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Measurement of Cell Volume Changes by Fluorescence Self-Quenching

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At high concentrations, certain fluorophores undergo self-quenching, i.e., fluorescence intensity decreases with increasing fluorophore concentration. Accordingly, the self-quenching properties can be used for measuring water volume changes in lipid vesicles. In cells, quantitative determination of water transport using fluorescence self-quenching has been complicated by the requirement of relatively high (m*M*) and often toxic loading concentrations. Here we report a simple method that uses low (μM) loading concentrations of calcein-acetoxymethyl ester (calcein-AM) to obtain intracellular concentrations of the fluorophore calcein suitable for measurement of changes in cell water volume by self-quenching. The relationship between calcein fluorescence intensity, when excited at 490 nm (its excitation maximum), and calcein concentration was investigated *in vitro* and in various cultured cell types. The relationship was bell-shaped, with the negative slope in the concentration range where the fluorophore undergoes fluorescence self-quenching. In cultured retinal pigment epithelial cells, calcein fluorescence and extracellular osmolarity were linearly related. A 25-mOsm hypertonic challenge corresponded to a decrease in calcein fluorescence with high signal-to-noise ratio ($>$15). Similar results were obtained with the fluorophore BCECF when excited at its isosbestic wavelength (436 nm). The present results demonstrate the usefulness of fluorescence self-quenching to measure rapid changes in cell water volume.

KEY WORDS: Water transport; cell volume; calcein; self-quenching; epithelial cells.

INTRODUCTION

Fluorophore self-quenching occurs even in simple dye solutions and has been known since 1888, when Walter observed that fluorescence increased with dilution of solutions of fluorescein, Magdala red, or eosin [1,2]. Walter proposed that "aggregation" was the cause of quenching, and that fluorescence arose only from single molecules (monomers) [3]. However, the mechanisms of self-quenching are still not entirely understood. It appears that the mechanism is a combination of dimerization of the dye, energy transfer to non-fluorescent dimers, and collisional quenching interactions between dye monomers [4]. Self-quenching of entrapped fluorophores has been used as a marker of water volume in liposomes and vesicles [5–9]. Self-quenching occurs at relatively high (m*M*) concentrations [5], and it has been considered impossible to load fluorescent indicators into living cells at high-enough concentrations for fluorescence self-quenching to take place [10].

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Measurement of water transport across cell membranes, with high temporal resolution and sensitivity, is essential for determining osmotic water permeability and changes in cell volume associated with stimulation or inhibition of ion transport mechanisms under isotonic conditions. Various invasive and non-invasive techniques have been employed to study cellular volume changes (for reviews see [10,11]). Most of these techniques measure the total cell volume, which is the sum of cell water volume (the osmotically active volume fraction) and the volume of the cell solids (the inert volume fraction). Among the non-invasive techniques, the use of fluorescent dye-dilution, based on an inversely proportional relationship between relative fluorescence intensity and relative cell water volume, provides excellent sensitivity for measuring volume changes in single cells [12].

It would be useful to have a non-invasive technique for measuring rapid changes in cell water volume in confluent epithelia or tissue slices to study the osmotic behavior of cell populations. In confluent epithelial cells, the "spatial filtering technique" [13] has proved to be suitable in terms of sensitivity and time resolution to measure rapid cell volume changes. However accurate, this technique is relatively difficult to employ and may not work in all systems [10]. Recently, studies based on decrease of fluorescence with cell shrinkage, have been presented in cultured confluent monolayers of hepatocytes [14–16], as well as in isolated dogfish rectal gland tubules [17]. The directly proportional relationship between fluorescence intensity and cell volume changes was interpreted as a result of fluorescence self-quenching [14–16]. However, in all these studies, few methodological considerations were presented and the method was not validated.

In the present study, we investigated in detail the relationship between the emitted fluorescence and the concentration of the water-soluble fluorescent dye calcein, in free solution and inside cells. A simple method based on calcein self-quenching to measure relative changes in cell water volume is described and validated.

