



# Measurement of $[Cl^-]_i$ unaffected by the cell volume change using MQAE-based two-photon microscopy in airway ciliary cells of mice

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Received: 5 December 2017 / Accepted: 4 January 2018 / Published online: 13 January 2018  
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## Abstract

MQAE is a ‘non-ratiometric’ chloride ion ( $Cl^-$ )-quenched fluorescent indicator that is used to determine intracellular  $Cl^-$  concentration ( $[Cl^-]_i$ ). MQAE-based two-photon microscopy is reported to be a useful method to measure  $[Cl^-]_i$ , but it is still controversial because a change in cell volume may alter the MQAE concentration, leading to a change in the fluorescence intensity without any change in  $[Cl^-]_i$ . In an attempt to elucidate the effect or lack of effect of cell volume on MQAE concentration, we studied the effects of changes in cell volume, achieved by applying different levels of osmotic stress, on the intensity of MQAE fluorescence in airway ciliary cells. To study solely the effect of changes in cell volume on MQAE fluorescence intensity, i.e., excluding the effect of any change in  $[Cl^-]_i$ , we first conducted the experiments in a  $Cl^-$ -free nitrate ( $NO_3^-$ ) solution to substitute  $NO_3^-$  (non-quenching anion for MQAE fluorescence) for  $Cl^-$  in the intracellular fluid. Hypo- (– 30 mM  $NaNO_3$ ) or hyper-osmotic stress (+ 30 mM  $NaNO_3$ ) effected changes in cell volume, but the stress did not result in any significant change in MQAE fluorescence intensity. The experiments were also carried out in  $Cl^-$ -containing solution. Hypo-osmotic stress (– 30 mM  $NaCl$ ) increased both MQAE fluorescence intensity and cell volume, while hyper-osmotic stress (+ 30 mM  $NaCl$ ) decreased both of these properties. These results suggest that the osmotic stress-induced change in MQAE fluorescence intensity was caused by the change in  $[Cl^-]_i$  and not by the MQAE concentration. Moreover, the intracellular distribution of MQAEs was heterogeneous and not affected by the changes in osmotic stress-induced cell volume, suggesting that MQAEs are bound to un-identified subcellular structures. These bound MQAEs appear to have enabled the measurement of  $[Cl^-]_i$  in airway ciliary cells, even under conditions of cell volume change.

**Keywords** MQAE · Intracellular  $Cl^-$  concentration · Two-photon microscopy · Cell volume ·  $NO_3^-$

## Introduction

Intracellular chloride ( $Cl^-$ ) is the major anion in living cells and plays a crucial role in maintaining cellular functions in many cell types [1–8], such as cell volume [3, 6], intracellular pH [9] and fluid secretion [10, 11]. MQAE, a fluorescent  $Cl^-$  indicator, has been found to be a useful fluorescence dye for noninvasive measurements of the intracellular  $Cl^-$  concentration ( $[Cl^-]_i$ ) [12–17], with a number of advantageous properties. First, it is membrane permeable and only a short incubation time is needed to stain cells. Second, it is insensitive to pH or bicarbonate ( $HCO_3^-$ ) [15, 16]. Third, it is highly sensitive to  $Cl^-$  [13] and rapidly quenched by  $Cl^-$  (< 1 ms) [17]. Moreover, the use of MQAE in combination with the two-photon confocal microscopy has been shown to reduce photochemical damage to the cells [12, 14, 16].

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MQAE therefore appears to be a very useful fluorescence dye to measure the  $[Cl^-]_i$ . However, there are some limitations associated with the use of MQAE due to its characteristics: it is a 'non-ratiometric' dye [single wavelength excitation (350 nm) and emission (460 or 510 nm)] [12, 16]. Decreasing or increasing MQAE concentration decreases or increases, respectively, the fluorescence intensity even without any change in the  $[Cl^-]_i$ . Therefore, in studies involving changes in cell volume, great care must be taken in interpreting changes in MQAE fluorescence intensity due to changes in  $[Cl^-]_i$  or MQAE concentration caused by the changes in cell volume.

In many cell types, including airway ciliary cells, various agonists or antagonists evoke cell shrinkage or swelling even under isosmotic conditions [1–3, 6]. Iso-osmotic cell shrinkage, which decreases the  $[Cl^-]_i$  [18], appears to increase the MQAE concentration, leading to increases in MQAE fluorescence intensity, possibly via both an increase in the MQAE concentration and a decrease in  $[Cl^-]_i$ , and vice versa. Therefore, it is unclear whether the change in MQAE fluorescence intensity is due to the change in  $[Cl^-]_i$  or the change in MQAE concentration. To solve this problem, a method to measure  $[Cl^-]_i$  quantitatively during an isosmotic cell shrinkage has been established by measuring both cell volume and MQAE fluorescence [2]. However, it is not easy to measure both cell volume and MQAE fluorescence simultaneously using the two-photon confocal microscopy method.

The airway ciliary cells show heterogeneous staining for MQAE, as do many types of cells [4, 14, 16, 19]. Surprisingly, this heterogeneous distribution is barely affected by the changes in cell volume induced by osmotic stress, which suggests that MQAE molecules may be bound to unidentified subcellular structures [14] and that the number of MQAE molecules in a fixed local area may be unaffected by the changes in cell volume. If this is the case, it may be possible to measure the changes in MQAE fluorescence intensity due to the  $[Cl^-]_i$  because the MQAE concentration in the local area under study is not altered by the change in cell volume. To clarify this question, in this study, we changed the osmotic stress in a  $Cl^-$ -free nitrate ( $NO_3^-$ ) solution to achieve changes in cell volume without changing the  $[Cl^-]_i$ . The aim of this study was to confirm that  $[Cl^-]_i$  measurements by MQAE-based two-photon microscopy are not modified by the effect of changes in MQAE concentration on its fluorescence intensity during changes in cell volume in airway ciliary cells.

