Pendrin Overexpression Affects Cell Volume Recovery, Intracellular pH and Chloride Concentration after Hypotonicity-induced Cell Swelling

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Key Words

Pendrin • Cell volume regulation • RVD • Cl⁻ transport
• Cell volume measurements

Abstract

The pendrin (*SLC26A4* or PDS) gene is responsible, when mutated, for the Pendred syndrome, a recessive disorder characterized by sensorineural hearing loss often accompanied by thyroid dysfunctions. Pendrin protein is an anion exchanger and we focused on a still unexplored function that it might play in view of its importance in the inner ear: Cl⁻ fluxes regulation during cellular volume control. We challenged HEK-293 Phoenix cells over-expressing wild type pendrin (PDS HEK cells) together with the EYFP (Enhanced Yellow Fluorescent Protein) or over-expressing the EYFP alone (control HEK cells) with hypo-osmolar solutions. Taking advantage of the confocal optical sectioning we measured the cell volume. In addition, we determined the intracellular pH and chloride concentration with fluorescent probes (EYFP and seminaphthorhodafluor-5F, SNARF-5F). Consequently, we could estimate simultaneously Cl⁻ fluxes, cellular volume and intracellular pH

variations. Cl⁻ movements markedly differed between PDS and control HEK cells upon hypotonic shock and are accompanied by an attenuation of the swelling induced pH drop in PDS HEK cells. The contemporary measurements of the three variables not yet reported in living cells, allowed to assess a possible influence of pendrin upregulation in volume homeostasis and evidenced its participation to Cl⁻ fluxes.

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Introduction

The pendrin (*SLC26A4*) gene was cloned in 1997 by Everett and collaborators [1]. It is responsible, when mutated, for the recessive disorder known as Pendred Syndrome (PS) characterized by sensorineural hearing loss often accompanied by thyroid dysfunctions. Since its discovery, the role of this anion exchanger (transported

anions: Cl^- , I^- and OH^- , among others) [2, 3] has been functionally associated to different pathologies besides those involving the inner ear and thyroid. Disturbances involving acid base balance [4, 5], blood pressure, and respiration (asthma) have also been linked to SLC26A4 [6, 7]. Pendrin (PDS) is expressed in different tissues but only one isoform is known; its exact function within the various organs where it resides is the focus of much research. In the inner ear, pendrin is involved in pH maintenance of the endolymphatic fluids owing to its function as a $\text{Cl}^-/\text{HCO}_3^-$ exchanger [8]. In *SLC26A4* knock-out (KO) mice, dramatic morphological alterations, such as a severe dilation of the endolymphatic compartment, are observed similarly to that seen radiologically in deaf individuals with PDS mutations [9].

Since pendrin promotes electroneutral anion exchange [10], its participation in osmotic homeostasis could appear negligible. However, considering that pendrin can also exchange OH^- for chloride [3] and that transepithelial fluid movement is associated with chloride transport, it is possible that pendrin may have an active role in cellular and extracellular volume control.

Chloride is fundamental for driving water movements in response to osmotic challenges. Yet, there is little information on variation in its intracellular concentration following osmotic challenges, such as during regulatory volume decrease (RVD), partly owing to technical difficulties in measurement of the intracellular anion concentration during volume variations [11]. Monitoring of the intracellular concentration of ions or dyes can be done with quantitative fluorescence microscopy [12] or ion-sensitive microelectrodes [13].

To investigate the physiology related to cell volume regulation, a reliable technique should allow characterization of cell volume in a proper time scale, with good spatial resolution while retaining cell functionality. Many of the most frequently used methods to evaluate cell volume are based on continuous monitoring of the concentration of intracellularly loaded reagents (previously loaded fluorescent dyes or ions naturally present within the cell) [12, 13]. For example, impedance methods allow fast evaluation of cell volume from large populations of cells, but can only be applied to cells in suspension and of similar shape. Moreover, these measurements give only an average estimation of cell volume [14]. Light microscopy methods, such as video-enhanced contrast optical microscopy [15], light microscopy with spatial filtering [16], and laser

light-scattering systems [17], have been used to estimate cell shape and, consequently cell volume. Currently, one of the most advanced techniques used for estimating cell volume is laser scanning confocal microscopy (LSCM). With this technique, it is possible to reconstruct a three-dimensional model of individual cells, thereby allowing for volume measurements [18].

In this manuscript, we present a simplified confocal microscopy-based procedure to measure cell volume. Moreover, by using the enhanced yellow fluorescent protein (EYFP) and seminaphthorhodafluor-5F (SNARF-5F) pH fluorescent probe, the intracellular chloride ($[\text{Cl}]_{\text{in}}$) concentration and intracellular pH (pH_{in}) values were measured in living cells following hypo-osmotic challenge.

Using this novel method, we show that pendrin has an important role in regulating $[\text{Cl}]_{\text{in}}$ with possible implications on cell volume, thus opening to the hypothesis that the transporter intervenes in the osmotic control of the extracellular environment by influencing transepithelial water fluxes.