EXPERIMENTAL

Sheets of porcine retinal pigment epithelial (RPE) cells were isolated from freshly enucleated eyes of specific pathogen free (SPF) Danish landrace pigs (4 months old, 30 kg). The sclera was carefully removed from the eyes, leaving the uvea intact. Hereafter the eyes were incubated for 60 min at 37°C with 2 mg/ml collagenase (Sigma, USA) dissolved in culture medium (DME-H16, Life Technologies, Taastrup, Denmark). The uvea/Bruch's membrane/RPE-complex was then isolated, and RPE could be removed as small sheets by the water-jet method. Contamination of choroidal cells was avoided by washing the cells in calcium-magnesium–free phosphate-buffered saline (CMF-PBS). The isolated sheets were then seeded directly into Lab-Tek 8-well chambered coverglasses (CCGs) (Life Technologies) coated with 10 μg/cm^2 mouse laminin (Beckton Dickinson, Broendby, Denmark). The culture medium was DME H16 supplemented with 15% fetal calf serum (FCS), 300 μg/ml glutamine, 50 μg/ml gentamicin, and 2.5 μg/ml fungizone, all from Life Technologies. The media were changed every second day. Cells were used for experiments between 2 and 14 days after plating.

For microinjection experiments, confluent plates of primary RPE cells were passaged and plated onto glass cover slips at about 0.5×10^3 cells/cm^2. Microinjection was performed in an Eppendorf micromanipulator 5171 and transjector 5246 system mounted on a Leica DMIRBE inverted research microscope. Microcapillaries (borosilicate with filament, catalogue no. BF120-94-10, Sutter Instruments Company, Novato, California, USA) were pulled to an outer diameter of 0.25 μm on a Sutter P-97 Micropipette Puller. The dye-loaded cells were visualized by excitation at 470 nm (±20 nm) and recorded at 509-nm emission using Haupage version 3.3.18038 software and Kappa CF 15/4 MC-S camera (Leica).

For non–steady-state experiments, CCGs with primary cultures of RPE cells were mounted in a customized chamber on the stage of a Zeiss Axiovert 10 inverted microscope (Zeiss, Oberkochen, Germany). The chamber allowed perfusion only of the retinal side of the monolayer. Solutions were fed into the chamber via a Minipuls 3 peristaltic pump (Gilson, Villiers-le-Bel, France) and collected by aspiration. The rate of perfusion was adjusted so that the volume of the chamber (600 μL) was exchanged in 15 s. The excitation light source was a 75-W xenon-arc lamp (Osram, Berlin, Germany). The light was directed through a 490-nm (±5 nm) excitation filter (Zeiss). Light pulses lasting less than 100 ms were delivered at a frequency of 1 Hz. To this end, we used a fast shutter that was closed between the excitation pulses to reduce photobleaching and photodynamic damage. To further reduce exposure of cells to UV light, a neutral density filter was used that transmitted only 3% of the light. In addition, an iris diaphragm confined the illuminated field to a light spot. A 40× oil immersion objective (Nikon) with a relatively shallow depth of field (N.A. 1.3) was used in these experiments. The fluorescence emission was measured between 503 and 530 nm using a photomultiplier tube (Zeiss), and the output signals were stored in a computer.

Control solution contained (in m*M*): 119 Na^+, 5 K^+, 0.8 Mg^{2+}, 1.8 Ca^{2+}, 114 Cl^-, 0.8 SO_4^{2+}, 25 HEPES, 5.6 glucose, and 44 mannitol. Anisosmotic solutions were prepared by mannitol addition or removal to obtain the desired osmolality, thus maintaining the ionic strength. The salts were of analytical quality, and the osmolarities were checked by a freezing point depression osmometer (Labex-Roebling). All solutions were titrated to pH 7.4 and bubbled with atmospheric air.