## Materials and methods

The procedures and protocols for the experiments reported here were approved by the Animal Research Committee of Kyoto Prefectural University of Medicine, and the

experiments were carried out in accordance with the guidelines of this committee, which is identical to those of the Physiological Society of Japan.

## Solution and Chemicals

The control solution contained (in mM) NaCl, 146; KCl, 4.5;  $MgCl_2$ , 1;  $CaCl_2$ , 1.5; Na-HEPES, 5; H-HEPES, 5; glucose, 5. The  $Cl^-$ -free  $NO_3^-$  solution was prepared by replacing  $Cl^-$  in the control solution with  $NO_3^-$ . The hyper-osmotic stress was applied by adding 30 mM NaCl or  $NaNO_3$  to the control solution or to the  $Cl^-$ -free  $NO_3^-$  solution, and the hypo-osmotic stress was applied by removing 30 mM NaCl or  $NaNO_3$  from the control solution or from the  $Cl^-$ -free  $NO_3^-$  solution. The solutions were aerated with 100%  $O_2$ . The pH of each solution was adjusted to 7.4 by adding 1 N HCl or 1 N  $HNO_3$ , as appropriate. The experiments were carried out at 37 °C.

## Cell preparation

Female mice (C57BL/6J, 5 weeks of age) were purchased from Simizu Experimental Animals (Kyoto, Japan) and fed standard pellet food and water ad libitum. Airway ciliary cells were isolated from the lungs as previously described [6, 20–26]. Briefly, the mice were anesthetized by 3% isoflurane (inhalation) and then further anesthetized by an intraperitoneal injection (ip) of pentobarbital sodium (40 mg/kg) and heparinized (1000 U/kg) for 15 min. The mice were then sacrificed by a high-dose of pentobarbital sodium (100 mg/kg, ip) and the airway ciliary cells isolated by an elastase treatment [6, 20, 21, 26].

## Measurement of MQAE fluorescence intensities

MQAE was dissolved in a mixture of acetonitrile and water (1:1; stock solution), and the stock solution (500 mM) was stored at  $-20$  °C. Isolated airway ciliary cells were incubated with 10 mM MQAE for 60 min at 37 °C [2]. MQAE at 5 mM is widely used for intracellular loading in many cell types [4, 9, 14, 19], but at this concentration the airway ciliary cells in our study were not sufficiently stained for MQAE within 60 min. We thus used a 10 mM MQAE to obtain sufficient staining to measure the MQAE fluorescence intensity in the airway ciliary cells. The same concentration (10 mM) was used to measure  $[Cl^-]_i$  in A6 cells [2]. The MQAE-loaded cells were set on a coverslip pre-coated with Cell-Tak (Becton–Dickinson Labware, Bedford, MA), which was set in a micro-perfusion chamber (20  $\mu$ l) mounted on an inverted light microscope equipped with a confocal laser scanning system (model LSM 510META; Carl Zeiss, Jena, Germany). MQAE was excited at 780 nm using a two-photon excitation laser system (MaiTai; Spectra-Physics, Santa

Clara, CA), and emission was at 510 nm. The normalized value of fluorescence intensity ( $F_t/F_0$ ) was calculated; the subscripts “0” or “t” indicate the time just before or just after the start of application of osmotic stress, respectively.

The airway ciliary cells were observed in the optical sections (thickness 0.9  $\mu\text{m}$ ) using the confocal laser scanning microscope. The cell volume was measured by tracing the outline of a ciliary cell on the phase contrast image of each optical section, and the area ( $A_n \mu\text{m}^2$ ) was measured; “n” shows the number of optical sections. We also measured the MQAE fluorescence intensity ( $F_n$ ) in the intracellular area of the cell in each optical section. The image analysis system reported  $F_n$  as intensity per micron<sup>2</sup>. The cell volume ( $V$ ) was calculated by the sum of  $A_n$  in each section. We also calculated the total MQAE fluorescence intensity of the all cell areas by summing the total fluorescence intensity ( $A_n \times F_n$ ) in each section. The total MQAE fluorescence intensity in all cell areas indicates  $[\text{Cl}^-]_i$ , if the number of MQAE molecules does not change. We obtained 18–22 optical sections from each cell. The normalized value of cell volume ( $V_t/V_0$ ) was also calculated using the sum of  $A_n$ , whereby the subscripts “0” or “t” indicate the time just before or after the application of osmotic stress, respectively. We also measured the changes in MQAE fluorescence intensity ( $F_t/F_0$ ) in the selected local area of the selected cell using the identical focal plane throughout the experiment; the subscripts “0” or “t” indicate the time just before or after the osmotic stress, respectively.

## Statistical analysis

Data are expressed as the mean  $\pm$  standard error of the mean. Statistical significance between means was assessed by analysis of variance (ANOVA) or the Student *t* test (paired or unpaired), as appropriate. Differences were considered to be significant at  $p < 0.05$ .

