Acta Physiologica Hungarica, Volume 102 (2), pp. 125–130 (2015)
DOI: 10.1556/036.102.2015.2.2

Application of atomic force microscopy for investigation of Na^+,K^+-ATPase signal-transducing function

MM Khalisov[1,2], AV Ankudinov[2,3], VA Penniyaynen[1], D Dobrota[4], BV Krylov[1]

[1]Pavlov Institute of Physiology Russian Academy of Sciences, St. Petersburg, Russia
[2]St. Petersburg National Research University of Information Technologies, Mechanics and Optics, St. Petersburg, Russia
[3]Ioffe Physical-Technical Institute Russian Academy of Sciences, St. Petersburg, Russia
[4]Comenius University Jessenius Faculty, Department of Medical Biochemistry, Martin, Slovak Republic

Received: December 3, 2014
Accepted after revision: February 20, 2015

The Young's modulus of 10–12-day-old chick embryos' sensory neurons cultivated in dissociated cell culture was measured using a PeakForce Quantitative Nanomechanical Mapping atomic force microscopy. The native cells were tested in control experiments and after application of ouabain. At low "endogenous" concentration of 10^{-10} M, ouabain tended to increase the rigidity of sensory neurons. We hypothesize that this trend resulted from activation of Na^+,K^+-ATPase signal-transducing function.

Keywords: sensory neurons, Na^+,K^+-ATPase signal-transducing function, ouabain, atomic force microscopy, PeakForce QNM

The signal-transducing function of Na^+,K^+-ATPase is triggered by specific binding of ouabain. The crosstalk between the ouabain-activated pathways modulates expression of a number of genes (18). That is why ouabain affects the growth of cardiac myocytes and nerve tissue as well as the proliferation of the smooth muscle cells. Moreover, it also provokes apoptosis in various malignant cells. On the other hand, it can control the voltage sensitivity of slow sodium $Na_v1.8$ channels in sensory neurons that are responsible for pain sensation in mammals (1, 6). The coupling between Na^+,K^+-ATPase and $Na_v1.8$ channels was demonstrated earlier (11). The hypothesis that the signal-transducing function of Na^+,K^+-ATPase can play an important role in nociception was investigated in detail at the membranous, cellular and behavioral levels (12). Supposedly, it is the signal-transducing function of Na^+,K^+-ATPase which is controlled by the nanomolar concentrations of ouabain detected in human blood (8). Application of the atomic force microscopy (AFM) method to register the responses of native cells after ouabain-induced activation of Na^+,K^+-ATPase signal-transducing function is a delicate and efficient technique to investigate the physiological consequences of this process.

AFM is a unique tool yielding the high-resolution imaging topographical data. It can apply a controlled force to cells, measure the cellular elastic properties, and monitor the variations in the elastic (Young's) modulus before and after activation of cellular signaling processes. The pioneer papers on eukaryote cells employing AFM appeared at the end of the last century (3, 7). The AFM method has been used to evaluate the elasticity of various cells (neurons, fibroblasts, cardiomyocytes, etc., 2, 4, 5, 9, 14, 16, 17).

Corresponding author: Boris V. Krylov, Sci. Dr, PhD, Professor
Pavlov Institute of Physiology, Russian Academy of Sciences
199034 nab. Makarova 6, St. Petersburg, Russia
Phone/Fax: +7(812)3281301; Fax: +7(812)3280501; E-mail: krylov@infran.ru

The PeakForce Quantitative Nanomechanical properties Mapping (PeakForce QNM) mode was recently developed by Bruker Corporation as an optimal approach to cell imaging. It explores topography of intact cells with submicron resolution and can monitor the cell elasticity. The PeakForce QNM AFM mode is more effective in cell scanning than any other AFM mode due to a high-accuracy control of the tip-sample interaction force in normal and lateral directions. In this mode, the tip touches the sample and then moves back until it reaches the safe distance; thereafter the probe moves to the next measuring point, and the cycle is repeated again. This concept of PeakForce QNM enables to maintain the cell integrity during AFM imaging.