All experiments were performed at room temperature. 2′,7′-bis (2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethylester (BCECF-AM), calcein-AM, and calcein (free acid) were obtained from Molecular Probes (Leiden, The Netherlands). Results are expressed as mean ± SEM. Unless otherwise stated, the number of observations given in the parentheses (*n*) is the number of experiments. The number of cultures tested in all cases was larger than three.

RESULTS AND DISCUSSION

Relationship Between Fluorescence and Concentration of Dye in Solution

To assess the self-quenching properties of calcein *in vitro*, the fluorescence of control solution containing various concentrations of free calcein was determined using the described experimental setup for non–steady-state experiments. The relation between fluorescence intensity vs. concentration of calcein was linear in the range 0–2 m*M*, peaked at 3.1 ± 0.4 m*M* ($n = 11$) and then decreased, reaching 50% maximum fluorescence at 8.4 ± 0.9 m*M* ($n = 11$). An example is shown in Fig. 1. A similar bell-shaped relationship was observed in microtiter plate experiments in a fluorometer (not shown). In the range between 6 and 10 m*M* the slope of the descending part of the curve was steepest. Hence, this is taken as the calcein concentrations for optimal dynamic range for measurement of changes in cell water volume by fluorescence self-quenching. With our present optical setup, using an estimated total volume of a confluent RPE monolayer of 400 nL (3.2×10^6 cubic cells with cell height 5 μ*M*), it can be calculated that a low external loading concentration of 5 μ*M* (i.e., 3 nmol/600 μL loading solution) corresponds to an internal concentration of calcein of 7.5 m*M*, provided that all dye is cleaved, retained, and accumulated. This rough estimate shows that even if the cell height changes ±50%, the calculated intracellular dye concentration will still be within the linear part of the negative slope segment of the bell-shaped relation shown in Fig. 1.

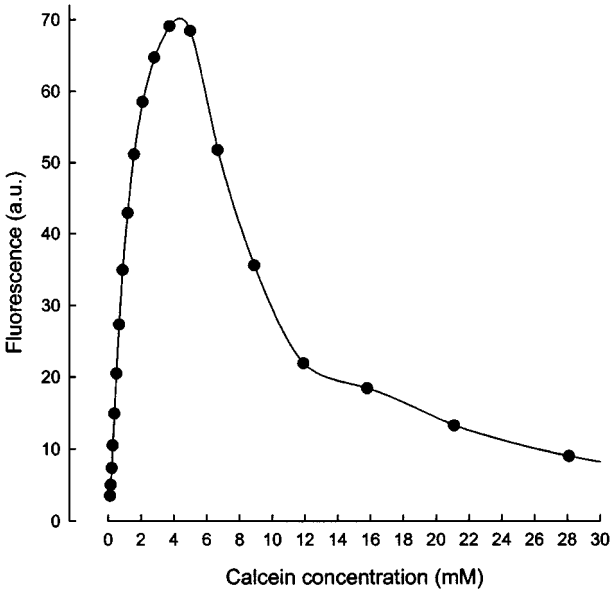

Fig. 1. Fluorescence intensity plotted as a function of calcein concentration. A 50-m*M* solution of calcein dissolved in control solution was prepared. The solution was stepwise diluted using control solution and maintaining a constant fluid volume in the chamber. Fluorescence was monitored using the same experimental setup as that employed for experiments with cells. The positive slope of the bell-shaped curve indicates that the fluorescence increases with fluorophore concentration up to 4 m*M*. Then, fluorescence intensity decreases with increase in calcein concentration, yielding the negative slope of the bell-shaped relation. The negative slope is due to calcein self-quenching.

A similar bell-shaped relationship with a maximum fluorescence intensity at about 2.3 m*M* calcein was also obtained when calcein was diluted in H_2O instead of control solution. This indicates that the quenching observed in Fig. 1 is not affected by the ionic composition and ionic strength of the control solution.

