Relationship between Fluorescence Intensity of GFP and the Expression Level of Prestin in a Prestin-Expressing Chinese Hamster Ovary Cell Line

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Outer hair cells (OHCs) in mammals can elongate and contract at frequencies up to 100 kHz in response to changes in their membrane potential. The origin of this unique motility is the motor protein prestin, which is densely packed in the lateral membrane of the OHCs. In a previous work, we constructed a prestin-expressing cell line using Chinese hamster ovary (CHO) cells to obtain a stable supply of prestin. When we research prestin using constructed cells, it is necessary to estimate the expression level of prestin in the cells easily and non-invasively. As the prestin gene and a green fluorescent protein (GFP) gene were introduced into constructed cells using the same vector, the expression level of prestin and fluorescence intensity of GFP are possibly correlated. Since this correlation is not clear, however, in this study, we therefore investigated whether the expression level of prestin evaluated by patch-clamp recording and the fluorescence intensity of GFP obtained from fluorescence images are correlated or not. As a result, it was demonstrated that they were correlated. The expression level of prestin can therefore be evaluated by measuring the fluorescence intensity of GFP.

Key Words: Acoustic, Biomechanics, Measurement, Sound, Prestin, Green Fluorescent Protein, Chinese Hamster Ovary Cell, Outer Hair Cell, Patch Clamp Technique

1. Introduction

The high sensitivity and sharp tuning of mammalian hearing originates from a mechanical amplification mechanism known as the 'Cochlear amplifier' (Fig. 1). The outer hair cells (OHCs) in the organ of Corti, which alter their longitudinal length in response to changes in their membrane potential, are involved in this mechanism. This unique type of cell motility, called 'electromotility' (2)–(6), operates at frequencies up to 100 kHz and does not require ATP hydrolysis (7). The molecular mechanism of electromotility is thought to be a voltage-dependent conformational change of a motor protein densely embedded in the lateral membrane of the OHCs (8) (Fig. 2).

In 2000, this motor protein was identified by a cDNA library subtraction procedure and termed prestin (9). Since its identification, prestin has been researched intensively to understand its function. Cultured kidney cells transfected with prestin cDNA were found to show voltage-dependent membrane capacitance (9), (10) and electrically evoked changes in cell shape (9), similar to OHCs. The importance of prestin for the auditory mechanism was demonstrated by the fact that prestin knock-out mice exhibited a loss of outer hair cell electromotility in vitro and a significantly elevated hearing threshold of 40–60 dB in vivo (11).

For further explication of the function of prestin, it is necessary to study prestin at the molecular level. As a springboard for such study, access to a stable supply of prestin is essential. For this reason, we constructed a prestin-expressing Chinese hamster ovary (CHO) cell line and confirmed that the constructed cells expressed...
Fig. 1 A schematic of the cochlea and a cross section of the organ of Corti. When the basilar membrane vibrates, shear motion occurs between the reticular lamina and the tectorial membrane. The stereocilia of the IHC and OHCs are deflected by this shear motion, and ions flow into the cells, resulting in intracellular depolarization which causes auditory nerve fiber activation in the IHC. Simultaneously, the OHCs show a motile response and apply force to the basilar membrane.

Fig. 2 Lateral wall of the OHC(12). The OHC lateral wall consists of three layers: plasma membrane, cortical lattice and subsurface cisternae. The motor protein is believed to be embedded in the plasma membrane. prestin in their plasma membrane and exhibited prestin-originated voltage-dependent nonlinear membrane capacitance(12). With this cell line, we can obtain prestin-expressing cells anytime without killing animals.

Generally, proteins are expressed and resolved dynamically in a living cell, and each cell has a different cycle of protein expression. As a result, the expression level of prestin in constructed cells may change with time and differ among cells. An indicator of the expression level of prestin in constructed cells is therefore required. The expression level of prestin in prestin-expressing cells is usually determined by analyzing the voltage-dependent nonlinear membrane capacitance, which is derived from a conformational change of prestin, using the whole-cell patch-clamp recording technique. However, the procedure of such recording is complicated and requires making an injurious hole in the cell membrane. It is therefore necessary to develop a method by which the expression level of prestin in each cell can be easily and non-invasively investigated.

