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Fast Fluorometric Method for Measuring Pendrin (SLC26A4) Cl^-/I^- Transport Activity

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Key Words
Pendrin • Chloride measurements • Chloride transport • EYFP

Abstract
Malfunction of the SLC26A4 protein leads to Pendred syndrome, characterized by sensorineural hearing loss, often associated with mild thyroid dysfunction and goiter. It is generally assumed that SLC26A4 acts as a chloride/anion exchanger, which in the thyroid gland transports iodide, and in the inner ear contributes to the conditioning of the endolymphatic fluid. Here we describe a fast fluorometric method able to be used to functionally scrutinize SLC26A4 and its mutants described in Pendred syndrome. The validation of the method was done by functionally characterizing the chloride/iodide transport of SLC26A4, and a mutant, i.e. $SLC26A4_{S28R}$, which we previously described in a patient with sensorineural hearing loss, hypothyroidism and goiter. Using the fluorometric method we describe here we can continuously monitor and quantify the iodide or chloride amounts transported by the cells, and we found that the transport capability of the $SLC26A4_{S28R}$ mutant protein is markedly reduced if compared to wild-type SLC26A4.

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Introduction

Functional studies scrutinizing the anion transport activity of the SLC26A4 protein and its mutants are hampered by the use of radioactive uptake-studies. Two isotopes are commonly used in these studies, i.e. chloride ($^{36}Cl^-$)[1-3], whose low specific activity leads to technical limitations, or iodide ($^{125}I^-$) [4-7].

In order to measure SLC26A4 anion exchange activity, without the need of radioisotopes, we applied an easy to use, nowadays readily available and fast fluorometric method that has been used previously for the characterization of CFTR induced chloride movements across the cellular membrane [8].

It has been demonstrated that SLC26A4 can exchange anions as different as Cl^-, HCO_3^-, OH^-, I^-, or formate [9]. The SLC26A4 protein is mainly expressed in the inner ear, the kidney, and the thyroid gland. In the inner ear, the SLC26A4 protein is involved in the conditioning of the endolymphatic fluid, most likely by acting as an anion transporter [10].

In the kidney, SLC26A4 is located in the apical membrane of β-intercalated cells [11]. There, SLC26A4 seems to be involved, amongst other important transporters like AE1 and AE4 [12, 13], in bicarbonate transport. In the thyroid gland, SLC26A4 is expressed in the thyrocytes, where it has been shown that SLC26A4 resides in the apical membrane [14]. This membrane is facing the follicular lumen into which the exchanger transports iodide, which then will be organified on thyroglobulin [15].

The malfunction of the SLC26A4 protein leads to the Pendred syndrome (PS; OMIM 274600). Pendred syndrome is considered one of the most common forms of syndromic deafness accounting for up to 7.5% of cases of childhood deafness [16]. More than 150 different mutations of the SLC26A4 gene have been found in humans so far (www.medicine.uiowa.edu/pendredandbor). Clinically, the patients have severe or profound sensorineural hearing loss, associated with enlargement of the vestibular aqueduct and of the endolymphatic duct and sac [17]. The hearing symptoms are associated with positive perchlorate test, whereas goiter and hypothyroidism can be diagnosed in about half of the cases [18]. Since the modified anion exchange capability of the mutated SLC26A4 proteins in patients suffering of PS can explain the observed pathological phenotypes, the development of an easy-to-use technique able to quantify the SLC26A4 transport capability is of utmost importance. Here we show a fast and easy-to-use fluorometric method able to disclose hampered SLC26A4 chloride/iodide exchange.

Materials and Methods

Cloning of SLC26A4 cDNA

Standard procedures were used for DNA preparation, cloning, purification, and sequencing. The human wild-type SLC26A4 cDNA was obtained by RT-PCR, using total RNA from normal human thyroid tissue and Taq PLATINUM Pfx DNA polymerase (Invitrogen,-Life Technologies). The SLC26A4 open reading frame was cloned into pTARGET vector, where six histidines were added to the C-terminus of SLC26A4 for localizing the overexpressed protein by using anti-His-tag antibodies. For this procedure the primer-pairs were: sense, 5'-GTT GGA TCC GCG AGC AGA GAC AGG TCA-3'; antisense, 5'-CTG CGC GGC CGC TCA *GTG GTG GTG GTG GTG GTG* GGA TGC AAG TGT ACG CAT A -3'. The primers contained appropriate linkers for cloning the SLC26A4 cDNA into the *Bam*H I and *Not* I sites of the pTARGET plasmid (Promega).