## Results

### Osmotic stresses under the $\text{Cl}^-$ -free condition

To examine the effects of cell volume on MQAE fluorescence intensity, we applied osmotic stress to the MQAE-loaded airway ciliary cells to alter cell volume. To exclude any change in MQAE fluorescence intensity caused by any changes in  $[\text{Cl}^-]_i$ , we carried out the initial experiments using the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution because  $\text{NO}_3^-$  does not quench MQAE fluorescence [2]. The substitution of  $\text{NO}_3^-$  for  $\text{Cl}^-$  in the intracellular space limits the measurement of changes in MQAE fluorescence intensity to only those that are due to changes in MQAE concentration caused by increases/decreases in cell volume as there can be no

change in  $[\text{Cl}^-]_i$  in the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution. Hypo- or hyper-osmotic stress was applied by removing 30 mM  $\text{NaNO}_3$  from or adding it to the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution, respectively.

Figure 1A shows the phase contrast images (Fig. 1A,a, A,b) and the MQAE fluorescence images (Fig. 1A,c, A,d) in an optical section during application of the hypo-osmotic stress ( $-30$  mM  $\text{NaNO}_3$ ) to MQAE-loaded airway ciliary cells in the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution. An optical section was selected to be the identical focal plane of the selected cell throughout the experiment. Figure 1A,a and A,b shows the phase contrast images just before and 10 min after the application of the hypo-osmotic stress, respectively. The white line in each figure shows the outline of the cell. The cell outlines obtained from the phase contrast images shown in Fig. 1A,a and A,b clearly indicate that hypo-osmotic stress induced cell swelling. The cell area just before application of the hypo-osmotic stress ( $-30$  mM  $\text{NaNO}_3$ ) was  $136 \mu\text{m}^2$  (Fig. 1A,a) and that 10 min after application of the hypo-osmotic stress was  $165 \mu\text{m}^2$  (Fig. 1A,b). Thus, the application of hypo-osmotic stress increased the  $A/A_0$  to 1.22, and the value of  $V/V_0$  calculated from total slice area of this cell was 1.15. The cell outlines shown in Fig. 1A,a and A,b are superimposed in the MQAE fluorescence image in Fig. 1A,c and A,d, respectively. The fluorescence image of MQAE molecules that were heterogeneously distributed in the cell was barely affected by the application of the hypo-osmotic stress (Fig. 1A,c, A,d).

Figure 1B shows the phase contrast images and MQAE fluorescence images in an optical section just before and 10 min after the application of the hyper-osmotic stress ( $+30$  mM  $\text{NaNO}_3$ ) in the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution. The area just before application of the hyper-osmotic stress ( $+30$  mM  $\text{NaNO}_3$ ) was  $85 \mu\text{m}^2$  (Fig. 1B,a) and that 10 min after application of the hyper-osmotic stress was  $71 \mu\text{m}^2$  (Fig. 1B,b). Thus, the hyper-osmotic stress decreased the  $A/A_0$  to 0.82, and the value of  $V/V_0$  calculated from total slice area of this cell was 0.86. The optical section was selected to be the identical focal plane of the selected cell throughout the experiment. The application of the hyper-osmotic stress decreased the cell volume (compare Fig. 1B,a and B,b). The cell outlines were also superimposed on the MQAE fluorescence images (Fig. 1B,c and B,d). The fluorescence image of MQAE molecules, which were heterogeneously distributed in the cell, was little changed by the application of the hyper-osmotic stress in spite of the decrease in cell volume (Fig. 1B,c, B,d).

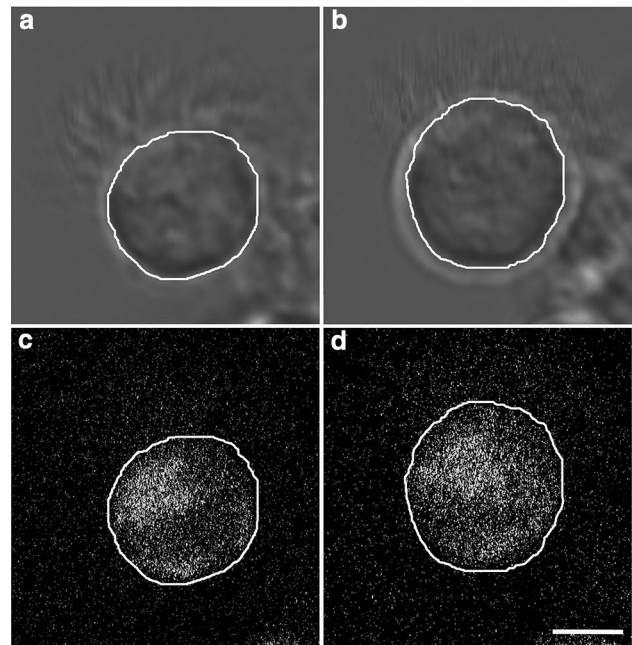
The images shown in Fig. 1 suggest that MQAE distribution would not change even under the conditions associated with changes in cell volume. To confirm this point, we attempted to quantitatively clarify the effect of cell volume changes on the intracellular MQAE distribution by measuring the MQAE fluorescence intensity under conditions that