In the construction of a prestin-expressing CHO cell line in our previous study(12), the prestin gene was transfected into CHO cells using a mammalian expression vector. The humanized Renilla reniformis green fluorescent protein (hrGFP) gene, which is a subspecies of the green fluorescent protein (GFP) gene, was inserted as a marker in the vector to confirm the expression of prestin in the cell. GFP is widely used as a marker protein because it yields a green fluorescence, whose intensity is proportional to the amount of GFP, when it is exposed to blue light(13). When the vector is transfected into CHO cells, the prestin gene and the hrGFP gene are transcribed into sequential mRNA. Prestin and hrGFP are then translated from this mRNA and localized to the plasma membrane and the cytoplasm of the cell, respectively (Fig. 3). As the prestin gene and the hrGFP gene are transcribed into the same mRNA, the expression level of prestin and fluorescence intensity of hrGFP are possibly correlated. If they do have a correlation, the expression level of prestin
can be evaluated by the fluorescence intensity of hrGFP. However, this correlation has not been clarified yet.

In this study, an attempt was therefore made to determine the relationship between the fluorescence intensity of hrGFP and the expression level of prestin. The fluorescence intensity of hrGFP and the expression level of prestin were evaluated using fluorescence micrographs and whole-cell patch-clamp recordings, respectively.

2. Materials and Methods

2.1 Cell preparation

A prestin-expressing CHO cell line, cultured in flasks with RPMI-1640 medium containing 10% fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin at 37°C with 5% CO2, was used. This cell line had been previously constructed(12). When the prestin gene was transfected into CHO cells, the mammalian expression vector pRES-hrGFP-1a (Stratagene, La Jolla, CA) was used. In the constructed cells, prestin-coding and hrGFP-coding sequences of the vector are transcribed into the same mRNA. Prestin and hrGFP are then independently translated from this mRNA and are localized in the plasma membrane and the cytoplasm of the cell, respectively.

To transfer the cells to an experimental chamber, they were detached from the flasks by incubating them with 100 µM EDTA in PBS solution for 5–10 min. The EDTA/PBS solution containing CHO cells was then transferred to a conical tube and centrifuged at 250 × g for 5 min. After centrifugation, all of the supernatant was removed, and the cell pellet was resuspended in culture medium. This medium containing the cells was plated on a glass base dish, i.e., the experimental chamber, and incubated for 2–15 hours at 37°C with 5% CO2 for cell adhesion to the base of the dish. After the incubation, the medium was replaced by an external solution composed of 145 mM NaCl, 5.8 mM KCl, 1.3 mM CaCl2, 0.9 mM MgCl2, 10 mM HEPES, 0.7 mM Na2HPO4, and 5.6 mM glucose, adjusted to pH 7.3, and the prepared samples were then used for the following experiment.

2.2 Quantification of hrGFP fluorescence intensity

2.2.1 System for measuring fluorescence intensity

The hrGFP fluorescence intensity of prestin-expressing CHO cells was quantified using the following system (Fig. 4). Samples were observed using an inverted microscope (TE300, Nikon, Tokyo, Japan) with a filter cube (a excitation filter for wavelengths of 450–490 nm, a dichroic mirror for wavelengths of 505 nm or more and a emission filter for wavelengths of 520 nm or more). All of the optical components of the microscope, such as a mercury lamp, filters, iris diaphragms and lenses, were kept under the same conditions during the measurements. Micrographs were detected by a black and white CCD camera (C2400-77, Hamamatsu Photonics, Shizuoka, Japan) with a contrast gain of 10.0, image signals being transmitted to a digital recorder (DCR-PC110, Sony, Tokyo, Japan) at 30 frames per second. One frame of the transmitted signals was stored in the digital recorder as a static image. The average intensity of pixels in the target area was calculated using Eq. (1).