The current report includes data obtained with the PeakForce QNM mode of AFM method. It revealed a pronounced upward trend in the Young's modulus of intact sensory neurons by approximately 30% after activation of the Na^+,K^+-ATPase signal-transducing function.

Materials and Methods

Drugs

Ouabain was purchased from Sigma-Aldrich (St. Louis, USA).

Animals

Dorsal root ganglia (DRG) were obtained by microdissection from White Leghorn chicken embryos (Sinyavino Poultry, Leningrad District, Russia). Experiments were performed on cultured isolated DRG neurons of 10–12-day chicken embryos.

Culture of Dissociated DRG

DRG were dissected from the L5–S1 regions of 10–12-day-old chicken embryos and dissociated by mechanical pipetting under physiological conditions. The culture fluid was added to the cell suspension in order to obtain the desired cell density in a plastic Petri dish. The non-neuronal cells were removed by allowing them to settle onto the surfaces of plastic 90-mm Petri dishes for 25 min at 37 °C. The medium did not contain any proteolytic enzymes and consisted of 45% Hank's solution, 40% Eagle's medium, and 15% fetal calf serum, and was supplemented with insulin (0.5 U/ml), glucose (0.6%), glutamine (2 mM), gentamicin (100 U/ml), and 7S nerve growth factor (10 ng/ml) (Sigma). The cell suspension was plated onto fibronectin- and collagen-coated 40-mm Petri dishes. Their bottoms were covered with 7 μg/ml solution of collagen. After an hour, the formed collagen film was coated with fibronectin (10 μg/ml). The cells were cultured for three days at 37 °C and 5% CO_2. Ouabain was added to the cell culture medium. The cells cultured without ouabain were used as the control.

AFM measurements

A Bruker Bioscope Catalyst AFM setup integrated with a standard Carl Zeiss Axio Observer D1m inverted optical microscope was used. Bright field or phase contrast optical images were used to visualize the dissociated neurons for AFM examination and to monitor the neuronal morphology and viability during experiments. All experiments were conducted using a LakeShore 335 controller to keep the temperature of the fluid in the Petri dish at 37 °C. AFM study was performed in the PeakForce QNM mode with Bruker SNL-10 (C)

probes, whose nominal spring constant was 0.12 N/m. Before the experiments, the actual spring constant for each probe was calibrated using the thermal noise method. The AFM image frame sizes were varied from 20 × 20 μm to 40 × 40 μm in order to encompass the cell body and the neurite regions. Usually the height of the examined regions was below 3 μm (approximately equal to the cantilever tip height). Thus, the cells were protected from contact with the cantilever beam, which could otherwise detach the cell from the substrate. To reduce the hydrodynamic forces acting on AFM probe moving in liquid, the measurements were conducted at low scanning frequency (0.1 Hz) and at minimum tip vertical motion frequency (250 Hz). To minimize the probe-induced destruction of neuronal membrane, a decreased number of pixels forming the AFM image (128×128) and peak forces below 2 nN were used.

In the PeakForce QNM mode, the force curve was registered at each point of the AFM measurement, which is the dependence of interaction force on the probe-sample distance. This curve provides information on mechanical properties of the sample, the Young's modulus included. The Young's modulus data of neurons were extracted automatically using the Sneddon model to process the force curves. In this model, the probe is considered as a cone interacting with the sample's surface, and the Young's modulus E of the sample is calculated according to:

$$F = \frac{2}{\pi} \frac{E}{\left(1-\nu^2\right)} \tan(\alpha)\delta^2 \quad (1),$$

where F is the probe-sample force, v is the Poisson's ratio (sample dependent, typically 0.2–0.5), α is the half-angle of the indenter, and δ is the indentation depth.

The programs NanoScope Analysis 1.5 and Gwyddion 2.28 were used to process the AFM data.