Relationship Between Fluorescence and Concentration of Dye in Cells

The relationship between fluorescence and intracellular dye concentration was investigated by microinjecting calcein (free acid) into the cytoplasm of single first-passage RPE cells. By stepwise increases in intracellular calcein concentration, the bell-shaped relationship in free solutions (Fig. 1) could be reproduced in a living cell (Fig. 2). No leakage of dye into the extracellular space was observed in the time scale of the microinjection experiments (seconds). Because of the variability in size between subconfluent RPE cells, it was not possible to provide a quantitative estimate of the concentration at which the intracellular self-quenching occurred. For large compared to smaller cells, however, it was necessary to

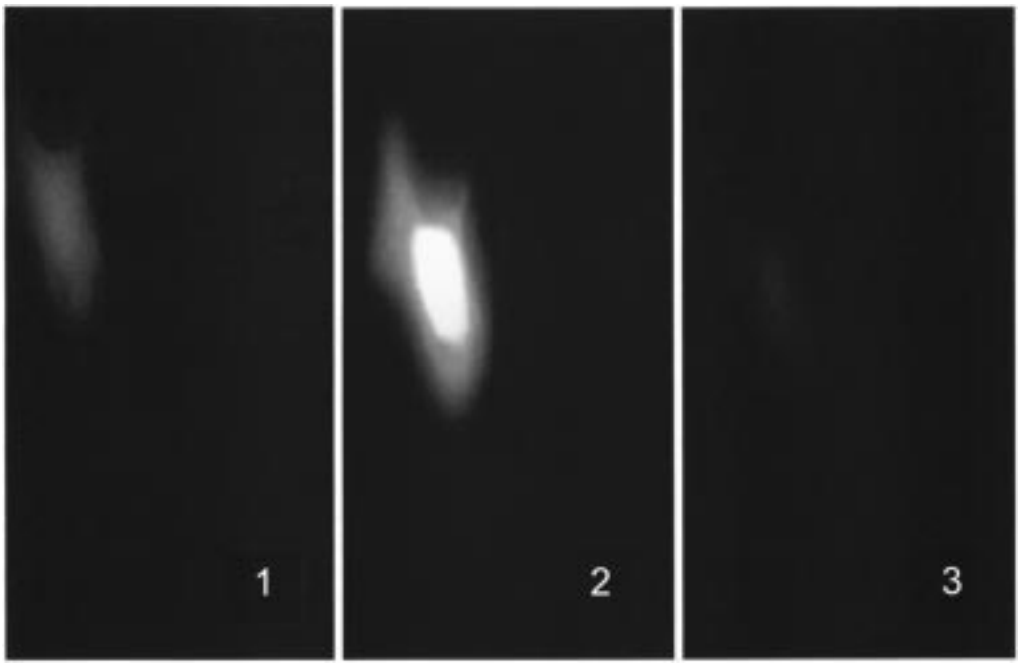

Fig. 2. Relationship between fluorescence intensity and intracellular concentration of calcein. Calcein was introduced into a single RPE cell by microinjection. 15 fl of a 20-m*M* calcein stock solution was injected before picture 1, another 15 fl between pictures 1 and 2, and yet another 15 fl between pictures 2 and 3. A bell-shaped relationship is observed.

inject several times before self-quenching was observed, in agreement with the theoretical assumptions underlying Fig. 1.