![Fig. 4 Measurement system of hrGFP fluorescence intensity.](image)

Before evaluation of the hrGFP fluorescence intensity of the constructed cells, the relationship between the intensity of the light entering the object lens and the obtained pixel intensity was calibrated within the range of hrGFP fluorescence intensity. Transmitted light for bright field observation, the output voltage of which was fixed, was used as incoming light. Incoming light intensity was altered by passing the transmitted light through neutral density (ND) filters (Olympus, Tokyo, Japan) located on a stage. The relative light intensity in the absence of a filter was defined as 100%, and intensities with ND50, ND25, ND12 and ND6 filters were defined as 50%, 25%, 12% and 6%, respectively. When light was completely shielded, relative incoming light intensity was defined as 0%. The filter cube was used to obtain images. The same range of wavelengths as that of the hrGFP fluorescence is included in the transmitted light and is detected by the CCD camera. Images were taken of the respective in-
coming light intensities. The obtained average intensity of pixels within the area, corresponding to a circle with a diameter of 20 µm located in the center of the image, was calculated using Eq. (1) and plotted against relative incoming light intensities controlled by the ND filters. Measurements were made in a dark room and room temperature was maintained at 23–25°C throughout the experiment.

2.2.2 Quantification of hrGFP fluorescence intensity of prestin-expressing CHO cells

After replacement of the medium by the external solution, prestin-expressing CHO cells plated on the experimental chamber were observed using the microscope under exposure of excitation light at a wavelength of 450–490 nm to detect brightly fluorescent cells with spherical shape. The focus plane was set at the center of a target cell. The fluorescence image of the cell was then stored as mentioned above. Furthermore, a bright field image was taken under white light exposure in the same area as that of the fluorescence image.

To obtain the hrGFP fluorescence intensity of a cell, first, the outline of the target cell was determined from the bright field image. Next, in the fluorescence image, the average intensity of pixels within the determined outline of the target cell was calculated (Fig. 5). The obtained value represents the hrGFP fluorescence intensity of the cell and was termed ‘GFP intensity’. This GFP intensity is a relative value depending on the equipment.

2.3 Electrophysiological measurements

It is well known that prestin-expressing cells exhibit voltage-dependent nonlinear membrane capacitance against membrane potential. This nonlinear membrane capacitance reflects the charge movements across the plasma membrane of the cell due to prestin. To evaluate the expression level of prestin in prestin-expressing CHO cells, the membrane capacitance of each target cell was measured by the whole-cell patch-clamp method just after its fluorescence image was taken.

Measurements of membrane capacitance were conducted according to the previously reported procedure (see Ref. (12)) using the ‘membrane test’ feature of pCLAMP 8.0 acquisition software (Axon Instruments, Foster City, CA). Measurements were continuously repeated three times, and when maximum variations of membrane capacitance at each membrane potential obtained from three sequential measurements were 2% or less, the obtained data were used for the following analysis.

After the measurements, the values of membrane capacitance were plotted against membrane potential and fitted to the derivative of a Boltzmann function,

\[ C_m(V) = C_{\text{lin}} + \frac{Q_{\text{max}}}{a e^{-\frac{V-V_{1/2}}{\alpha}} \left(1 + e^{-\frac{V-V_{1/2}}{\alpha}}\right)^2}, \]

where \( C_{\text{lin}} \) is linear capacitance, \( Q_{\text{max}} \) is maximum charge transfer, \( V \) is membrane potential, \( V_{1/2} \) is the voltage at which the maximum charge is equally distributed across the membrane and \( \alpha = kT/ze \) is the slope factor of the voltage dependence of the charge transfer where \( k \) is Boltzmann’s constant, \( T \) is absolute temperature, \( z \) is valence and \( e \) is electron charge. Fitting was performed using the Levenberg-Marquardt method.

As \( Q_{\text{max}} \) is the total amount of charge transferred by prestin and \( e \) is electron charge, which equals the charge transferred by one prestin molecule, the number of prestin molecules in the cell is given by \( Q_{\text{max}}/e \). As \( C_{\text{lin}} \) expressed in picofarads indicates the total capacitance of the plasma membrane of the cell, and the membrane capacitance of the cell per unit surface area is known to be 0.01 pF/µm²(14), the surface area of the cell is expressed by \( C_{\text{lin}}/0.01 \) µm². The expression level of prestin per unit surface area, i.e., charge density, of the cell is therefore obtained from

\[ \text{Charge density} = \frac{Q_{\text{max}}}{e} \frac{C_{\text{lin}}}{0.01}. \]
3. Results

3.1 Relationship between average intensity of pixels and incoming light intensity

The obtained relationship between average intensities of pixels and relative incoming light intensities controlled by ND filters is shown in Fig. 6. The solid line represents the regression line obtained by the least squares method. The correlation coefficient was above 0.99. This result indicates that the calculated average intensity of pixels and the light intensity entering the object lens are in proportion.