**Fig. 1** Phase contrast and MQAE fluorescence images of airway ciliary cells. The images in **A,a**, **A,b**, **B,a** and **B,b** were obtained from the same optical focal plane of a cell. Experiments were carried out in the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution. **A** Hypo-osmotic stress ( $-30 \text{ mM NaNO}_3$ ) condition: **a**, **c** Phase contrast image (**a**) and MQAE fluorescence image (**c**) just before the application of the hypo-osmotic stress; **b**, **d** phase contrast image (**b**) and MQAE fluorescence image (**d**) 10 min after the application of the hypo-osmotic stress. The white line shows the outline of the cell. The application of the hypo-osmotic stress induced cell swelling, but there was little change in MQAE fluorescence intensity. **B** Hyper-osmotic stress ( $+30 \text{ mM NaNO}_3$ ) condition: **a**, **c** Phase contrast image (**a**) and MQAE fluorescence image (**c**) before the application of the hyper-osmotic stress; **b**, **d** phase contrast image (**b**) and MQAE fluorescence image (**d**) 10 min after the application of the hyper-osmotic stress. The white line shows the outline of the cell. The application of hyper-osmotic stress induced cell shrinkage, but there was little change in MQAE fluorescence intensity

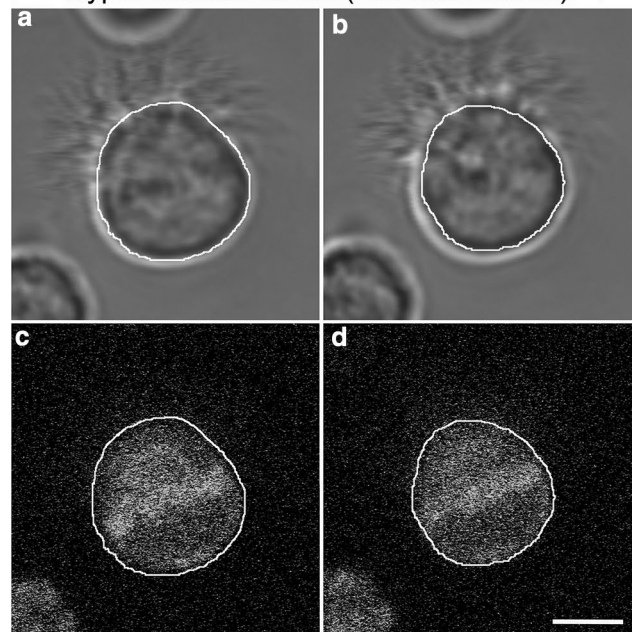
excluded any change in  $\text{Cl}^-$  concentration, which is known to affect MQAE fluorescence intensity. In this experiment we used the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution and measured MQAE fluorescence intensity using the image analysis program of the two-photon laser scanning confocal microscope. The airway ciliary cells were perfused with the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution for 20 min prior to the application of osmotic stress. The switch from the control  $\text{Cl}^-$ -containing solution to the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution increased MQAE fluorescence, which reached a plateau level within 10 min, indicating that the intracellular  $\text{Cl}^-$  was almost completely replaced with  $\text{NO}_3^-$  within 10 min after the switch in extracellular solution. The switch to the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution increased the  $F/F_0$  to  $1.22 \pm 0.02$  ( $n = 5$ ). Figure 2 shows the normalized MQAE fluorescence intensity ( $F/F_0$ ), cell volume ( $V/V_0$ ) and total MQAE fluorescence intensity during the osmotic stress application in the MQAE-loaded airway ciliary cells perfused with the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution. The effects of the hypo-osmotic stress ( $-30 \text{ mM NaNO}_3$ ) on the MQAE fluorescence intensity and the cell volume were shown in Fig. 2a–c. The application of the hypo-osmotic stress increased the cell volume in the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution ( $V/V_0$  10 min after the hypo-osmotic stress =  $1.17 \pm 0.01$ ;  $n = 4$ ) (Fig. 2b), nevertheless it did not change MQAE fluorescence intensity in the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution ( $F/F_0$  10 min after the hypo-osmotic stress =  $0.99 \pm 0.01$ ;  $n = 4$ ) (Fig. 2a). These results indicate that the MQAE concentration at local areas would not be affected by changes in cell volume. The total MQAE fluorescence intensities were also calculated and found not to be changed by the hypo-osmotic stress (Fig. 2c). The normalized value of the total MQAE fluorescence intensity 10 min after the hypo-osmotic stress ( $-30 \text{ mM NaNO}_3$ ) was  $1.00 \pm 0.01$  ( $n = 4$ ). These observations suggest that MQAE content and activity in the cell were constant during the experimental time period.

We also studied hyperosmotic effects ( $+30 \text{ mM NaNO}_3$ ) on MQAE fluorescence intensity and cell volume (Fig. 2d–f). The application of hyper-osmotic