For non–steady-state experiments on whole epithelia, the calcein loading procedure was almost identical to the one described by Alvarez-Leefmans *et al.* [11]. Briefly, cells were incubated at room temperature in control solution containing 2–5 μM calcein-AM. Inside the cells, esterases cleave off the acetoxymethyl groups, producing the membrane-impermeant fluorescent dye calcein. After 40–60 minutes, the loading solution was washed out with control solution for at least 1 h before starting the experiments. The loading and subsequent washout was recorded continuously; one example is shown in Fig. 3. A bell-shaped curve is seen, in which the positive slope of the ascending limb corresponds to the increase in dye concentration and the negative slope of the descending limb corresponds to a combination of photobleaching, calcein self-quenching, and leakage/pumping of dye. Photobleaching is probably of minor importance, because the cells were dye-loaded only during brief (<100 ms) light exposures once every 30 s, and the excitation light passed through a 3% neutral density filter. Calcein only leaks out of cells very slowly, as confirmed by confocal microscopy (not shown) and in agreement with observations in other cell types [18]. The estimated intracellular concentration of calcein is within the region where self-quenching occurs (see above). Therefore, the majority of the negative slope could be due to (cell volume-independent) calcein self-quenching. A similar bell-shaped curve was also noted for calcein loading and washout in cultured human fetal pigmented ciliary epithelial cells (not shown).

Correction for Drift and Relative Background

Before transforming the fluorescence signals into relative changes in cell water volume, it is necessary to correct for two effects: drift, caused by cell water volume independent reduction in fluorescence intensity, and relative background, a result of the fraction of intracellular fluorescence insensitivity to changes in external osmolarity. The measured fluorescence is corrected for drift as described by Muallem *et al.* [19]. Briefly, when a fluorophore is excited intermittently and the fluorescence is monitored over time, the fluorescence gradually decreases. This drift follows a single exponential course. The rate constant of this curve, however, varies between experiments, and therefore it is determined for every experiment 3–5 min of fluorescence intensity recording at the excitation maximum before the first solution

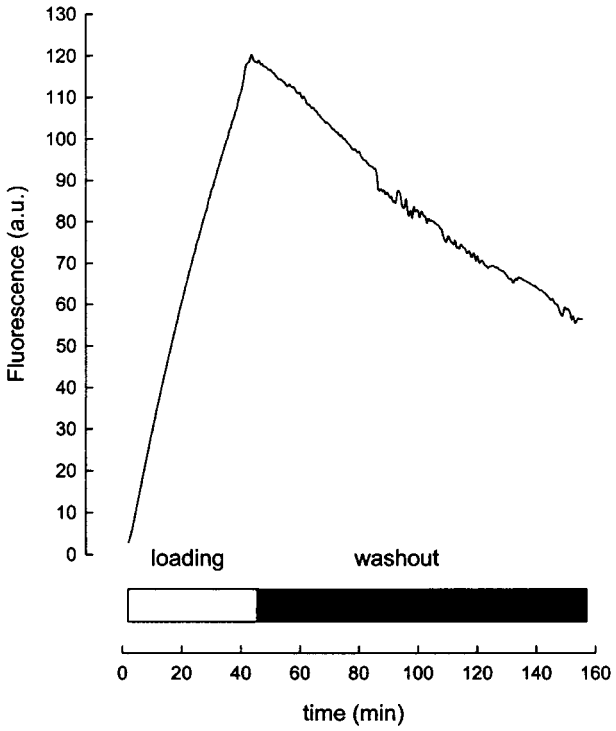

Fig. 3. Fluorescence intensity recorded during calcein loading and washout. A confluent layer of RPE cells was loaded with 2 μM calcein-AM for about 40 min and subsequently superfused with control solution to washout extracellular dye. Fluorescence was recorded every 30 s at 490-nm excitation. During the loading phase the fluorescence increases as a result of intracellular conversion of non-fluorescent calcein-AM to fluorescent calcein. During the washout phase the fluorescence decreases mainly because of cell volume-independent calcein self-quenching (see text).