Results

An example of a sensory neuron examined with AFM is presented by optical image in Fig. 1a. The neuron consists of a 10 mm-wide soma and two neurites possessing the growth cones separated by approximately 100 mm from each other. The typical set of AFM experimental data used to characterize the neuron is presented in Figs 1b–1f. The AFM topography image shown in Fig. 1b reveals the soma of the neuron rising by about 3 mm above the surrounding substrate. The top part of the soma is transformed into the long and relatively low (the height less than 0.5 micron) process, a neurite. The Young's modulus map (Fig. 1c), the profile (Fig. 1e), and the calculated Young's modulus distribution (Fig. 1d), revealed pronounced heterogeneity in the neuron elastic properties.

The sensory neuron shown in Fig. 1c corresponds to the dark contrast area of image, while the white contrast background represents the relatively rigid substrate. According to these data, Young's modulus of the soma was about 5 kPa, while for the whole neurite, this parameter was about 15 kPa. In addition, there was a small 30 kPa area at the bottom part of the neurite. It is worth noting that this area corresponds to the homogeneous slope of the cell topography (Fig. 1e). Hence, it cannot be a scanning artifact due to variation in the probe-cell contact area on the sample surface places characterized with different topography slopes. Thus, this location could be identified as an axon hillock expected to be the most rigid part of the cell. On the whole, we investigated fourteen of control and the same number of ouabain-treated sensory neurons. However, only nine of twenty eight neurons had a distinct axon hillock location.

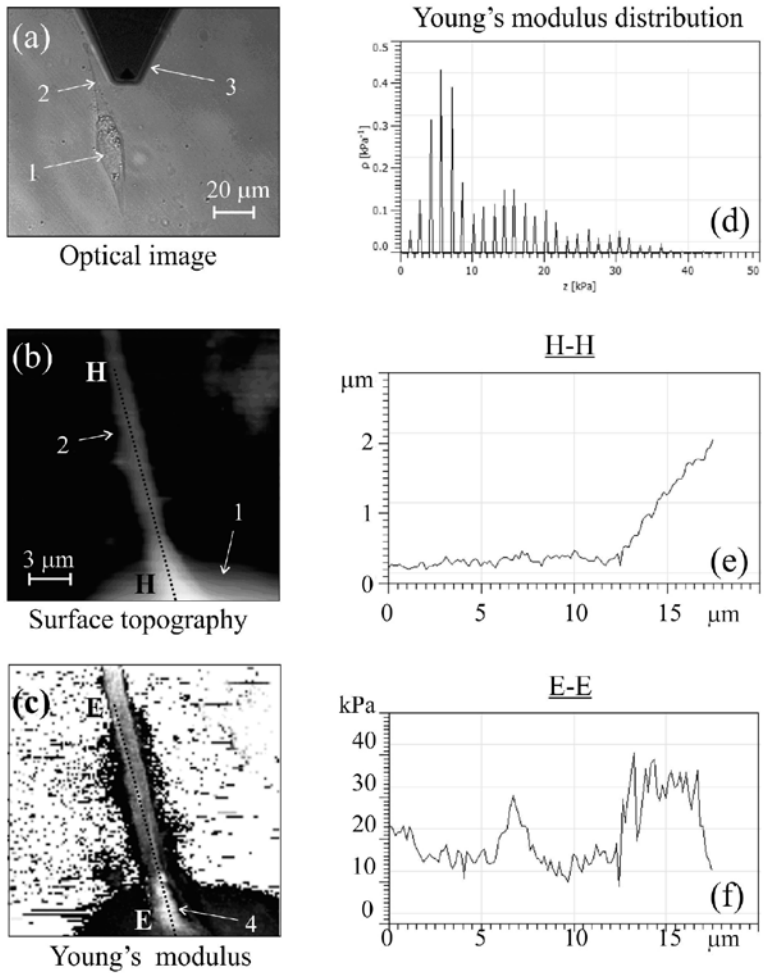

Fig. 1. Control sensory neuron. (a) An optical image. (b) An AFM surface topography image, gray scale difference is 3.2 mm. (c) A Young's modulus image measured simultaneously with (b), gray scale difference is 35 kPa. (d) A histogram of the Young's modulus for the examined area of the cell. (e) The height profile is given along H-H line (b). (f) The Young's modulus profile was calculated along E–E line (c). The numbers denote: 1 – the soma, 2 – the neurite, 3 – the tip of the triangular pyramidal cantilever with a pyramidal probe; 4 – the axon hillock. The averaged value of the Young's modulus is 10 ± 10 kPa