Mutagenesis

The $SLC26A4_{S28R}$ mutant was made using the Quik Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol, using the following primers: sense 5'-CGG CCG GTC TAC AGA GAG CTC GCT TTC CA-3'; antisense 5'-TGG AAA GCG AGC TCT CTG TAG ACC GGC CG-3'. The mutant was fully sequenced before testing at the functional level.

Cell culture and transient transfection

HEK 293 Phoenix cells (this is a second generation retrovirus producing cell line for the generation of helper free ectopic and amphotropic retroviruses; [19]) were grown in Minimum Essential Medium Eagle (Sigma) supplemented with 10% foetal bovine serum (Cambrex BioScience), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 1 mM pyruvic acid (sodium salt). The cells were maintained at 37°C in a 5%CO_2: 95% air humidified incubator; subcultures were routinely established every second to third day seeding cells in Petri dishes after trypsin/EDTA treatment.

Fluorescence measurements

A) Chloride and iodide measurements. For in vivo fluorometric chloride/iodide measurements, HEK 293 Phoenix cells grown on coverslips (∅ 40 mm) were transiently transfected (calcium-phosphate precipitation

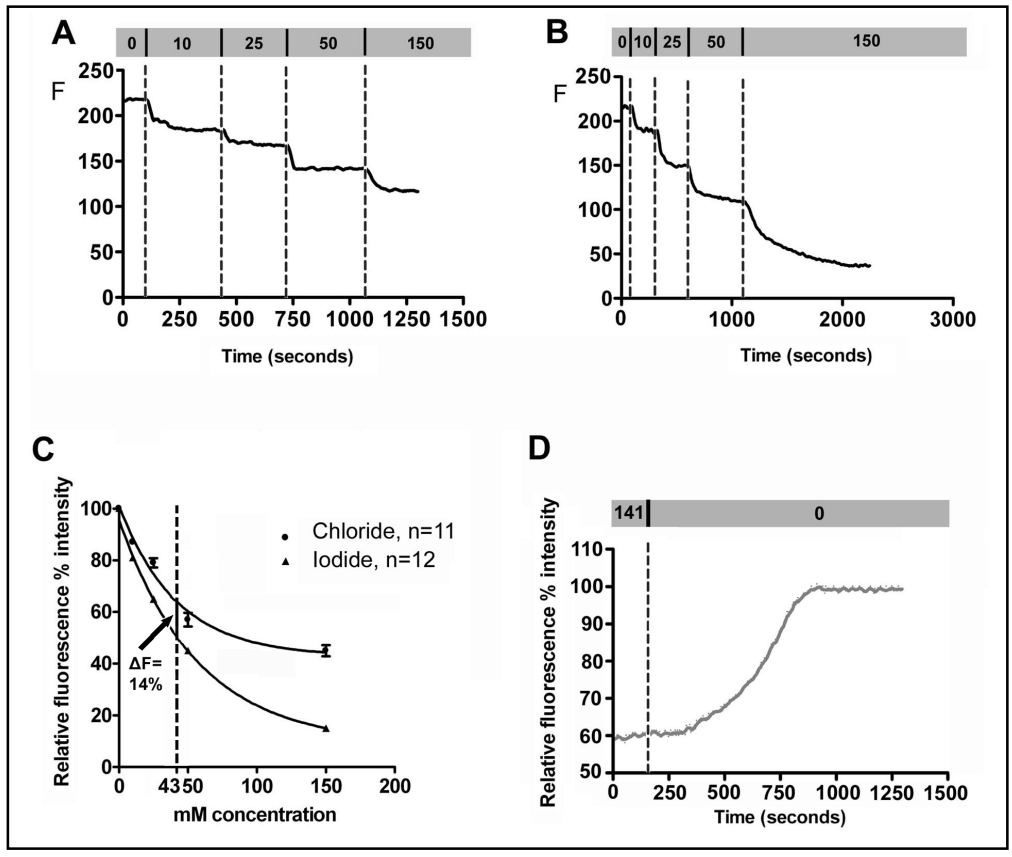

Fig. 1. The EYFP signal is sensitive to chloride and iodide. Fluorescence measurements in HEK 293 Phoenix cells transfected with EYFP and exposed to different chloride, as well as iodide solutions in the presence of nigericin and tributyltin chloride (both 10 µM). Since the halides quench the EYFP signal, their increased concentration leads to a diminished fluorescence signal. A) Fluorescence measurements in the presence of increasing chloride concentrations (from 0 mM to 150 mM; the dashed lines indicate the respective changes of the extracellular chloride or iodide concentrations). B) Fluorescence measurements in the presence of increasing iodide concentrations (from 0 mM to 150 mM). Compared to chloride, iodide is able to quench the EYFP fluorescence more efficiently, leading to a more pronounced decrease of the fluorescence signal at the respective iodide concentrations if compared to chloride (1A). C) Summary of the experiments shown in A and B. Fluorescence intensities are normalized in order to give a value, in the absence of chloride or iodide, equal to 100%. The data points are well fitted (r^2 = 0.98 for chloride and r^2= 0.99 for iodide) with an one phase decay exponential equation (see methods) with the following parameters for chloride and iodide, respectively: α= 58,42±1,5 and 86,83±0,37; β = 42,72±1.13 and 8,824±0.39; K = 0,02409±0,0016 and 0,01757±0,00024 (n = 4). The line indicates the respective relative fluorescence intensity at an intracellular halide concentration of 43 mM, which is the intracellular chloride concentration according to panel D. Due