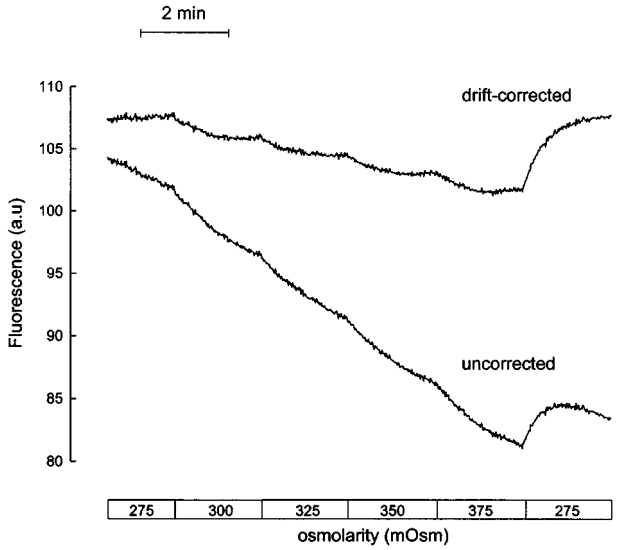

Fig. 4. Relationship between external osmolarity and fluorescence intensity recorded in a confluent monolayer of RPE cells loaded with calcein. The epithelium was superfused initially with a control solution of 275 mOsm, and then the osmolarity was changed as indicated by adding or removing mannitol. The fluorescence intensity was continuously recorded at 490-nm excitation (lower trace). From this curve, the rate constant of volume-independent reduction in fluorescence intensity (drift) was estimated from the values before the first solution change and after the last solution change, and used to obtain the volume-dependent change in fluorescence intensity (upper trace).

change, or, for long series of experiments, for individual segments between experimental challenges.

The relation between relative (drift corrected) fluorescence and external osmolarity of calcein-loaded cells is characterized as follows. At the beginning of the experiment, the cells are exposed to hypoosmotic and hyperosmotic solutions or to a series of hyperosmotic challenges (Fig. 4). The drift-corrected steady-state fluorescence change (F_t/F_0) is then plotted as a function of the reciprocal of the relative osmotic pressure of the superfusion solution (π_0/π_t). This yields a linear relation (Fig. 5, solid line), indicating that the change in fluorescence reflects changes in calcein self-quenching resulting from changes in cell water volume. The slope of a regression line fitted to the data points is less than 1, which is the slope expected for ideal osmometric behavior (Fig. 5, dashed line), according to the equation

$$F_t/F_0 = (\pi_0/\pi_t) \quad (1)$$

where F_0 is the fluorescence of the cells in control solution having an osmotic pressure π_0, and F_t is the fluorescence from the same region in equilibrium with a solution having an osmotic pressure π_t. The departure from ideal behavior is due to a fraction of intracellular calcein that is insensitive to changes in external osmolarity; the fluorescence from this dye fraction (the relative background, f_b) has to be subtracted. It is reasonable to assume that this parameter corresponds to the *y* intercept of a plot of F_t/F_0 versus π_0/π_t, which is the relative fluorescence when no osmolarity change is performed. f_b ranged from 0.5–0.9; i.e., 50–90% of the intracellular dye seems to be insensitive to changes in external osmolarity. The possible reasons for this behavior have been previously discussed [11,12]. Because of the large variation in the value of f_b, it has to be assessed and subtracted in each experiment. It should be noted that when subtracting the f_b one also corrects for any autofluorescence that the cells might exhibit.