Materials and Methods

Cell culture and transfection

Human embryonic kidney (HEK) 293 Phoenix cells (HEK cells) were grown as previously described [19]. For *in vivo* functional studies, HEK cells were seeded on 40 mm Ø glass coverslips and transiently transfected for 48 hours with either wild type pendrin (PDS) cloned into the pIRES2-EYFP vector or the empty plasmid (pIRES2-EYFP, control) using the calcium phosphate method [20].

pIRES2-EYFP contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) between the MCS and the EYFP coding region. This permits both the open reading frame (ORF) of interest (cloned into the MCS) and the EYFP ORF to be translated as separated proteins from a single bicistronic mRNA.

Cell volume and EYFP fluorescence measurements

Transiently transfected HEK cells were perfused in a laminar-flow chamber (FCS2 System, Biopetech, USA) at 0.3 ml/min (room temperature) and Z-stacks were acquired in isotonic (Iso) extracellular solution (in mM: NaCl 90, KCl 5, CaCl_2 2, MgCl_2 2, glucose 5; Mannitol 80, Hepes 10; pH 7.4; 295 mOsm) and in hypotonic extracellular solutions (Hypo, obtained by total mannitol omission or Hypo 40, obtained by 40 mM mannitol omission) at 3.5, 5, 10 or 20 minutes after substitution. In the case of the experiments reported in Fig. 1, 5 μM nigericin (K^+/H^+ ionophore), 5 μM valinomycin (K^+ ionophore) and 10 μM tributyltin (halide/ OH^- ionophore) were added to the extracellular solutions to clamp K^+ , Cl^- and H^+ intracellular concentrations to those of the external solutions: (i.e. $\text{K}^+ = 5$ mM, $\text{Cl}^- = 103$ mM, pH = 7.4). The hypotonic

shock was obtained by exposing the cells to Hypo or Hypo 40 for 5 min.

Z-stacks were acquired at a pixel size of 465 nm and constant Z-step size of 500 nm. EYFP fluorescence was excited at 514 nm and imaged between 525 and 600 nm with a confocal microscope (TCS SP2, Leica Microsystems GmbH, Wetzlar, Germany).

The method applied for measuring cell volume relies on the possibility to automatically evaluate the area of the different optical cell sections using EYFP as the fluorescent marker of the entire cell. The analysis was performed using the “analyze particle” plug-in of ImageJ (v.1.45) software (<http://rsbweb.nih.gov/ij/download.html>) imposing a threshold above the background fluorescence.

Since the distance from one section to the other is constant and known, the Cavalieri principle [21] (i.e. adding all section areas multiplied by the constant height on the z axis) was applied, and the cell (or cluster of cells) volume was calculated with the following equation:

$$V_{\text{cell}} = \sum_1^n z(x,y) \times \text{section area}$$

where n is the number of scanned sections, z(x,y) is the constant height of each section scanned on the X, Y plane, and V_{cell} is the cell volume.

With the chosen resolution, cells contained within a field were scanned in a time ranging from 15 to 20 sec (depending on the cell height). Comparison of volumes obtained by increasing resolution or number of sections did not give different results.

EYFP fluorescence was measured as the total cell fluorescence, considering (adding) the mean fluorescence multiplied by the pixel number of each cell section and was corrected for the photobleaching-induced decay measured in control cells exposed to Iso extracellular solution and treated with the same acquisition protocol at 3.5, 5, 10, 20 min (n=8, not shown).

Intracellular Cl⁻ or H⁺ titration experiments

The clamping of ionic concentrations in cells was achieved with nigericin, valinomycin and tributyltin. Chloride and pH titration of EYFP fluorescence intensity was carried out by exposing cells to a high-K⁺ solution (in mM: Hepes 20, K⁺-gluconate 130, Ca²⁺-gluconate 0.7, Mg²⁺-sulphate 2, glucose 10) at pH 6.6 to 7.4 in the presence of nigericin (5 μM), valinomycin (5 μM), and tributyltin (10 μM). Specific chloride concentrations (0-60 mM) were achieved through isotonic replacement of K⁺-gluconate with KCl.

For the construction of calibration curves, EYFP fluorescence at each halide concentration (0, 10, 20, 40 and 60 mM) and at clamped pHs (6.6, 6.9, 7, 7.25 and 7.4) was acquired with the same hardware settings as for cell volume and EYFP fluorescence measurements. The EYFP fluorescence at intracellular pH 7.4 and [Cl⁻] 0 mM was considered 100%.

After completion of all experiments involving tributyltin, the perfusion system and imaging chamber were extensively rinsed with ethanol (the solvent for tributyltin) and all

disposable components were replaced.

Intracellular SNARF calibration and pH measurements

For pH measurements, SNARF-5F (5-(and-6)-carboxylic acid, acetoxymethyl ester, acetate) (Invitrogen S.r.l) was used due to its high pH sensitivity, its ratiometric method and because its emission spectra do not overlap with those of EYFP.