to the fact that iodide is a better quencher of the EYFP signal compared to chloride, at the same halide concentration the relative fluorescence given by iodide is 14% lower compared to chloride (solid part of the line). D) Normalized relative fluorescence intensity given by HEK cells in the presence of "high Cl^-" (see method) and after omitting the extracellular chloride. According to panel C the 63% relative fluorescence amounts to 43 mM intracellular chloride.

method) with the vector pEYFP-N1 coding for a modified YFP protein (EYFP), which can be used for measuring the intracellular halide amount (Clontech, CA, USA) [20]. For this, 3 µg of pEYFP plasmid dissolved in 175 µl H_2O were mixed with 25 µl of buffer A ($CaCl_2$ 2 M), and 200 µl of buffer B (140 mM NaCl, 1,5 mM Na_2HPO_4, 50 mM HEPES, pH 7 adjusted with NaOH) and then added to each coverslip. For measuring the halide (chloride and iodide) effects on cells expressing pendrin or its mutants, cells were co-transfected with 2 µg of pEYFP-N1 and the pTARGET (6 µg) plasmid bearing the cDNA of the respective SLC26A4 protein, i.e. wild-type SLC26A4 or $SLC26A4_{S28R}$. The experiments were performed 24-48 hours after transfection.

For the chloride/iodide titration experiments (figure 1) needed for the quantification of the halides signals, HEK 293 Phoenix cells transiently transfected with pEYFP-N1 were perfused in a laminar-flow chamber (FCS2 System, Bioptechs, USA) at 0,3 ml/min (room temperature) and EYFP fluorescence changes (excitation at 515 nm; emission at 525-600 nm) were measured in the presence of (i) different concentrations of Cl^- or I^- (0, 10, 25, 50 and 150 mM respectively; in order to avoid changes in the osmolality, potassium gluconate was added accordingly), (ii) nigericin (10 µM), (iii) tributyltin chloride (10 µM), and (iv) high potassium (150 mM), in order to allow equilibration of the intracellular and extracellular halides concentrations, to avoid pH variations and to dissipate the membrane potential. The relative fluorescence intensities at the different chloride (figure 1a) or iodide

(figure 1b) concentrations are summarized in figure 1c, and best fitted by an one phase decay exponential equation:

$$F_{rel}=\alpha^{(-K[x])}+\beta \qquad EQ1$$

where F_{rel} is the measured relative fluorescence intensity (%), [x] is the halide concentration (mM) and α, β and K are the fitting parameters. Figure 1d shows the measurement of F_{rel} at "high Cl^-" (see below) and after omitting chloride from the extracellular solution. The F_{rel} under "high Cl^-" amounts to 63 ± 0.6 % (n=14), which corresponds, according to equation 1 (EQ1), to an intracellular chloride concentration of ≈43 mM (figure 1c), which is in good agreement with the 45 ± 4 mM reported by Gillen and Forbush [21].

