Analysis of cellular morphology by confocal microscopy and a membrane-intercalating fluorescent dye PKH67

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Abstract

Cells of the body are exposed to anisosmotic environments under various physiological and pathological conditions, which cause mobilization of water and changes in cellular volume and morphology; these changes provide an indication of the cellular environment changes and predict the subsequent cellular damage. We have constructed confocal microscopy-based methods to analyze osmotic stimuli-induced changes in cell dimension and morphology using a membrane-bound fluorescent probe, PKH67. Low osmolarity stimulation of the renal tube derived MDCK cells resulted in an initial increase in their cell volume, which was followed by a subsequent decrease towards initial value. Transient exposure to high osmolarity formamide solution of mouse ventricular cardiomyocytes resulted in complete loss of their transverse tubules (T-tubules). Confocal microscopy in combination with a membrane-intercalating dye was shown to be simple, accurate and free of cellular damage, and would be useful for further studies on the regulation of cellular volume and morphology.

1. Introduction

Cells of the body are exposed to anisosmotic environments under various physiological and pathological conditions, which cause mobilization of water and changes in cellular volume and morphology[1-4]. The renal tubular epithelium is involved in the concentration of urine and faces a large difference in osmolarity ranging from 100mOsm to 1500mOsm. Under the pathological states of renal disorders, diabetes mellitus or cardiac ischemia, cells of the tubular epithelium or myocardium are subject to disturbances in the osmotic environment and exhibit changes in their volume and morphology. When exposed to osmotic stress, cells are forced to activate compensatory mechanisms to maintain its volume and morphology, a process known as regulatory volume decrease or increase[5,6]. In some cases, osmotic stimuli exert irreversible effects on cellular morphology.

To study the cellular effects of osmotic stimuli, analyzing systems for cell volume are indispensable. A practical but indirect method is to load the cell with membrane impermeable fluorescent dyes and calculate cell volume from changes in cytoplasmic fluorescence[7]. Major disadvantages of this method are dye leakage and compartmentalization, which leads to inaccurate estimation of cell volume. A powerful tool for direct measurement of cell volume is confocal microscopy combined with fluorescence labeling of the cell membrane[8,9]. Staining the cell membrane with fluorescent probes followed by two or three dimensional scanning allows direct measurement of the cell volume and morphology. This method is in principle applicable to various cell types.

In the present study, we applied confocal

microscopy and a membrane-intercalating dye, PKH67, to two cell types with different properties, the Madin-Darby canine kidney (MDCK) cells and isolated ventricular cardiomyocytes. The MDCK cell line is of renal tubular epithelial origin and is widely used as a model to study volume regulation[10,11]. Ventricular cardiomyocytes are routinely used for the studies of excitation-contraction mechanisms and cellular damage accompanying diseases such as ischemia and diabetes mellitus[12,13].

2. Methods

MDCK cells (National Institute of Health Sciences) were cultured in Eagles modified essential medium (EMEM) supplemented with 1% penicillin, 0.4% streptomycin and 5% fetal bovine serum (FBS) under humidified 5% CO_2 atmosphere. The cells were plated on glass coverslips 48 to 72 hours before the experiments. Mouse ventricular cardiomyocytes were obtained by Langendorff perfusion of the heart with collagenase solution as previously described[13].

For fluorescence labeling of the cell membrane, PKH67 (Sigma-Aldrich) was dissolved in the loading buffer supplied by the manufacturer and applied to the cells at a final concentration of 5μM. Then the coverslips with the cells were placed in a chamber on the stage of inverted microscopes and perfused with normal extracellular medium to washout the excess fluorescent probe. All experiments were performed at 37°C.

The osmolarity of the basic culture medium was 300mOsm as determined by a freezing point-based osmometer. The NaCl concentration was reduced by 100mM in the hyposmotic media, whose osmolarity was confirmed to be 100mOsm. The change from normal to hyposmotic extracellular solution was performed after the cells were equilibrated for 30 min in the normal solution.