Titration experiments were performed on control cells according to the manufacturer's instructions. In brief, to load SNARF-5F, cells were incubated in medium containing 10 μM SNARF-5F for 20 min at 37 °C. Following loading, cells were placed in standard medium for 20 min to ensure de-esterification of the fluorophore and then mounted in the microscope perfusion chamber. Cells were perfused at a rate of 0.3 ml/min for 5 min with the initial experimental solution at room temperature before starting the acquisitions.

For calibration, loaded HEK cells were exposed to isotonic solutions at known pH values in the presence of high-K⁺ (in mM: KCl 130, MgCl₂ 1, HEPES 30, mannitol 20) and 10 μM nigericin. SNARF-5F emission spectra were acquired in confocal lambda scan acquisition mode (xyλ, 5 nm acquisition window) between 565 and 695 nm exciting the fluorophore at 561 nm. The ratio (R) between fluorescence intensity at 595 nm (λ1) and 635 nm (λ2) was plotted against pH. pH values were obtained from SNARF-5F fluorescence ratio values by linear fitting.

To calculate the intracellular pH values in transiently transfected HEK cells (PDS or control), SNARF-5F loaded cells were exposed to Iso or Hypo solutions and the fluorescence was acquired in sequential scan mode between 590-600 nm (λ1) and 625-635 nm (λ2), exciting SNARF-5F at 561 nm. The obtained R values (fluorescence intensity at λ1 divided by fluorescence intensity at λ2) were transformed in pH values using the calibration curve and plotted against t (time, min) in Hypo solution.

Intracellular chloride concentration measurements

From the observed “Δfluorescence” values (% Fig. 2B), referring to EYFP fluorescence, we calculated the corresponding [Cl⁻]_{in} by using the titration curves shown in Fig. 3B that link [Cl⁻]_{in} (at each pH_{in}) with each EYFP Δfluorescence (%).

Fig 3B is the exponential transform (similar to one-phase decay) of the experimentally obtained sigmoidal curves (Fig. 3A). The exponential curves allow, for each pH value, to obtain [Cl⁻]_{in} values, represented by the curves, from the abscissa values. In other words, the two graphs give the same indications but in the form of Fig 3B are immediately available for [Cl⁻]_{in} calculation.

The equation used to fit the points in 3B was:

$$Y = (Y_0 - \text{Plateau}) * e^{(-k * X)} + \text{Plateau} \quad [1]$$

where Y represents the fluorescence % (at any [Cl⁻]_{in} value); Y₀ is the initial value of Y (%); Plateau is the end value of Y (%); K is the fitted decay constant; X stands for [Cl⁻]_{in}.

To obtain directly $[Cl^-]_{in}$ values (X) the equation [1] has been written as:

$$X = \ln [(Y - \text{Plateau}) / (Y_0 - \text{Plateau})] / -k \quad [2]$$

We applied the equation [2] considering in Iso condition the measured pH_{in} ($pH_{in(iso)}$) and a $[Cl^-]_{in(iso)}$ of 43 mM. This $[Cl^-]_{in(iso)}$ can be experimentally measured and we considered the $[Cl^-]_{in(iso)} = 43$ mM on the base of previous electrophysiological experiments in HEK cells (data not shown). The possibility that control and PDS HEK cells have a different $[Cl^-]_{in}$ must be taken into account, but the relative $[Cl^-]_{in}$ variations shown in Fig. 5A would not change significantly.

In evaluating EYFP fluorescence variations in Fig. 2B we assumed the initial experimental fluorescence value (in Iso condition) as 100%. However, considering the exponential curves (as in Fig. 3B) corresponding to the $pH_{in(iso)}$ (i.e. pH 7.37 in control HEK cells, Fig. 4A) the “normalized fluorescence intensity” (%) at $[Cl^-]_{in(iso)}$ (i.e. 43 mM) was smaller (i.e. it is 63.5% at pH = 7.37, control HEK cells) since it is referred to a 100%, that corresponded to $[Cl^-]_{in} = 0$.

Therefore, to calculate the actual $[Cl^-]_{in}$ in a certain time in hypo ($[Cl^-]_{in(hypo\ t)}$) the calculated “ Δ fluorescence” (%), Fig. 2B) was rescaled according to the “normalized fluorescence intensity” value (%), Fig. 3B) corresponding to the Iso condition (“normalized fluorescence intensity_(iso)”).

The “rescaled Δ fluorescence” (%) were then added to the “normalized fluorescence intensity_(iso)” values and the obtained normalized fluorescence intensity (%) allowed the calculation of the “ $[Cl^-]_{in(hypo\ t)}$ ” using equation [2] on the exponential curve corresponding to the measured $pH_{in(hypo\ t)}$.

Theoretical intracellular chloride concentration (Fig. 5A, grey dashed lines) has been calculated only considering the effect due to volume variations, according to the following equation:

$$[Cl^-]_{in(hypo\ t)} = [Cl^-]_{in(iso)} * V_{in(iso)} / V_{in(hypo\ t)}$$

Where $V_{in(iso)}$ and $V_{in(hypo\ t)}$ are the cell volume in Iso and in Hypo extracellular solution at each time point, respectively.