Analysis of surface topography of both sensory neurons groups did not show any significant differences. However, when the mean Young's modulus was taken as an indicator, the difference was discernible. The mean Young's modulus histograms for examined sensory neurons groups are presented in Fig. 2. Evidently, the distribution in the experimental group is broadened, and its center is shifted relative to that of the control group by about 30%.

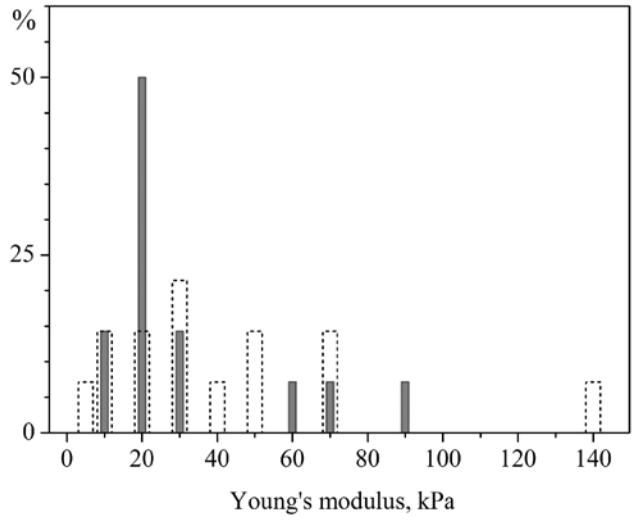

Fig. 2. Distributions of Young's modulus for the control neurons (gray bars) and the neurons grown in the presence of ouabain (empty dotted bars). More rigid (treated) neurons had the mean Young's modulus of 41 ± 34 kPa, while in the control neurons, this value was 31 ± 23 kPa

Discussion

The AFM method makes it possible to achieve a very high optical resolution for cell imaging. On the other hand, testing the mechanical cell properties by this method might shed light at the molecular structure and function of the cells. The neuron mechanical properties reflect the structure of its membrane, cytoskeletal components (microtubules and actin filaments), and the cytoplasm organelles. Due to this heterogeneity, the quantitative data obtained by AFM method are very much scattered. The values of the sensory neuron Young's moduli vary from 1 to 140 kPa (10, 13, 17). The elasticity of the cell body remained constant for 1 h and then degraded thereby indicating loss of cellular cytoskeleton rigidity. Our experiments never lasted for more than 1 h. The loss of rigidity can be also achieved by application of neurotoxins that can destroy the cytoskeleton structure (13). The current report describes the opposite effect (an increase in rigidity) after activation of Na^+,K^+-ATPase signal-transducing function in the sensory neuron membrane. Incubation of embryonic cells in the medium containing ouabain at very low "endogenous" concentrations resulted in an approximately 30% increase in the mean value of the Young's modulus, as compared to the control. Figure 2 shows that distribution function of the Young's moduli of ouabain-treated cells is slightly shifted to the region of higher values. This delicate effect of ouabain at endogenous concentrations, which specifically switches on the signal-transducing function of Na^+,K^+-ATPase, affects the elasticity characteristics of the neuron. It is tempting to suggest that activation of signal-transducing function of the sodium pump, which evokes the responses of Src- and MAP-kinase systems (15, 18), could increase the cell rigidity. Our results indicate that the AFM method is a very promising tool for investigation of molecular mechanisms of cell signaling.

Acknowledgements

The experiments were carried out using the equipment of shared-use Center "Confocal microscopy" at Pavlov Institute of Physiology, Russian Academy of Sciences. Authors are grateful to Dr. I.V. Rogachevskiy (Pavlov Institute of Physiology, Russian Academy of Sciences) for valuable discussions.

This project was supported by the Russian Science Foundation (grant No. 14-15-00677).