Calculation of Water Volume Changes

The change in cell water volume can now be calculated as

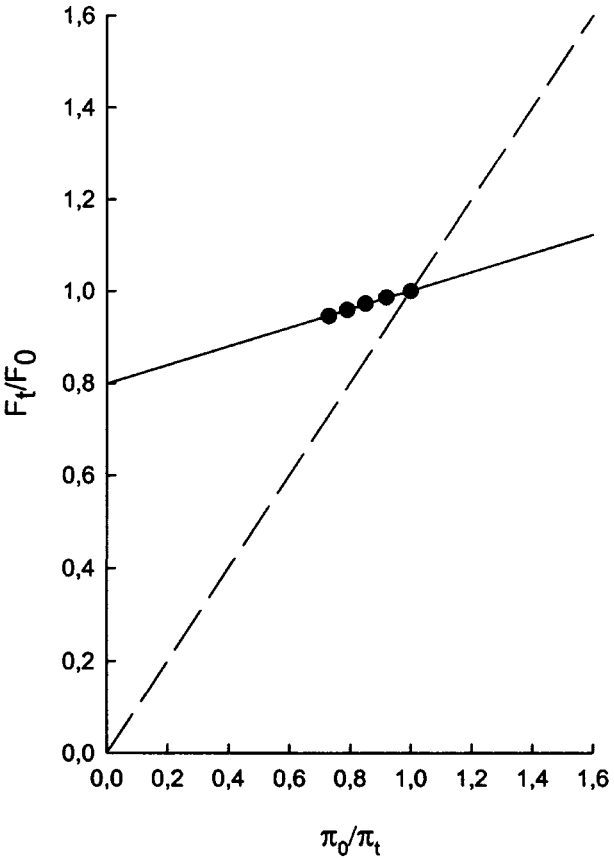

Fig. 5. Relationship between the drift-corrected changes in fluorescence (F_t/F_0) and the reciprocal of the relative osmotic pressure of the bathing solution (π_0/π_t). F_t is the fluorescence in steady-state in a solution of osmotic pressure, π_t. F_0 is the fluorescence of an isosmotic solution having an osmotic pressure π_0. Values were obtained from the experiment shown in Fig. 4. The slope of the solid line through the circles was 0.2. The slope expected for ideal osmometric behavior according to Eq. (1) is 1 (dashed line). The *y* intercept of the solid line is the relative background fluorescence, f_b (see text).

$$V_tV_0 = [(F_t/F_0) - f_b]/(1 - f_b) \quad (2)$$

where V_t is the epithelial cell water volume at time *t* and V_0 is V_t when $t = 0$. Eq. (2) yields the calibration curve to be used for converting the fluorescence changes into relative changes in cell water volume (Fig. 6).

Given the relationship between fluorescence intensity and cell water volume, we can now plot the relative changes in cell water volume as a function of the external osmolarity (Fig. 7). In the example we see that a change in external osmolarity from 275 to 300 mOsm (i.e., a 9.1% change) corresponds to a change in fluorescence from 107.6 (F_0) to 105.8 (F_t in steady state) (Fig. 4) and an f_b of 0.8 (Fig. 5). Inserting these parameters in Eq. (2) yields a $V_t/V_0 = 8.4\%$, (Fig. 7), which is very close to the value predicted from cells exhibiting ideal osmometric

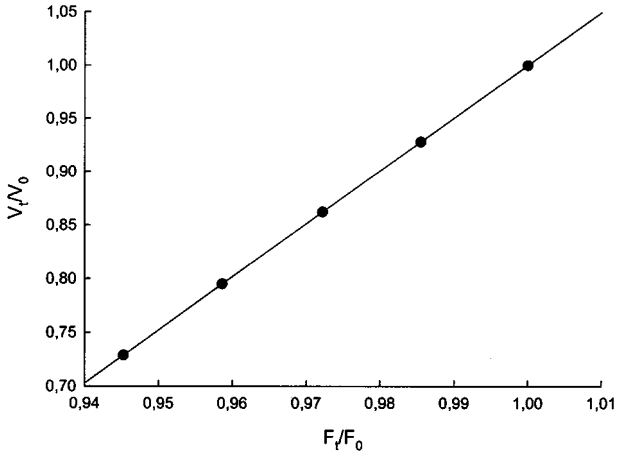

Fig. 6. Relationship between relative changes in fluorescence intensity (F_t/F_0) and relative changes in cell water volume (V_t/V_0). This serves as the calibration curve (see text). Values were obtained from the experiments in Fig. 4.

behavior. If the background fluorescence is not subtracted, the change in calcein fluorescence underestimates the actual volume change [17]. Eqs. (1) and (2) are analogous to those derived for single cells [11].