All these calculations can be easily implemented in Excel software and have been done for each analysed cell; then the $[Cl^-]_{in}$ at each time point has been expressed as $\Delta[Cl^-]_{in}$ (%) and the obtained values expressed as mean $\pm$ SEM (Fig. 5A).

Statistical analysis

Significant differences among groups were evaluated with the two-way Anova test followed by Bonferroni's multiple comparison test or Student's t-test. Significance was assumed at $p < 0.05$. Parameters assessing goodness of fit for linear or non-linear regression curves were calculated with the Prism v5 GraphPad Software (San Diego, California, USA).

Fig. 1. Volume and EYFP fluorescence variations (%) relative to isotonic conditions in HEK cells exposed to extracellular hypotonic solutions in the presence of 5 μ M nigericin, 5 μ M valinomycin, and 10 μ M tributyltin. Cell volume increased following exposure to extracellular hypotonicity. In the absence of net Cl^- or H^+ movement, the total fluorescence remained constant (**= $p < 0.01$, one-sample t-test for the statistical comparison with the value in Iso condition).

Results

In the absence of net Cl^- or H^+ movement cell volume increases upon a hypotonic shock but the total EYFP fluorescence remains constant

EYFP fluorescence is quenched both by halides and H^+ [22], making it a biosensor for these ions.

In the presence of the ionophores nigericin, valinomycin and tributyltin, which permeabilize the cell to Cl^- , H^+ and K^+ , the concentration of these ions should quickly equilibrate across the plasma membrane. As expected, the total EYFP fluorescence in HEK cells transfected with EYFP alone did not significantly change following hypotonic shock (1.2 ± 0.5 in Hypo 40 and -0.2 ± 0.7 in Hypo, $n=5$) (Fig. 1). On the other hand, cell volume significantly increased ($8.2 \pm 1.5\%$ in Hypo 40 and 12.8 ± 1.7 in Hypo, $n=5$).

PDS HEK cells exposed to a hypotonic extracellular solution show an impaired RVD and a higher EYFP fluorescence increase compared to control HEK cells

The average volume observed in control HEK cells (transfected with EYFP alone) and PDS HEK cells (transfected with PDS) in Iso extracellular solution was not significantly different from each other

Fig. 2. Volume and EYFP fluorescence variations (%) relative to isotonic conditions in HEK cells exposed to extracellular hypotonic solutions. In A the volume difference (%) ($(V_{\text{Hypo}(t)} - V_{\text{iso}})/V_{\text{iso}} \times 100$) has been plotted versus time in Hypo. The two cell types behave differently and the volume increase is higher for PDS HEK cells compared to control cells at 20 min. Also the EYFP fluorescence variation (%) shown in B ($(F_{\text{Hypo}(t)} - F_{\text{iso}})/F_{\text{iso}} \times 100$) is different for PDS HEK cells, with a higher fluorescence increase at 10 min in Hypo compared to control cells (*= $p < 0.05$; **= $p < 0.01$).

(3.66 ± 0.34 pl ($n=15$) and 3.15 ± 0.35 pl ($n=14$) for control and PDS HEK cells, respectively). In order to investigate the influence of pendrin on cell volume regulation, we simultaneously measured EYFP fluorescence and volume in control or PDS HEK cells following hypotonic stimulation in the absence of nigericin, valinomycin and tributyltin (Fig. 2). The volume increase was evident in cells transfected with EYFP alone and in cells transfected with pendrin (Fig. 2A). The increase in volume at 20 minutes following stimulation with Hypo was significantly greater in the cells over-expressing pendrin. The EYFP fluorescence in control HEK cells exposed to hypotonicity differed from that in isotonic conditions only at the 5-minute

Fig. 3. EYFP normalized fluorescence response to $[Cl^-]_{\text{in}}$ and pH_{in} . Control HEK cells were exposed to different pH and clamped $[Cl^-]_{\text{in}}$ in the presence of high- K^+ extracellular solutions and 10 μ M tributyltin, 5 μ M nigericin and 5 μ M valinomycin. The experimental points have been normalized to the $pH=7.4$, $[Cl^-]_{\text{in}}=0$ condition and have been fitted with sigmoidal curves (A). From the curves in (A), is possible to obtain the exponential relation between EYFP fluorescence intensity and $[Cl^-]_{\text{in}}$ for every pH value (B).

time-point. On the contrary, the EYFP fluorescence detected in PDS HEK cells was higher at every time point compared to Iso conditions, and the fluorescence in these cells was significantly greater from that in control HEK cells at 10 min.