Confocal imaging was performed with LSM 510 (Carl Zeiss) or LSM 5 LIVE (Carl Zeiss) with procedures described in our previous studies[14,15]. The cells loaded with PKH67 were excited at 488nm with an argon laser and the

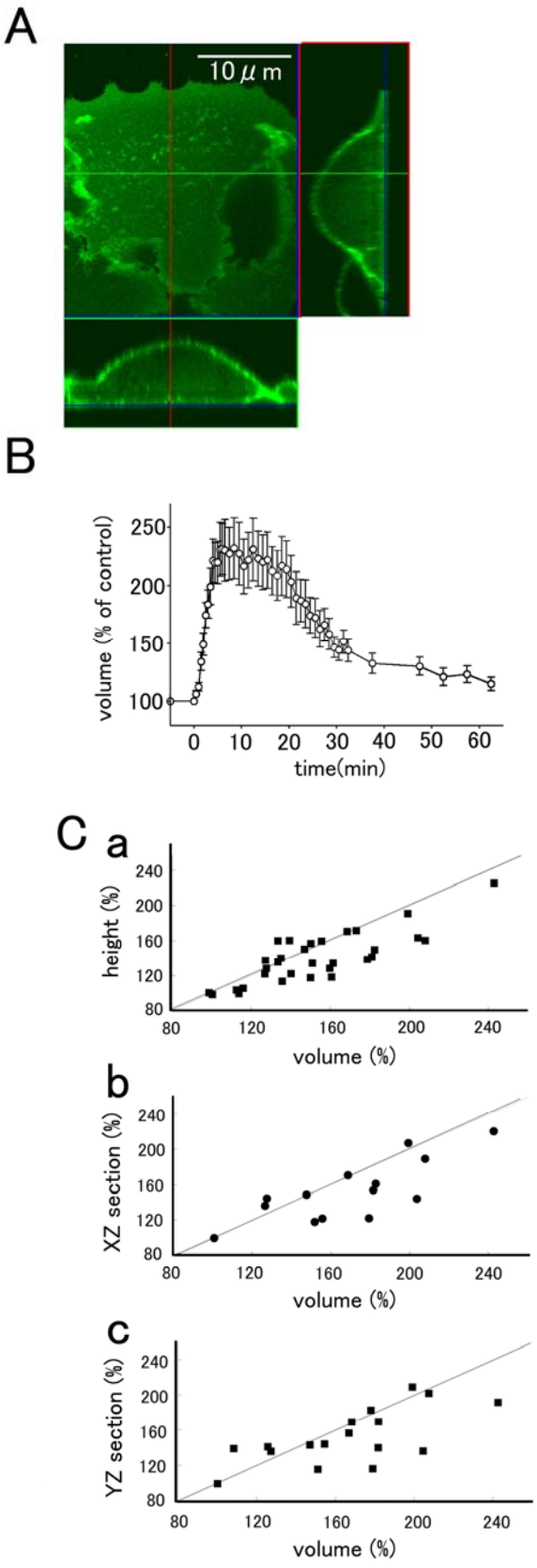

Figure 1. Osmotic stimuli-induced changes in cell dimension of MDCK cells labelled with a membrane dye PKH67. A: typical orthogonal two-dimensional images. Bars in the x-y panel indicate 10μm. B: Time course of the change in cellular volume before and after treatment with low osmolarity solution. Symbols and bars indicate the mean ± standard error of the mean from 10 cells. C: Correlation diagram of increases in cellular volume and cell height (a), area of the x-z plane (b), area of the y-z plane (c) measured at the peak of cell volume.

emission at wavelength between 500nm and 530nm was detected by a photomultiplier. The data obtained were assembled into two-dimensional images. The objective used was Plan-Apocromat 63x1.4 NA oil immersion (Carl Zeiss).

3. Results

The cell membrane of MDCK cells was clearly stained by PKH67 and the cell volume could be calculated (Fig. 1A). Low osmotic solution induced a transient increase in cell volume (Fig. 1B); at 10min after change to low osmotic solution, the volume reached a peak of 231.0 ± 22.8 % of the initial value. After which, a decrease toward the initial value was observed; the volume at 30min and 60min after change to low osmotic solution was 144.2 ± 9.0 % and 115.0 ± 5.6 % of the initial value, respectively. The height, and the area of the two vertical section of the cell correlated with the cell volume (Fig. 1C).

The cell membrane of ventricular cardiomyocytes was clearly stained by PKH67 (Fig. 2A). T-tubules, transverse invaginations of the cell membrane which occur perpendicular to the longitudinal axis, were also clearly stained in ventricular cardiomyocytes. Intracellular organella such as the mitochondria and endoplasmic reticulum were not stained. Treatment of the cells with a high osmolarity extracellular solution containing 1.5M formamide for 15 min followed by washout with normal extracellular solution caused a complete loss of their T-tubules (Fig. 2B).