In experiments aimed to evaluating the pendrin induced halide transport, HEK 293 Phoenix cells co-transfected with pEYFP-N1 and the pTARGET plasmid bearing the respective SLC26A4 cDNA were continuously superfused with "high Cl^-" (in mM: KCl 2, NaCl 135, $CaCl_2$ 1, $MgCl_2$ 1, D-glucose 10, HEPES 20, pH 7,4), or "high I^-" (in mM: KCl 2, NaI 135 mM, $MgCl_2$ 1, $CaCl_2$ 1, D-glucose 10, HEPES 20, pH 7,4) solutions. The EYFP fluorescence measurements were performed using a Leica TCS SP2 AOBS confocal microscope (Leica Microsystem, Heidelberg, Germany), using a 515 nm Ar/ArKr laser line for exciting the EYFP fluorescence. Transfection efficiency was determined by immunocytochemistry, counting the cells expressing EYFP and pendrin or its mutant in respect to the cells expressing only EYFP (for SLC26A4 the co-transfection efficacy was 79.0 ±2.5 %, n=7, and for SLC26A4S28R the co-transfection efficacy was 73.4 ± 4.2 %, n=7; both values are not statistically different from each other).

B) pH measurements. For intracellular pH measurements the fluorescent indicator BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) was used. HEK 293 Phoenix cells were transiently transfected with pTARGET-SLC26A4 plasmid and loaded (30 min, 37 °C) with 1,5 mM BCECF-AM (acetoxymethyl ester). BCECF fluorescence was excited using a 488 nm and a 458 Ar/ArKr laser lines during the continuous perfusion of the cells with the "high Cl^-" or the "high I^-" solutions (emission intensity evaluated at 535 ±20 nm; experiments were performed at RT). Intracellular pH was calculated following the standard ratiometric method (http://probes.invitrogen.com/media/pis/mp01150.pdf). Titration of the intracellular pH was performed in nigericin (10 µM) treated HEK 293 Phoenix cells in presence of 130 mM potassium (KCl 130 mM, $MgCl_2.6H_2O$ 1 mM, HEPES 30 mM, mannitol 20 mM), at pH's of 5.0 ,7.0 and 7.4.

Immunocytochemistry

For the immunofluorescence assays, cells were probed 32 hours after double transfection with pTARGET-SLC26A4 or pTARGET-SLC26A4$_{S28R}$ and pEYFP-N1 plasmid (3:1 ratio). Cells were washed with 2 ml PBS at room temperature, fixed for 15 min in 3% paraformaldehyde, permeabilized for 3 min with 0.1% Triton X-100 and blocked 1 hour at room temperature in PBS/3% BSA. Coverslips were incubated overnight at 4°C with an anti His-tag mouse monoclonal antibody (Roche; Germany), diluted 1:100 in PBS/0.1% BSA, recognized by an Alexa Fluor 568 coupled goat anti-mouse secondary antibody (diluted 1:200 in PBS/ 0.1% BSA, 1 hour incubation at room temperature).

Chemicals

All chemicals used are of *analysis* grade.

Statistical Analyses

Data are expressed as arithmetic means ± S.E. Statistical analysis was performed using the one way ANOVA with Bonferroni's multiple comparison test. Statistically significant differences were assumed at $p < 0.05$.

Results and Discussion

The EYFP signal is able to reveal changes in the intracellular chloride concentrations

Recently, variants of the yellow fluorescent protein (YFP) from *Aequorea* have been successfully used as a new class of biocompatible fluorescent dyes, sensitive for halides [8, 20, 22-26]. In the present study we use a readily available enhanced YFP protein as an indicator for the intracellular halide amount (EYFP; pEYFP-N1, Clontech) [20].