Quenching of EYFP fluorescence by Cl^- and H^+ : titration curves in HEK cells

To discriminate if the fluorescence increase in control and PDS HEK cells during cell swelling (Fig. 2B) is associated with a decrease in intracellular chloride concentration or an increase in intracellular pH, EYFP fluorescence calibration curves with respect to $[Cl^-]_{\text{in}}$ and pH_{in} have been experimentally obtained

Fig. 4. Intracellular pH measurements in control or PDS HEK cells. The cells were loaded with 10 μ M SNARF-5F and the pH was measured in Iso condition and during hypotonic cell swelling. In A the measured pH values were plotted versus time in Hypo. PDS HEK cells have a more acidic pH in Iso compared to control HEK cells. The pH response to cell swelling is different for the two cell types (two-way Anova test). In B the pH values were normalized with respect to the isotonic condition and plotted versus time in Hypo. PDS HEK cells have a less pronounced pH acidification due to cell swelling compared to control HEK cells (*= $p < 0.05$; **= $p < 0.01$).

(Fig. 3A). The exponential relation between normalized fluorescence intensity and $[Cl^-]_{in}$ at every wanted pH (Fig. 3B) can be used to calculate the actual $[Cl^-]_{in}$ in control or PDS HEK cells upon hypotonic cell swelling. To do this, however, the intracellular pH must be measured since EYFP fluorescence is also quenched by H^+ ions.

Fig. 5. % variations in intracellular Cl^- concentration during hypotonic cell swelling with respect to the Iso condition ($\Delta[Cl^-]_{in}$ (%)) in control or PDS HEK cells. Using curves similar to that in Fig. 3B, the Δ fluorescence from iso (%) observed in Hypo solution for control or PDS HEK cells have been transformed in $[Cl^-]_{in}$, considering the $[Cl^-]_{in}$ in Iso as 100%. The theoretical $[Cl^-]_{in}$ based only on the volume changes is also shown (grey symbols, dashed lines, A). In B the relative difference between the theoretical $[Cl^-]_{in}$ and the measured $[Cl^-]_{in}$ for control and PDS HEK cells has been plotted versus time in Hypo. PDS HEK cells experienced a more pronounced $[Cl^-]_{in}$ decreased during hypotonic shock compared to control cells (*= $p < 0.05$; ***= $p < 0.001$). The relative $[Cl^-]_{in}$ difference shown in B highlights that the PDS cells extrude Cl^- in the first 3.5 minutes in Hypo (one-sample t-test with respect to Iso, $p < 0.05$), then the fluxes become slower (no statistical difference between the values at 3.5 and 5 min was observed) and eventually cease between 10 and 20 minutes.

Reduced pH_{in} and swelling induced acidification in PDS HEK cells compared to control HEK cells

The intracellular pH was measured with SNARF-5F in control or PDS cells in Iso and Hypo extracellular solutions (Fig. 4A). It is known that during cell swelling an intracellular acidification takes place [23]. In isotonic condition, the intracellular pH

of control HEK cells was significantly higher than PDS HEK cells (7.37 ± 0.03 and 7.25 ± 0.01 , respectively, $n=5$, $p<0.01$, Fig. 4A). In addition, for both control and PDS HEK cells, a decrease in pH_{in} was observed following hypotonic shock. Interestingly, this decrease was more pronounced in control HEK cells compared to PDS HEK cells ($p<0.001$, Fig. 4A and $p<0.01$, Fig 4B, two-way Anova).

PDS HEK cells sustain a stronger decrease in $[Cl^-]_{in}$ upon hypotonic shock compared to control HEK cells

On the base of the measured pH_{in} values in each experimental condition (Fig. 4A), the corresponding exponential curves at each pH have been obtained from data in Fig. 3A. The actual $[Cl^-]_{in}$ have been calculated from the normalized EYFP fluorescence difference observed after exposing the cells to Iso or Hypo extracellular solutions and considering the same average $[Cl^-]_{in}$ in Iso condition for both cell types (see Material and Methods).

In the case of PDS HEK cells the $\Delta[Cl^-]_{in}$ (%) from isotonic condition decreased more compared to control HEK cells at 10 and 20 minutes in Hypo solution ($-7.75\% \pm 8.19\%$ at 10 min and $-10.44\% \pm 9.65\%$ at 20 min, $n=15$, and $-34.15\% \pm 7.44\%$ at 10 min and $-34.25\% \pm 7.56\%$ at 20 min, $n=14$ for control and PDS HEK cells respectively; $p<0.05$ in both cases, Fig. 5A). To discriminate if the diminished $[Cl^-]_{in}$ in both cell types has to be ascribed to the mere dilution due to cell swelling or to an exit of chloride from the cell, the theoretical $[Cl^-]_{in}$ calculated only on the bases of the dilution is also shown (grey dashed lines in Fig. 5A). The difference between the calculated $[Cl^-]_{in}$ and the theoretical values (%) is shown in Fig. 5B for both cell types. PDS HEK cells had a bigger difference at 5 and 10 minutes compared to control cells ($-7.40\% \pm 4.76\%$ at 5 min and $-1.55\% \pm 6.58\%$ at 10 min, $n=15$, and $-22.71\% \pm 5.32\%$ at 5 min and $-26.33\% \pm 7.90\%$ at 10 min, $n=14$, for control and PDS HEK cells respectively; $p<0.05$, t-test comparison at each time point).