### A Hypo-osmotic stress ( $-30 \text{ mM NaNO}_3$ )

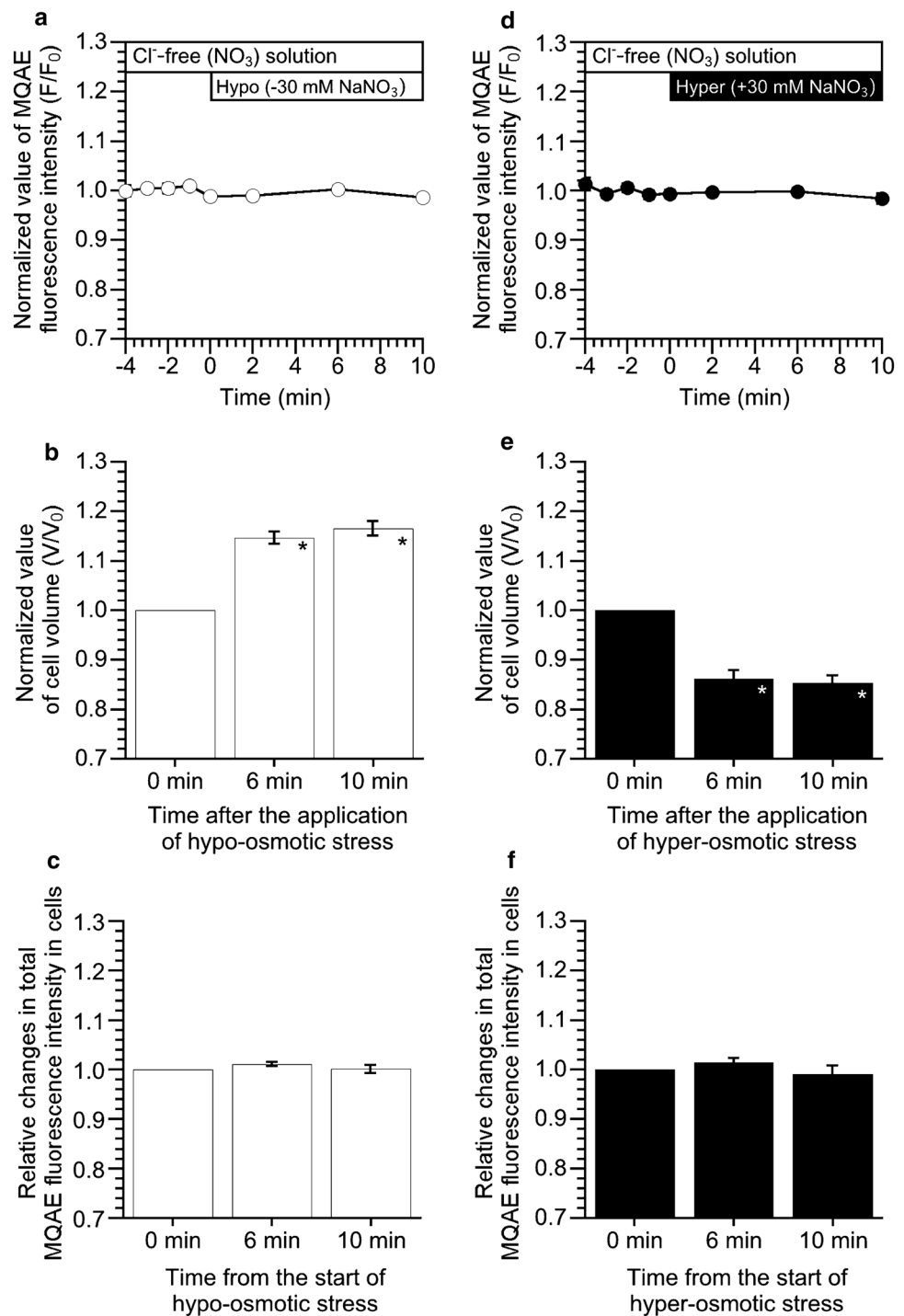


### B Hyper-osmotic stress ( $+30 \text{ mM NaNO}_3$ )



stress also did not change the intensity of MQAE fluorescence ( $F/F_0$  10 min after exposure to the hyper-osmotic stress =  $0.98 \pm 0.01$ ;  $n = 4$ ) (Fig. 2d), although it decreased cell volume ( $V/V_0$  10 min after exposure to the hyper-osmotic stress =  $0.85 \pm 0.01$ ;  $n = 4$ ) (Fig. 2e). Thus, a decrease in the cell volume ( $V/V_0$ ) also did not induce any change in MQAE fluorescence intensity ( $F/F_0$ ) despite the expectation that it would increase  $F/F_0$  due to an increase in the MQAE concentration. We also calculated the total MQAE fluorescence intensity in the cells

**Fig. 2** Changes in MQAE fluorescence and cell volume in airway ciliary cells in the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution induced by osmotic stresses. Cells were pre-treated with the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution for 20 min prior to the exposure to osmotic stress. To achieve hypo- or hyper-osmotic stress state,  $\text{NaNO}_3$  (30 mM) was removed from or added to the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution. **a–c** Hypo-osmotic stress (–30 mM  $\text{NaNO}_3$ ). Application of hypo-osmotic stress (–30 mM  $\text{NaNO}_3$ ) did not change the normalized value of MQAE fluorescence intensity ( $F/F_0$ ) (**a**), while it significantly decreased the normalized value of the cell volume ( $V/V_0$ ) (\*significant difference at  $p < 0.05$ ; **b**). Total MQAE fluorescence intensities were not changed by exposure to hypo-osmotic stress (**c**). **d–f** Hyper-osmotic stress (+30 mM  $\text{NaNO}_3$ ). Hyper-osmotic stress did not change the normalized MQAE fluorescence ( $F/F_0$ ) (**d**), while it significantly increased the normalized value of the cell volume ( $V/V_0$ ) (\*Significant difference at  $p < 0.05$ ; **e**). Total MQAE fluorescence intensities were not changed by the hyper-osmotic stress (**f**). Symbols in **a** and **d** represent the mean; bars and whiskers in **b**, **c**, **e**, **f** are the mean and standard error of the mean (SEM), respectively



and found that the total MQAE fluorescence intensity was also not changed by exposure to the hyper-osmotic stress (Fig. 2f). The normalized total MQAE fluorescence intensity 10 min after the application of hyper-osmotic stress was  $0.99 \pm 0.02$  ( $n = 4$ ). That the total MQAE fluorescence intensity remained unchanged even during application of the hyper- and hypo-osmotic stresses suggests that the leakage of MQAE from the cells or the quenching of

MQAE by the laser beam was negligibly small during the experimental time period.