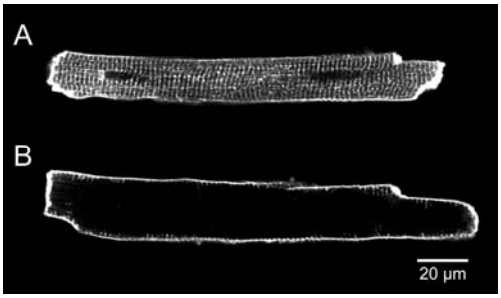

Figure 2. Osmotic stimuli-induced changes in mouse ventricular cardiomyocytes labeled with PKH67. Two-dimensional images before (A) and after (B) treatment with high osmolarity formamide solution. Note that the t-tubules are observed before treatment but not after.

4. Discussion

We constructed a confocal microscopy-based system for the measurement of cellular volume and morphology with a membrane-intercalated dye, PKH67. MDCK cells showed transient increases in cellular volume in response to low osmotic stimuli. The low osmolarity stimuli-induced increase in cell volume was transient; the increase was followed by a decrease towards the initial level. Such phenomenon is generally known as regulatory volume decrease, and is considered to be a mechanism to maintain cellular integrity under osmotic stress[5,6]. In the case of MDCK cells, the regulatory volume decrease was reported to be dependent on an increase in intracellular Ca^{2+} concentration[14]. The low osmotic stimuli-induced increase in the volume of the cell reconstructed by z stacking of horizontal sections (x-y) correlated to a certain extent with the increases in the area of the x-z and y-z vertical sections and also with the cell height. These parameters provided a practical means to measure the changes in cell volume over time.

In mouse ventricular cardiomyocytes, transverse invaginations of the cell membrane which occur perpendicular to the longitudinal axis[15] were clearly stained by PKH67 (Fig. 2). Treatment with high osmolarity formamide solution resulted in complete disappearance of the T-tubules. Earlier studies with ventricular cardiomyocytes showed that detachment of the T-tubules from the cell membrane occurs on washout of anisosmotic extracellular solution[16,17]. The

detubulation was attributed to osmolarity gradient-induced transient swelling or shrinking of the cell. Swelling of cardiomyocytes is a common complication in various pathological conditions such as ischemia and diabetes melitus, and whether detubulation of the ventricular cardiomyocytes occur under such conditions remain to be investigated. As the T-tubules are known to play a key role in excitation-contraction coupling of ventricular cardiomyocytes through stimulation of Ca^{2+} release from the sarcoplasmic reticulum[9,13], it is highly possible that the changes in myocardial function under various pathological conditions are partly mediated by changes in the T-tubules.

Use of the membrane-intercalating dye, PKH67, was shown to be of practical value in the measurement of cellular volume and morphology. The cell membrane could be easily stained by dye application to the extracellular medium, and non-specific staining of intracellular organella was not observed. Staining with PKH67 appeared to be free of cellular damage, and cellular morphology could be stably measured under severe osmotic stimuli. A similar dye with longer excitation and emission wavelengths, PKH26, is also available. Thus, confocal microscopy in combination with membrane-intercalating dyes would be useful for further studies on the regulation of cellular volume and morphology.

5. References

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共焦点顕微鏡法と細胞膜挿入型蛍光色素PKH67による細胞形態の解析

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キーワード：浸透圧刺激，共焦点顕微鏡法，細胞膜挿入型蛍光色素

抄録

生体内の細胞は様々な生理的および病的条件下で浸透圧の異なる環境にさらされ，これが水の移動と細胞の体積変化を引き起こす．細胞の体積変化は，このような細胞を取り巻く環境の変化を反映しており，細胞障害の予測にもつながると考えられる．我々は共焦点顕微鏡法と細胞膜挿入型蛍光色素PKH67を用い，浸透圧刺激による細胞形態の変化を計測する方法を確立した．低浸透圧刺激による腎尿細管上皮由来細胞株MDCKの体積の増大と減少，高浸透圧刺激による心室筋細胞の横行小管（T管）の消失を捉えることが出来た．本研究で用いた手法は，操作が簡便で正確な計測が可能であり，細胞への傷害も少ないという優れた特徴を有しており，細胞の体積および形態の制御機構解明に有用である．

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東邦大学大学院薬学研究科博士後期課程修了．
専門は循環薬理学．薬理学的・電気生理学的手法を基本に，細胞内事象の蛍光イメージング法と組み合わせた総合的検討により，心臓の薬物応答性の包括的理解を目指して研究を行っている．