In a first set of experiments, we verified whether HEK 293 Phoenix cells expressing EYFP are sensitive to changed intracellular chloride and iodide concentration. For this we incubated EYFP expressing cells with the anion ionophore tributyltin [27] and changed the extracellular chloride or iodide concentration in the presence of high potassium and nigericin in order to avoid pH variations and to dissipate the membrane potential, which affects the passive anion distribution across the cell membrane. As shown in figure 1a and b, cells were kept in a solution void of chloride or iodide (0 mM). Increasing the

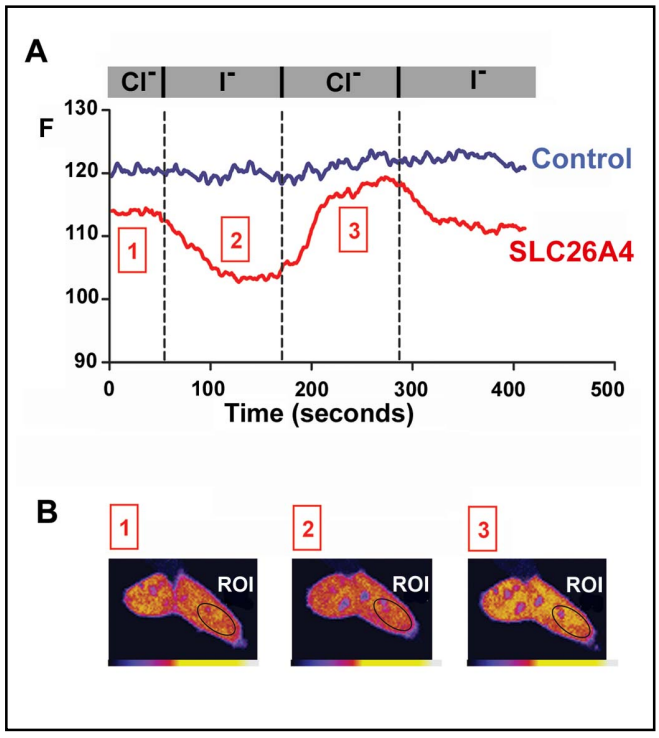

Fig. 2. HEK 293 Phoenix cells expressing SLC26A4 transport I^-. A) EYFP fluorescence (F) measured in HEK 293 Phoenix cells co-transfected with EYFP and SLC26A4 (SLC26A4) or with EYFP and the empty plasmid (control), and exposed to the "high Cl^-" or the "high I^-" solutions. Only cells transiently transfected with SLC26A4 respond to the change in extracellular I^- concentration with a decrease in intracellular fluorescence, evidencing a I^-/Cl^- exchange. Dashed lines indicate the respective changes of the extracellular solutions. B) The ROI (region of interest) used for the calculation of the mean fluorescence of the SLC26A4 trace shown in A).

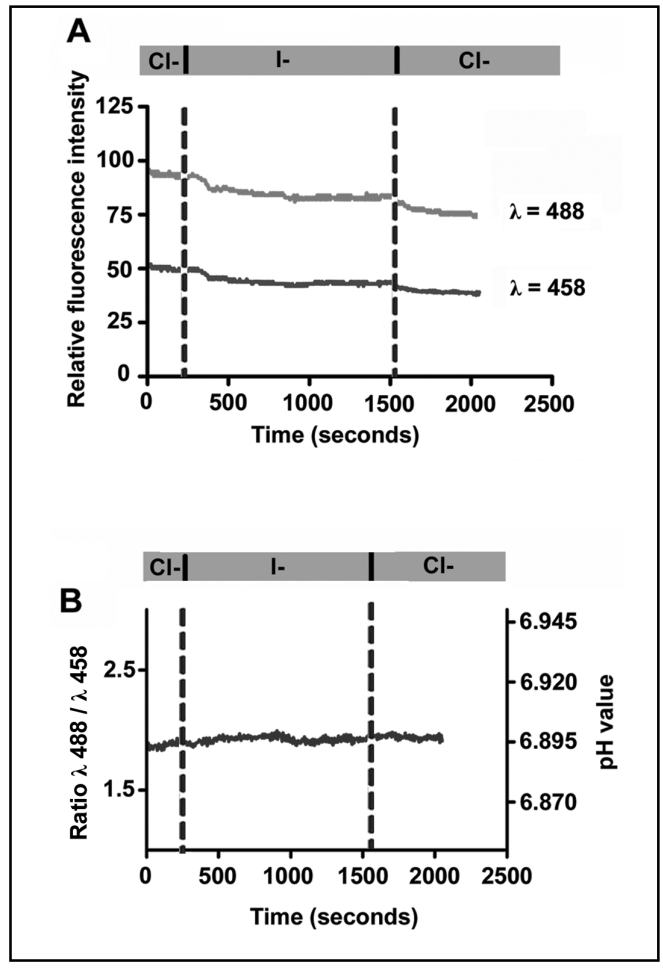

Fig. 3. Measurements of the intracellular pH by using BCECF. A) Original tracing showing the emission at the two wavelengths (488 nm and 458 nm) after changing the extracellular chloride ("high Cl^-") to iodide ("high I-"). B) The ratio of the two wavelengths showing that the intracellular pH does not change during the exchange of the extracellular halides.