### Osmotic stresses under the $\text{Cl}^-$ -containing condition

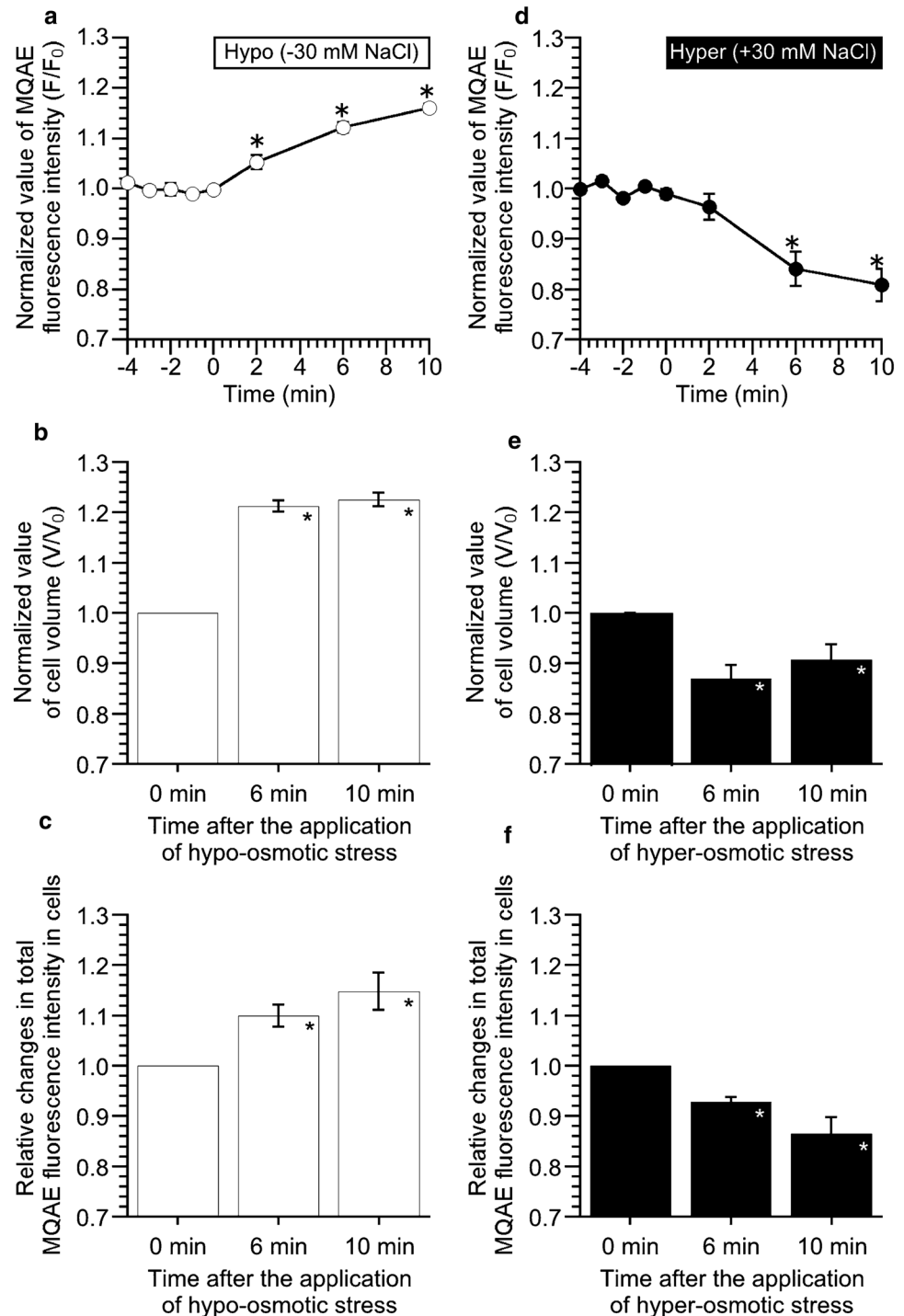
Osmotic stress was also applied to cells in the  $\text{Cl}^-$ -containing solution (Fig. 3) by removing 30 mM  $\text{NaCl}$  from (hypo-osmotic stress) or adding 30 mM  $\text{NaCl}$  to (hyper-osmotic

stress) the  $\text{Cl}^-$ -containing control solution. The effects of the hypo-osmotic stress ( $-30$  mM NaCl) on MQAE fluorescence intensity and cell volume are shown in Fig. 3a–c. Exposure of the cells to hypo-osmotic stress increased MQAE fluorescence intensity ( $F/F_0$ ) and cell volume ( $V/V_0$ ) (Fig. 3a, b). The  $F/F_0$  at 10 min after the hypo-osmotic stress was  $1.16 \pm 0.01$  ( $n = 4$ ) (Fig. 3a), and the  $V/V_0$  10 min after the hypo-osmotic stress was  $1.19 \pm 0.02$  ( $n = 4$ ) (Fig. 3b).

Relative changes in the total MQAE fluorescence intensity in cells are shown in Fig. 3c. The total MQAE fluorescence intensity significantly increased ( $p < 0.05$ ), and the normalized value at 10 min after the application of the hypo-osmotic stress was  $1.15 \pm 0.02$  ( $n = 4$ ).