3.2 Time dependence of fluorescence decay

To examine the time dependence of fluorescence decay\(^{(15)}\) of the object cells, GFP intensity was measured every one second in the same frame \((n = 10)\). Normalized GFP intensity, which was obtained from GFP intensity at each second divided by the initial GFP intensity, was plotted against exposure time to excitation light (Fig. 7). The result shown in Fig. 7 indicates that GFP intensity decreases to 30% of the initial GFP intensity with 20-second exposure to excitation light. As exposure to excitation light reduces fluorescence intensity, to obtain sharp photographs, the time interval between the start of exposure to excitation light and the capture of images should be shortened. In this study, this time interval was 1–2 seconds because it takes that long to set the optical path and capture an image.

3.3 GFP intensity and charge density of prestin-expressing CHO cells

Representative data of the hrGFP fluorescence image, a brightness histogram of the pixels included within the outline of the target cell and measured membrane capacitance against membrane potential of the target cell are shown in Fig. 8.

In a group of 20 cells, fitting parameters of \( C_{\text{lin}} = 18.6 \pm 5.5 \) pF, \( Q_{\text{max}} = 126.2 \pm 107.2 \) fC, \( \alpha = 41.3 \pm 10.7 \) mV and \( V_{1/2} = -57.9 \pm 13.2 \) mV, and charge density of \( 394 \pm 227 \) e\(^+\)/\( \mu \)m\(^2\) were obtained from patch-clamp recordings (mean \( \pm \) SD).

The obtained charge density was plotted against GFP intensity (Fig. 9). The regression line was calculated using the least squares method. The correlation coefficient obtained from least squares fit is 0.67. This correlation between charge density and GFP intensity is significant at the 0.05 level.

4. Discussion

The fitting parameters \( \alpha \) and \( V_{1/2} \) of Eq. (2), which reflect the characteristic properties of prestin, obtained from 20 cells, correspond well with findings of previous reports\(^{(10),(16)}\), supporting the validity of the measurements. The average value of charge density, i.e., 394 e\(^+\)/\( \mu \)m\(^2\), obtained from 20 cells, is larger than that previously reported, i.e., 196 e\(^+\)/\( \mu \)m\(^2\). In this study, cells showing bright fluorescence were selected, while the previous study, cells were chosen at random. It is therefore considered that the charge density of 394 e\(^+\)/\( \mu \)m\(^2\) obtained in this study is not indicative of the average charge density of the whole group of prestin-expressing CHO cells.

Membrane capacitance of cells, which did not fluoresce, was also measured. However, those cells did not show voltage dependent nonlinear capacitance. These results indicate that prestin was not expressed or was only slightly expressed in the cells which did not fluoresce. These results agree with the fact that there is a correlation between GFP intensity and the expression level of prestin.

As shown in Fig. 9, it was clarified that there was a correlation between the GFP intensity and the expression level of prestin although the correlation coefficient was 0.67. This result indicates that the expression level of prestin can be estimated by using GFP intensity. The cells which show high GFP intensity should be selected when high prestin-expressing cells are required.

To obtain GFP intensity with less error, the time interval between the start of exposure to excitation light and
the capture of images should be the same because GFP intensity is reduced with time due to fluorescence decay (Fig. 7). In this study, however, the time interval between the start of exposure to excitation light and the capture of images ranged from one to two seconds. According to Fig. 7, there is a possibility that the obtained GFP intensity has a margin of error of up to 20%. Another way to reduce error is to apply a weaker excitation light and to improve the detection sensitivity of fluorescence because the application of weaker excitation light restricts the fluorescence decay.

5. Conclusions

It was demonstrated that hrGFP fluorescence intensity increases linearly as charge density increases. As charge density is proportional to the expression level of prestin, this result indicates that such expression can be evaluated by relative hrGFP fluorescence intensity in the constructed cell line.

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