extracellular chloride or iodide concentration stepwise to 150 mM leads to a substantial and reversible (not shown) decrease of the EYFP fluorescence, which is more pronounced in the iodide experiments if compared to chloride (figure 1c). These experiments indicate that the EYFP we use has a higher affinity for iodide if compared to chloride, which is in agreement with the halide sensitivity of an other variant of EYFP, i.e. $I^- > SCN^- > NO_3 > Cl^- > Br^-$ [8, 22].

HEK cells expressing SLC26A4 show a marked halide transport activity

In order to test the halide transport activity of expressed SLC26A4 we measured the EYFP fluorescence before and after substituting extracellular chloride by iodide. The SLC26A4 protein is able to transport chloride [1-3] as well as iodide [4-7, 28], and therefore, since iodide is a much better EYFP quencher than chloride, an increase of intracellular iodide should lead to a decrease of EYFP fluorescence [8]. Indeed, as shown in figure 2, substituting 135 mM of chloride by the same amount of iodide leads to a marked and reversible decrease of EYFP fluorescence in cells expressing SLC26A4 by -17.98 ± 3.53 % (n=8), considering the starting level of the relative fluorescence in those experiments as 100%. However, if the fluorescence in the absence of chloride is considered as 100%, then the observed -17.98% amounts to -11.31 ± 2.22% (n=8). It is interesting that the observed reduction of the fluorescence is indicating that chloride and iodide are exchanged in a 1:1 way by the

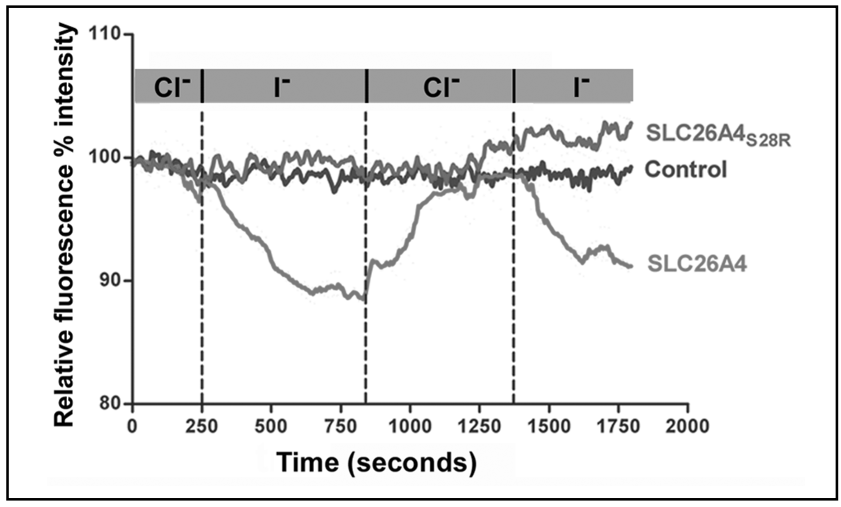

Fig. 4. HEK 293 Phoenix cells expressing $SLC26A4_{S28R}$ have an impaired I^- transport. Relative fluorescence intensity obtained in fields of about 10-20 cells and measured in HEK 293 Phoenix cells co-transfected with EYFP and pTARGET-SLC26A4 (n=3), pTARGET-$SLC26A4_{S28R}$ (n=3) or the empty (n=4) plasmids and exposed to the "high Cl^-" or the "high I^-" solutions. $SLC26A4_{S28R}$ transfected cells behave as the control cells. The dashed lines indicate the respective changes of the extracellular solutions.