REFERENCES

1. Borovikova LV, Borovikov DV, Ermishkin VV, Revenko SV: The resistance of cutaneous feline C-fiber mechano-heat-sensitive unit termination to tetrodotoxin and its possible relation to tetrodotoxin-resistant sodium channels. Primary Sensory Neuron 2, 65–75 (1997)
2. Braet F, Rotsch C: Comparison of fixed and living liver endothelial cells by atomic force microscopy. Appl. Phys. A. 66, 575–578 (1998)
3. Butt HJ, Wolff EK, Gould SA, Dixon Northern B, Peterson CM, Hansma PK: Imaging cells with the atomic force microscope. J. Struct. Biol. 105, 54–61 (1990)
4. Chen X, Feng L, Jin H, Feng S, Yu Y: Quantification of the erythrocyte deformability using atomic force microscopy: correlation study of the erythrocyte deformability with atomic force microscopy and hemorheology. Clin. Hemorheol. Microcirc. 43, 243–251 (2009)
5. Domke J, Parak WJ, George M, Gaub HE, Radmacher M: Mapping the mechanical pulse of single cardiomyocytes with the atomic force microscope. Eur. Biophys. J. 28, 179–186 (1999)
6. Gold MS, Reichling DB, Shuster MJ, Levine JD: Hyperalgesic agents increase a tetrodotoxin-resistant Na current in nociceptors. Proc. Natl Acad. Sci. USA 93, 1108–1112 (1996)
7. Häberle W, Herber JK, Binnig G: Force microscopy of living cells. J. Vac. Sci. Technol. 9, 1210–1213 (1991)
8. Hamlyn JM, Hamilton BP, Manunta P: Endogenous ouabain, sodium balance and blood pressure: a review and a hypothesis. J. Hypertens. 14, 151–167 (1996)
9. Henderson E, Haydon PG, Sakaguchi DS: Actin filament dynamics in living glial cells imaged by atomic force microscopy. Science 257, 1944–1946 (1992)
10. Jiang FX, Lin DC, Horkay F, Langrana NA: Probing mechanical adaptation of neurite outgrowth on a hydrogel material using atomic force microscopy. Ann. Biomed. Eng. 39, 706–713 (2011)
11. Krylov BV, Derbenev AV, Podzorova SA, Lyudyno MI, Kuz'min AV, Izvarina NL: Morphine decreases the voltage sensitivity of slow sodium channels. Neurosci. Behav. Physiol. 30, 431–439 (2000)
12. Lopatina EV, Yachnev IL, Penniyaynen VA, Plakhova VB, Podzorova SA, Shelykh TN, Rogachevsky IV, Butkevich IP, Mikhailenko VA, Kipenko AV, Krylov BV: Modulation of signal-transducing function of neuronal membrane Na^+, K^+-ATPase by endogenous ouabain and low-power infrared radiation leads to pain relief. Med. Chem. 8, 33–39 (2012)
13. Mustata M, Ritchiea K, McNally HA: Neuronal elasticity as measured by atomic force microscopy. J. Neurosci. Methods 186, 35–41 (2010)
14. Nowakowski R, Luckham P, Winlove P: Imaging erythrocytes under physiological conditions by atomic force microscopy. Biochem. Biophys. Acta 1514, 170–176 (2001)
15. Penniyaynen VA, Yachnev IL, Kipenko AV, Lopatina EV, Krylov BV: Probable role of Src-kinase in reception of infrared irradiation. Sensornye Sistemy 28, 90–94 (2014)
16. Rotsch C, Radmacher M: Drug-induced changes of cytoskeletal structure and mechanics in fibroblasts: an atomic force microscopy study. Biophys. J. 78, 520–535 (2000)
17. Spedden E, Staii C: Neuron biomechanics probed by atomic force microscopy. Int. J. Mol. Sci. 14, 16124–16140 (2013)
18. Xie Z: Ouabain interaction with cardiac Na/K-ATPase reveals that the enzyme can act as a pump and as a signal transducer. Cell Mol. Biol. 47, 383–390 (2001)