The effects of hyper-osmotic stress ( $+30$  mM NaCl) on MQAE fluorescence intensity and cell volume are shown in Fig. 3d–f. The application of hyper-osmotic stress decreased

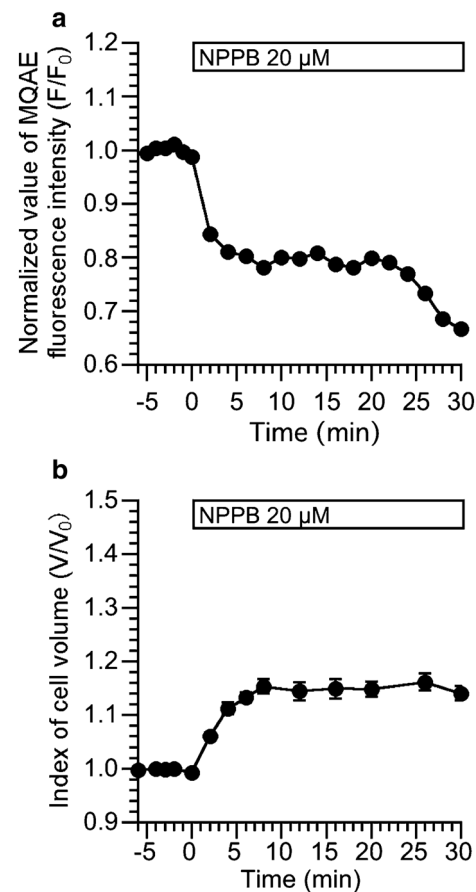
**Fig. 3** Changes in MQAE fluorescence and cell volume induced by osmotic stresses in the  $\text{Cl}^-$ -containing control solution. The hypo- or hyper-osmotic stress was applied by removing NaCl (30 mM) from or adding it to the  $\text{Cl}^-$ -containing control solution, respectively. **a–c** Hypo-osmotic stress ( $-30$  mM NaCl). **a** Changes in MQAE fluorescence intensity normalized by the value before the application of the hypotonic stress ( $F/F_0$ ). The application of hypo-osmotic stress increased the  $F/F_0$ , unlike its effect in the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution (\*significantly different at  $p < 0.05$ ; unpaired Student's  $t$  test). **b** Increases in cell volume normalized by the value before the application of the hypotonic stress ( $V/V_0$ ). The hypo-osmotic stress increased the  $V/V_0$  (\*significantly different at  $p < 0.05$ ; paired Student's  $t$  test). **c** Relative changes in total MQAE fluorescence intensity. The hypo-osmotic stress increased the total MQAE fluorescence intensity, unlike its effect in the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution (\*significantly different at  $p < 0.05$ ; paired Student's  $t$  test). **d–f** Hyper-osmotic stress ( $+30$  mM NaCl). **d** The application of hyper-osmotic stress decreased the  $F/F_0$  (\*significantly different at  $p < 0.05$ ). **e** Hyper-osmotic stress decreased the  $V/V_0$  (\*significantly different at  $p < 0.05$ ). **f** Hyper-osmotic stress decreased the total MQAE fluorescence intensity (\*significantly different at  $p < 0.05$ ). Symbols in **a** and **d** represent the mean; bars and whiskers in **b**, **c**, **e**, **f** are the mean and SEM, respectively



the intensity of the MQAE fluorescence and decreased the cell volume (Fig. 3d, e). The  $F/F_0$  10 min after the hyper-osmotic stress was  $0.81 \pm 0.03$  ( $n = 4$ ) (Fig. 3d), and the  $V/V_0$  10 min after the hyper-osmotic stress was  $0.80 \pm 0.05$  ( $n = 4$ ) (Fig. 3e). Relative changes in the total MQAE fluorescence intensity in cells are shown in Fig. 3f. The total MQAE fluorescence intensity significantly decreased ( $p < 0.05$ ), and the normalized value 10 min after the application of the hyper-osmotic stress was  $0.86 \pm 0.03$  ( $n = 4$ ). As confirmed for cells in the  $\text{Cl}^-$ -free  $\text{NO}_3^-$  solution, the leakage of MQAE from the cells or the quenching of MQAE by the laser beam was negligibly small during the experimental time period. These observations indicate that the total MQAE fluorescence intensity depends solely on the  $[\text{Cl}^-]_i$ , since the total MQAE content was kept constant. In the experiments performed in the  $\text{Cl}^-$ -containing control solution, the changes in  $F/F_0$  detected in the local fixed areas of cells were similar to those in the total MQAE fluorescence intensity in cells, meaning that the MQAE fluorescence intensity observed at the local fixed area ( $F/F_0$ ) indicates the  $[\text{Cl}^-]_i$ , but that it is not affected by the osmotic stress-induced cell volume change. Based on these results, we conclude that the change in the MQAE fluorescence intensity observed at the local fixed area ( $F/F_0$ ) can be an indicator for the change in  $[\text{Cl}^-]_i$ .

