the detailed interpretation of AFM images, the results of Schneider *et al.* (6) presented in this issue of the *Proceedings* are important because they demonstrate the potential for tracking the subcellular structures involved in exocytosis. An interesting possibility is that, through AFM measurements of the elasticity of the surface of a secretory cell, we can probe the status of the cortical cytoskeleton during exocytosis. An AFM image of the cytoskeletal elasticity during secretion may reveal to what extent these structures are involved in regulating vesicle fusion.

Future experiments may indeed demonstrate that the AFM images of Jena and collaborators correspond to the fusion pores of acinar cells undergoing exocytotic secretion, confirming once again the tremendous potential of AFM techniques to monitor cellular events in real time and *in vivo*.

1. Florin, E. L., Moy, V. T. & Gaub, H. E. (1994) *Science* **264,** 415–417.
2. Grubmüller, H., Heymann, B. & Tavan, P. (1996) *Science* **271,** 997–999.
3. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981) *Pflügers Arch.* **391,** 85–100.
4. Müller, D. J., Schabert, F. A., Büldt, G. & Engel, A. (1995) *Biophys. J.* **68,** 1681–1686.
5. Perez-Terzic, C., Pyle, J., Jaconi, M., Stehno-Bittel, L. & Clapham, D. E. (1996) *Science* **273,** 1875–1877.
6. Schneider, S. W., Sritharan, K. C., Geibel, J. P., Oberleithner, H. & Jena, B. P. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 316–321.
7. Chandler, D. E. & Heuser, J. E. (1980) *J. Cell Biol.* **86,** 666–674.
8. Ornberg, R. L. & Reese, T. S. (1981) *J. Cell Biol.* **90,** 40–54.
9. Monck, J. R. & Fernandez, J. M. (1994) *Neuron* **12,** 707–716.
10. Fernandez, J. M., Neher, E. & Gomperts, B. D. (1984) *Nature (London)* **312,** 453–455.
11. Breckenridge, L. J. & Almers, W. (1987) *Nature (London)* **328,** 814–817.
12. Zimmerberg, J., Curran, M., Cohen, F. S. & Brodwick, M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1585–1589.
13. Wightman, R. M., Jankowski, J. A., Kennedy, R. T., Kawagoe, K. T., Schroeder, T. J., Leszczyszyn, D. J., Near, J. A., Kilberto, E. J., Jr., & Viveros, O. H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10754–10758.
14. Alvarez de Toledo, G., Fernandez-Chacon, R. & Fernandez, J. M. (1993) *Nature (London)* **363,** 554–557.
15. Radmacher, M., Fritz, M. & Hansma, P. K. (1995) *Biophys. J.* **69,** 264–270.
16. Parpura, V. & Fernandez, J. M. (1996) *Biophys. J.* **71,** 2356–2366.
17. Liu, Z. Y., Young, J. I. & Elson, E. L. (1987) *J. Cell Biol.* **105,** 2933–2943.
18. Inoue, S. (1986) *Video Microscopy* (Plenum, New York).