In addition to the measurement of relative cell water volume changes, it is also possible to calculate the actual volume changes, if the anatomical height (h) of the epithelium is known. The influx of water per square centimeter of epithelium can then be expressed as

$$J_{H_2O} = (h/V) \times dV/dt \quad (3)$$

where *V* is the cell water volume, and *dV*/*dt* is the initial rate of change in cell water volume.

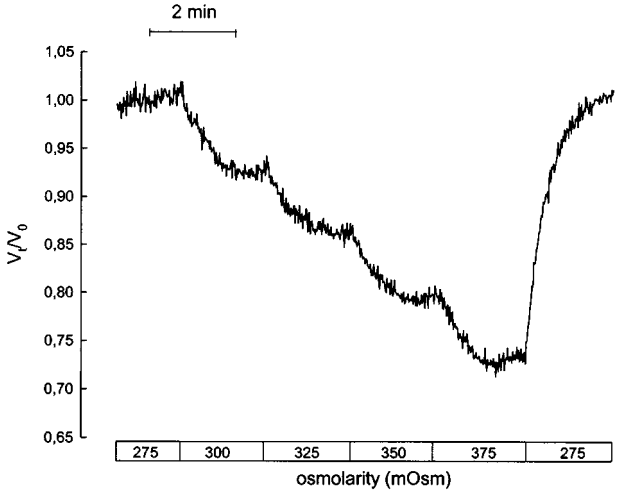

Fig. 7. Relative changes in cell water volume from the experiment in Fig. 4, calculated using Eq. (2).

Each time a calibration with hypoosmotic and a hyperosmotic shock is performed, an osmotic water permeability of the cell membrane can thus be calculated as

$$P_f = J_{H_2O}/\Delta Osm \quad (4)$$

where ΔOsm is the change in osmolarity.

Sensitivity of the Method

For comparison with other methods of cell volume measurement, the sensitivity of the method can be expressed in terms of signal-to-noise ratio (SNR)

$$SNR = \delta\mu_{2-1}/((\sigma_1 + \sigma_2)/2) \quad (5)$$

The magnitude of the change in the signal ($\delta\mu_{2-1}$) is the difference in the mean levels of fluorescence before (μ_1) and after (μ_2) a hyperosmotic shock under steady-state conditions. The noise level $(\sigma_1 + \sigma_2)/2$ is the average of standard deviations of the signal acquired over a short time period before and after the shock under steady-state conditions [20]. To calculate SNR, a 25-mOsm hyperosmotic challenge of mannitol was used. SNRs for RPE cells grown on CCGs were in all cases greater than 15. These values are acceptable for many physiological experiments. Changes in cell water volume were detectable down to 1%, which implies high sensitivity, although the SNR decreased, the smaller the volume change.

The self-quenching technique could also be used with BCECF as the fluorescent probe, recorded at the isosbestic wavelength of 436 nm. One advantage of this probe is that it permits simultaneous measurement of changes in cell water volume and intracellular pH. A disadvantage is that it is two to three times less fluorescent than calcein and therefore gives a lower SNR. Calcein is insensitive to changes in cellular ion concentrations within physiological ranges [5], which makes it suitable for measuring volume changes in ion-substitution experiments. It is also well retained inside cells, leaking very slowly [18].

CONCLUSIONS

In the present study, we have characterized the use of fluorescence self-quenching as a means of measuring changes in cell water volume in confluent epithelial monolayers. The technique is easy to use and applicable for rapid, low-noise measurements of volume changes in small epithelial cells. A quantitative method for transforming the fluorescence signals into relative cell water volume changes is presented. A large fraction of intracellular calcein does not contribute to the observed changes in fluorescence, and the value of this fraction must be subtracted in order not to underestimate the changes in cell water volume.

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