It is up to note that control cells apparently did not significantly decrease their $[Cl^-]_{in}$ (no significant difference at any time from the Iso condition) whereas PDS HEK cells had a significantly lower $[Cl^-]_{in}$ (with respect to control) at every time point in Hypo (one-sample t-test comparison at each time vs Iso, $p<0.05$).

Discussion

Cell volume measurement

Measure of cell volume “in vivo” represents an invaluable tool for functional characterization of membrane transporters involved in regulatory volume control. The procedure we present with this paper exploits the characteristics of the EYFP protein both for volume measurements (by using the fluorophore as marker of the cellular borders) and for intracellular pH and chloride measurements, by taking advantage of its dependence on Cl^- and H^+ activities, a common property of all the YFP family [24]. The advantages offered by the method comprise the non-toxicity of the fluorophore (EYFP) and the fact that it cannot leave the cell. Our method for volume measurement is direct, while most of the reported procedures are indirect (as illustrated in Table 1). The procedure, based on a confocal microscopy protocol, allows a precise measure of the entire cell in relatively short times (about 20 seconds), that can be lowered either by reducing the number of sections and/or by using confocal microscopes with faster scan heads.

The obtained volume average data of cells in isotonic conditions are in good agreement with those reported in literature [25]. Analyzing data of Table 1, it is clear that comparison of rates of swelling, volume decrease and percentage of recovery to initial volume, also considering the same cellular type (see for instance HEK-293 cells), is hampered by the different procedures adopted. Moreover the various methods for volume measurement, the way to obtain osmotic changes (simple dilution with distilled water, mannitol substitution etc.), growth conditions (cells to confluence, cells in suspension etc), fluxes variations and so on, can only allow analysis within each reported study and generalization of results is not possible.

For this purpose, it would be undoubtedly useful to adopt for studies of volume regulatory mechanisms standardized protocols (especially regarding solutions). Yet, some common features can be extracted: RVD onset is clearly distinguishable and takes place soon after the swelling period (generally comprised between 2 and 5 minutes). The recovery is usually slow and is never complete in the experimental period (the 100% recovery reported by Heo [26] occurs after restoring initial conditions).

Our data (Fig. 2A) fit these general features: in control cells (that do not express pendrin), maximal

Cell or tissue	Osmotic gradient	Swelling onset and duration after hypotonic shock	Max (%) volume variation after hypotonic shock	RVD beginning after swelling	Average recovery rate (as % of the max $\Delta\%$ swelling)	Total observed RVD	Volume measurement method
HEK 293 (transfected with K channels)	-100 mosm	Immediately for 2 min	+ 5%	Immediately after max swelling	2.7% min	40%	fura dilution [34]
Hek 293	-100 mosm	Immediately for 4-5 min	+40%	Immediately after max swelling	-0.6%	Not reported	Calcein dilution [35]
Hek 293	-84 mosm mannitol (symmetric experimental conditions)	Immediately for about 5 min	+ 30%	Recovery Immediately after max swelling (not RVD)	2 phase exp decay ($\tau_1 = 69$ sec $\tau_2 = 69$ sec) symmetric to swelling	100% recovery in about 5 min due to return to isotonicity	Impedance microchamber [26]
COS-7 early embryonic monkey kidneys NaK trf	-50 mosm	Immediately for 5 sec	+14%	Immediately after max swelling	22% min	n.r.	Calcein dilution -[36]
Rabbit Renal RCCD1	-100 e -200 mosm	Immediately with a +10% increase rate	+45% (at -100 mosm)	5 - 15 min after max swelling	2% min	about 15%;	BCECF dilution [37]
Rat CCD cells	-100 mosm mannitol	Immediately for 7 min	+54%	After about 4.20 min	2,1%	-55% in 30 min	BCECF dilution [38]
Intestine 407 human	-30% osm	Immediately for 1 min	+28%	Immediately	4.8% min	48% in 10 min	Coulter-type cell-sizing apparatus [39]
Mz-Cha-1 biliary epithelial cells	-70 mM	Immediately for 2 min	+ 33%	Immediately	52% min	70% in 80 sec	Ind: measure of cell diameter [40]
Mouse cholangiocytes	-110% osm - saccharose	Immediately for 10 min	+20%	Immediately	0,6% min	50% in 30 min	Cross section area microscopy [41]
A549 (Hum Alveolar basal ep. cells)	-50% osm	Immediately for 1- 2 min	+87%	Immediately	7.25% min	94% in 13 min	Image anal. Via side lateral view [42]
16HBE14o (Hum Bronch Epit Cells)	-50% osm	Immediately for 1-2 min	+75%	After 5 min	9.1% min	73% in 8 min	Image anal. Via side lateral view [42]
Human bronchial cells	-120 mosm	Immediately for 2.5 min	+38%	After 10 sec	6-> 3% min	70%	Cellular height with fluorescent labels [43]
NIH/3T3 (mouse embr fibroblast)	-50% osm	Immediately for 1-2 min	+60%	Never	0.0%	0%	Image anal. Via side lateral view [42]
3T3 Mouse fibroblast cells	-30% osm	Immediately for 10-20 sec	+35%	Immediately	3.6% min	29 % in 8 min	Ind: 1/light scattering signal [44]
Mouse fibroblast 3T3	-100 mosm	Immediately for about 10 sec	+ 27%	After 10 sec	5%	33%	Light scattering inside cuvette. [45]
Human mammary MCF-7	-150 mM mannitol	Immediately for 3 (at 37°) or 5 min (at 21°)	+70% (37°) +55% (21°)	Immediately	4,8% (37) 2,8% (21)	58% 33%	Microscopy evaluating cell area [46]
Murine mammary C127 cells	-20% osm	Immediately for 1 min	+28%	Immediately	1% min	20% in 20 min	Coulter-type cell-sizing apparatus [47]
CHO-K1 cells TRPV4 transfected	From 300 to 200 mosm with water	Immediately for about 45 sec	+20%	Immediately for about 4 min, then stable up to 15 th min	5%	75% in about 4 min	Laser Scanning microscopy; area section for volume [48]
Human corneal epithelial cells	50% osmolarity reduction	Immediately for about 1 min	About 45%	Immediately after	8% min for about 10 min (exp behavior)	Almost 100% in 40 min	Calcein fluorescence variations [49]
C6-BU-1 rat glioma cells (Role of AQP1 and sodium)	40% dilution from about 340 mosm by NaCl or sucrose subtraction	Immediately for about 1 min	+43% (less NaCl) +25% (less sucrose)	Immediately after	7% (first 5 min with less NaCl) About 2%(first 5 min with less sucrose)	About 50% at the 20 th min (NaCl) About 10% at the 20 th min (sucrose)	Coulter or Calcein fluorescence dilution [50]