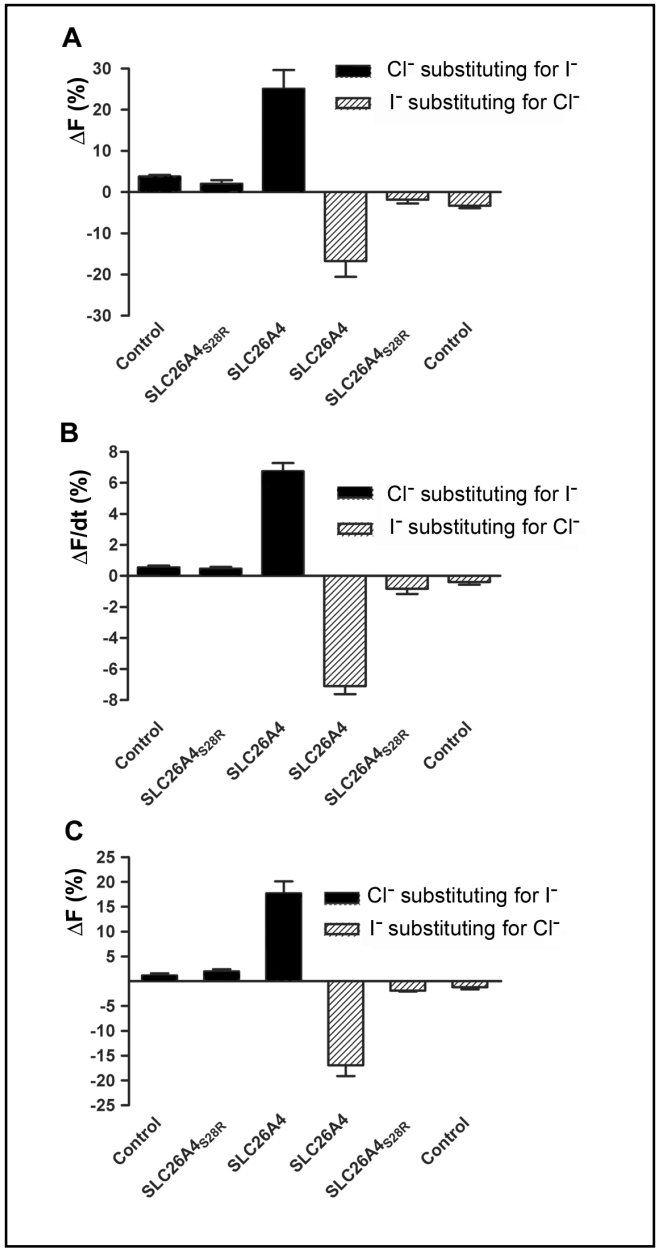

Fig. 5. ROI and whole-field fluorescence measurements. A) Maximal fluorescence % variation after the ionic substitution calculated as average of different ROIs inside the cells (Cl^- substituting for I^-: control n= 8, SLC26A4 n= 10, $SLC26A4_{S28R}$ n=7; I^- substituting for Cl^-: control n= 8, SLC26A4 n= 8, $SLC26A4_{S28R}$ n=7). B) Maximal fluorescence % variation per minute ($\Delta F/dt$) after the ionic substitution calculated as average of single ROI's inside the cells (Cl^- substituting for I^-: control n= 13, SLC26A4 n= 8, $SLC26A4_{S28R}$ n=7; I^- substituting for Cl^-: control n= 7, SLC26A4 n= 8, $SLC26A4_{S28R}$ n=7). C) Maximal fluorescence % variation calculated as average of different fields of about 10-20 cells (control n= 4, SLC26A4 n= 3, $SLC26A4_{S28R}$ n=3). In every panel the maximal fluorescence variation of the SLC26A4 is significantly different from the $SLC26A4_{S28R}$ and from the control.