### Decrease in MQAE fluorescence intensity

Figure 4a shows a typical case of decreasing MQAE fluorescence intensity in the airway ciliary cell. In this case, the experiments were carried out under the iso-osmotic condition. The airway ciliary cells were treated with NPPB ( $20 \mu\text{M}$ ), a  $\text{Cl}^-$  channel blocker, to increase cell volume. The addition of NPPB immediately decreased MQAE fluorescence intensity and it plateaued within 5 min; this decrease indicates that the addition of NPPB increased  $[\text{Cl}^-]_i$  coupled with the cell swelling [18]. However, the MQAE fluorescence intensity suddenly decreased 20 min after the addition of NPPB (second decrease in MQAE fluorescence intensity). The value of  $F/F_0$  30 min after the addition of NPPB was 0.67. We also measured cell volume ( $V/V_0$ ) using the same protocol. Following the addition of NPPB, the  $V/V_0$  immediately increased and plateaued (Fig. 4b). The values of  $V/V_0$  at 5 and 30 min after the NPPB addition were  $1.13 \pm 0.01$  and  $1.14 \pm 0.01$  ( $n = 11$ ), respectively. No further cell swelling during NPPB addition was noted. Although, in this case the sudden decrease in MQAE fluorescence intensity was detected at 20 min after the NPPB addition, such sudden decreases in MQAE fluorescence intensity were noted to occur on occasion during a long exposure to agonists, blockers or osmotic stresses changing the cell volume for periods longer than 30 min. The second decrease in MQAE fluorescence intensity appears to have been caused by the



**Fig. 4** **a** A typical case showing the decrease in MQAE fluorescence intensity induced by leakage. The airway ciliary cells were treated with NPPB ( $20 \mu\text{M}$ ), a  $\text{Cl}^-$  channels blocker, to increase cell volume under the iso-osmotic condition. The addition of NPPB immediately decreased MQAE fluorescence intensity, which reached a plateau within 5 min. However, 15 min after the plateau had been reached, MQAE fluorescence intensity suddenly decreased. This sudden decrease in MQAE fluorescence intensity started at 22 min after addition of the NPPB addition. **b** Cell swelling induced by NPPB. The addition of NPPB immediately caused an increase and plateau in cell volume ( $V/V_0$ ). No further cell swelling was occurred during NPPB addition

leakage of MQAE from the airway ciliary cell because no further cell swelling occurred (indicating no further increase in  $[\text{Cl}^-]_i$ ).

### Discussion

The results of our study demonstrate that changes in the volume of MQAE-loaded mouse airway ciliary cells had little effect on the intensity of MQAE fluorescence measured in the local area of these cells. These findings indicate the MQAE is applicable to measure the  $[\text{Cl}^-]_i$  in the airway ciliary cells under conditions of changing cell volume. Moreover, as shown in a previous study, the use of

a combined MQAE and two-photon confocal microscopy protocol allows for the continuous measurement of  $[Cl^-]_i$  with minimal photochemical damage [12, 16]. Moreover, the  $[Cl^-]_i$ -detecting method using MQAE is easily available without special equipment.

However, there are some limitations to MQAE-based  $[Cl^-]_i$  measurements. These include: (1) the selection of the local region in the intracellular space (the specific area of the identical optical section throughout the experiment); (2) the short time for measuring  $[Cl^-]_i$  (< 30 min) and (3) a small cell-volume change which does not alter the subcellular structures and MQAE binding to the subcellular structure. To measure MQAE fluorescence present in the selected local region of the intracellular space throughout the experiment, the focal plane is frequently adjusted to obtain the same focal plane. In the experiments applying a hypo-osmotic stress or NPPB (a  $Cl^-$  channel blocker), the long exposure times, such as > 20–30 min, sometimes resulted in decreased MQAE fluorescence intensity (unpublished observations), possibly due to the leakage of MQAE from the intracellular space and/or the quenching of the dye. Despite of these limitations, the use of MQAE in combination with two-photon confocal microscopy is of importance for measuring the dynamic changes in  $[Cl^-]_i$  in airway ciliary cells, since intracellular  $Cl^-$  plays crucial roles in regulation of ciliary beating [8, 22, 23].

In our study, the intensity of MQAE fluorescence was heterogeneously distributed throughout the cell body of the MQAE-loaded airway ciliary cells. Similar heterogeneous distributions of MQAE fluorescence intensities have previously been shown in the MQAE-stained neurons of brain slices [13, 16], neural cells [4] and salivary acinar cells [19]. The heterogeneity in MQAE fluorescence distribution in the intracellular space suggests that MQAE molecules are bound to the unidentified subcellular structures [14]. However, MQAE molecules, even the bound forms, would be easily accessible to  $Cl^-$  [16]. The binding of MQAE to subcellular structures is a well-known characteristic of methoxyquinoline compounds, including MQAE [14].

Another limitation in MQAE fluorescence measurements is leakage of the dye from the intracellular space. The leakage rate depends on the preparation (ranging from 3% per hour in liposomes [17] to 30% per hour in brain slices [16]) and is also affected by the experimental procedure, such as a large hypo-osmotic stress. Miyazaki et al. have shown that the leakage rate is 30% per 30 min in renal A6 cells upon application of 50% hypo-osmotic stress [2]. However, the MQAE leakage rate of airway ciliary cells in our study, even during changes in cell volume induced by the mild osmotic stress, appeared to be low, similar to that of liposomes.

Based on the results of this study, we conclude that MQAE-based two-photon confocal microscopy is a useful tool to measure the dynamic changes in  $[Cl^-]_i$  in living

airway ciliary cells even under conditions of changing cell volume caused by the mild osmotic stress and by various agonists activating ion channels and transporters, and their inhibitors.

**Acknowledgements** This work is partly supported by Grants-in Aid for Scientific Research from the Japan Society of the Promotion of Science to SH and TN (No. 17K08545).

## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** The procedures and protocols for the experiments were approved by the Animal Research Committee of Kyoto Prefectural University of Medicine (No. 26-263), and the experiments were carried out in accordance with the guidelines of this committee, which are identical to those of the Physiological Society of Japan.

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