Table 1. The table summarizes published data of cell volume measurements. Only mammalian epithelial cells have been considered. A big volume variations heterogeneity, which span from +5% (HEK cells) to +87% (Human Alveolar epithelial cells) can be noted. The cell volume recovery is also extremely different among cell types, ranging from 0% (mouse embryo fibroblasts) to 100% (human corneal epithelial cells).

swelling is reached within 5 minutes; followed by a recovery of the volume that is the effect of the activation of RVD mechanisms. As shown in Fig. 2, PDS HEK cells show a different behavior with respect to controls. Even if two way ANOVA statistical analysis gives a significant difference only at min 20, volume recovery seems to be very small for pendrin transfected cells: difference percentage from the 5 min value (max cell swelling) is minimal both at min 10 (-3,5% of the 5 min value) and 20 (-10% of the 5 min value) while, at the same times, control drops of about 60% and 70%. This might imply that pendrin overexpression alters the osmotic response of the cell, as it will be further discussed in the next paragraph.

Chloride movements during RVD in control and pendrin transfected cells

Chloride and pH simultaneous measurement is an interesting topic addressed by many investigators. The method we adopted is based on the discrimination of the pH effects on EYFP fluorescence intensity from those due to chloride. A similar approach has already been used by other investigators that used the YFP protein variant YFP-H148Q/I152L in a two step procedure with independent calibration curves for intracellular Cl^- (using the procedure we previously applied [27]) and pH (BCECF method), then evaluating the relative effects on EYFP fluorescence intensity [28]. Thanks to the SNARF-5F fluorophore, while measuring volume, we have been able to simultaneously monitor variations of pH and EYFP fluorescence intensity due to volume changes thus keeping into account their contribution for intracellular chloride evaluation. Up to now, to our knowledge, there is only one molecular sensor that can give both measurements of chloride and pH at the same time and is based on a GFP linked sensor [29]. Surely interesting, this method could be a valid alternative but it presents one main disadvantage considering our purposes. The use of pIRES2-EYFP, a bicistronic vector, is more suited in studies based on the overexpression of heterologous proteins, since it avoids the need to perform a double transfection, with the difficulty of discriminating the cells carrying the investigated protein. To bypass this problem, the method of Arosio D. and collaborators [29] should be implemented by inserting an IRES sequence between the GFP-based sensor and the investigated protein.

Our results indicate that chloride movements markedly differ between PDS and control HEK cells (Fig. 5). Up to the fifth minute, a period that comprises the

swelling phase and the beginning of RVD, control HEK cells show a rapid decrease of $[\text{Cl}^-]_{\text{in}}$. From the 5th minute (when RVD begins) to the 20th minute, the $[\text{Cl}^-]_{\text{in}}$ slightly recovers in control HEK cells probably due to the parallel volume recovery. Comparison of the measured $[\text{Cl}^-]_{\text{in}}$ with the theoretical one, calculated on the base of the effect of the sole volume variations (see control vs control theor in Fig 5A), indicates that the measured variations mostly reflects volume changes. This doesn't necessarily imply that chloride fluxes (considered as the $[\text{Cl}^-]_{\text{in}}$ multiplied for the cell volume in a certain time interval) are null, but it's possible that the method might be not sensitive enough to discriminate the effects of those movements.