transporter. The evidence for that is seen in figure 1c. As explained in the former chapter, the two curves shown in figure 1c indicate the reduced fluorescence of EYFP at increasing intracellular chloride as well as iodide concentrations. The relative fluorescence level obtained under "high Cl^-" is, as mentioned above, 63% (figure 1d), which corresponds to an intracellular chloride concentration of 43 mM (figure 1c). If the exchanger works in a 1:1 coupling-ratio then we should expect a decrease of the fluorescence by exchanging chloride with iodide of 14%, which is not statistically different from the observed -11.31% mentioned above. In control cells not overexpressing SLC26A4, the exchange of extracellular chloride with iodide leads to a significantly smaller decrease of the relative fluorescence of -3.33 ± 0.55% (n=8; again, considering the relative fluorescence level obtained

under "high Cl^-" as 100%), pointing to the fact that transporters or channels able to pass iodide from the extracellular space towards the cytosol are scarcely active in the native HEK 293 Phoenix cell line we use. This cell line is therefore particularly suited to test SLC26A4 induced halide transport activity after overexpressing the respective proteins. Since EYFP is also sensitive to pH [8, 29-31], in order to exclude any unpredictable pH changes under the conditions we chose for our experiments, we made control experiments measuring the intracellular pH by measuring the BCECF fluorescence (figure 3). The intracellular pH at "high Cl^-" is 6.91 ± 0.01 (n=8) and does not change significantly after changing the extracellular solution to "high I^-" ($\Delta pH = 0.01 \pm 0.01$; n=8) and again back to "high Cl^-" ($\Delta pH = 0.01 \pm 0.01$; n=8). These results indicate that using the conditions we described, neither HCO_3- nor OH- is substantially transported by SCL26A4 expressing cells.

The halide transport evidenced by the EYFP fluorescence is annihilated in cells expressing a SLC26A4 mutant

Recently we identified a mutant of SLC26A4, i.e. $SLC26A4_{S28R}$, in a patient with sensorineural hearing loss, hypothyroidism and goiter [32]. We demonstrated that this mutant can be expressed in HEK 293 Phoenix cells and is translocated towards the cellular membrane, however, the chloride transport evidenced by radioactive uptake-studies revealed an impaired function [2]. We used the same mutant of SLC26A4 in order to validate the EYFP-fluorometric method described here. As can be seen in figure 4, the marked change in EYFP fluorescence measured after exchanging chloride for iodide in cells expressing SLC26A4 is abolished in cells expressing $SLC26A4_{S28R}$, and therefore indistinguishable from the effect seen in control cells. The lost effect in $SLC26A4_{S28R}$ expressing cells is not due to a lacking transposition of the transport-protein towards the cellular membrane, as it was described for several SLC26A4 mutants [6]. As mentioned above, we have shown earlier that $SLC26A4_{S28R}$ is indeed transposed towards the membrane [2].

The fluorescence measurements shown in figures 1-2 were made using a region-of-interest (ROI) within single cells. These experiments are summarized in figure 5a. The data reported in figure 5a are taken after steady state conditions, however, as can be seen in figure 5b, qualitatively the same results can be obtained one minute after switching to the respective solutions under the different conditions tested, underlining the robustness of the EYFP fluorescence measurements in the context of SLC26A4 chloride/iodide transport testing. However, as expected, the absolute values after one minute are smaller if compared to the steady state conditions summarized in figure 5a. It is, however, not essential to use ROI's for the measurements. As evidenced in figure 5c, also whole field measurements reveal the observed differences between native SLC26A4 and the $SLC26A4_{S28R}$ mutant, and are therefore suitable for scrutinizing SLC26A4 dependent chloride/iodide transport in HEK 293 Phoenix cells.

Conclusions

The SLC26A4 protein is a chloride/anion exchanger capable of transporting also iodide. Malfunction of the protein leads to Pendred syndrome, a disease-complex characterized by deafness and impaired iodide organification. Here, we show, an easy to use and fast fluorometric method able to substantiate the chloride/iodide transport of SLC26A4. It is important to note that the use of a confocal microscope as described here, is not a prerequisite and that also more readily available fluorescence imaging devices can be used. Furthermore, we show for the first time that, by using this method, it is possible to determine functional deterioration of the SLC26A4 mutant ($SLC26A4_{S28R}$) we described previously, showing that its transport capacity (chloride/iodide exchange) is markedly reduced compared to wild-type SLC26A4, consistent with the clinical phenotype observed in the patient. In addition, we show that by titrating the cellular model system either with chloride or iodide, and measuring the respective fluorescence values allows, by using a simple exponential fit, to determine the time-course of intracellular halide concentrations. Therefore, the fluorometric method we describe here can be used for screening SLC26A4 mutants expressed in HEK 293 Phoenix cells, without the need of cumbersome and difficult to use radioactive uptake studies.

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