In the case of PDS HEK cells the presence of pendrin is accompanied by a faster and much larger drop in $[\text{Cl}^-]_{\text{in}}$. This is likely due to a larger chloride efflux in the first five min in Hypo, as evidenced by comparing the measured $[\text{Cl}^-]_{\text{in}}$ variations with those expected for the sole effect of volume changes (see PDS vs PDS theor in Fig. 5A and Fig. 5B). Surprisingly, even if PDS appears to significantly increase swelling induced Cl^- exit (at 3.5 and 5 min), this does not translate into a more efficient RVD. Further investigation is indeed needed to elucidate this aspect; anyway a few hypotheses can be made on the base of our data. This discrepancy might be a consequence of the initial major drop in $[\text{Cl}^-]_{\text{in}}$ induced by PDS over-expression, a drop concentrated in the first 3.5-5 min of hyposmotic challenge, when RVD mechanisms are in the initial phase (see Table 1). At the moment of their full activation this reduction of $[\text{Cl}^-]_{\text{in}}$ would cause a decrease of the chemical gradient favoring Cl^- exit, resulting in a less efficient RVD.

Moreover PDS over-expression, by affecting both pH_{in} and $[\text{Cl}^-]_{\text{in}}$ variations caused by cell swelling, might induce other direct or indirect changes into the transport properties of other channels/transporters involved in volume regulation. In particular, the major drop of intracellular chloride induced by pendrin during the swelling phase might influence the activity of other carriers. It has been demonstrated that low intracellular chloride, by acting as a sort of second messenger that coordinates different transport systems, positively regulates ENaC [11, 30] and NKCC [31, 32] that mediate RVI (Regulatory Volume Increase) [23], while conversely inhibits KCC cotransport [32], involved in RVD [23]. The result of these changes of activities would cause a decrease in the efficiency of volume recovery after swelling. Similarly, the reduced

variation of pH_{in} measured in PDS HEK cells (Fig. 4) might alter the function of other transporters/channels [23].

pH time-course during RVD in control and pendrin transfected cells

In the majority of vertebrate cells, swelling induces an intracellular acidification [33]. In accordance, our results (Fig. 4) show an increase in $[\text{H}^+]_{\text{in}}$ after exposure to hypotonic conditions, both in controls and PDS HEK cells.

The reduced pH percentage decrease of PDS cells ($\Delta\text{pH}_{20-0\text{min}} = -0.97\%$ of vs -2% in controls) is in accordance with the known transport properties of pendrin. Among the main roles attributed to PDS is the Cl^- (I^-)/anion exchange, with both HCO_3^- and OH^- being exchanged for Cl^- (or I^-). The subsequent role of pendrin in pH modulation is well documented [5]. In our case, the pendrin-dependent chloride efflux measured during RVD is likely to be accompanied by a OH^- influx (bicarbonate is not present in our solutions), resulting in an attenuation of the swelling induced pH drop. Similarly, the lower pH measured in the Iso condition in PDS HEK cells might be a consequence of a PDS-driven HCO_3^- (OH^-) efflux, owing to its $\text{Cl}^-/\text{HCO}_3^-$ (OH^-) transport activity [5]. According to this consideration we cannot exclude that also the starting $[\text{Cl}^-]_{\text{in}}$ of PDS HEK cells in isotonic condition could be different from that of control HEK cells, even if the variation expected on the base of the difference in the pH (7.37 and 7.25, in control and PDS HEK cells, respectively) would be in the μM range. It's anyway important to underline that even if PDS cells have a higher $[\text{Cl}^-]_{\text{in}}$ in Iso compared to

control HEK cells, the relative $[\text{Cl}^-]_{\text{in}}$ variations shown in Fig. 5A would not change significantly and the increased chloride exit in PDS cells would be confirmed in any case.

Final considerations

The experimental data presented in this paper underlie the usefulness of the procedure we set up to contemporarily evaluate volume, pH and chloride variations. In this way it is possible to discriminate between the effect on $[\text{Cl}^-]_{\text{in}}$ determined by volume variation and those related to the ion movement.

Moreover, even if the OH^-/Cl^- transport properties of pendrin are already documented, this is the first time they are investigated in connection with volume control. The presented data, besides confirming pendrin action on pH and Cl^- homeostasis, suggest that pendrin over-expression (or possibly, up-regulation) may impair the RVD mechanisms and provide some hints for further investigations. The potential alteration of volume homeostatic response exerted by pendrin, that might imply a possible cross talk with other Cl^- transporters, could be of particular relevance in the inner ear, where it is widely expressed.

Acknowledgements

The authors wish to thank Dr. Charity Nofziger for her useful suggestions and Dr. Silvia Dossena for kindly providing the PDS plasmids.

This study was supported by a grant from Ministero Ricerca Scientifica (PRIN 2008 – prot. 2008W5AZEC_002).

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