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mOrange2, a Genetically Encoded, pH Sensitive Fluorescent Protein, is an Alternative to BCECF-AM to Measure Intracellular pH to Determine NHE3 and DRA Activity

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Abstract

Background/Aims: NHE3 (Na⁺/H⁺ exchanger3) and SLC26A3 (Cl⁻/HCO₃⁻ exchanger, DRA) are the major components of the intestinal neutral NaCl absorptive process and based on the intestinal segment, contribute to HCO₃⁻ absorption and HCO₃⁻ secretion. NHE3 and DRA are highly regulated by changes in second messengers, cAMP, cGMP and Ca²⁺. Precise and convenient measurement of exchanger activity is necessary to allow rapid study of physiologic and pharmacologic functions. Some epithelial cells are difficult to load with AM ester dyes and loading may not be uniform.

Methods: The use of a genetically modified fluorescent protein, mOrange2 was explored as an intracellular pH sensor protein to measure exchange activity of NHE3 and DRA. The model used was FRT cells stably expressing NHE3 or DRA with intracellular pH measured by changes of mOrange2 fluorescence intensity. Intracellular pH was monitored using a) Isolated single clones of FRT/mOrange2/HA-NHE3 cells studied in a confocal microscope with time-lapse live cell imaging under basal conditions and when NHE3 was inhibited by exposure to forskolin and stimulated by dexamethasone, b) coverslip grown FRT/mOrange2 cells expressing NHE3 or DRA using a computerized fluorometer with a perfused cuvette with standardization of the mOrange2 absorption and emission signal using K⁺/Nigericin as an internal standard in each experiment.

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Author Contributions

R. Sarker, M. Tse, R. Lin, and V. Singh performed the experiments, analyzed the data and planned additional studies. R. Lin development methods to generate the monolayers and prepared the monolayers used. G. McNamara helped standardize the fluorometric studies and establish best way to quantitate intracellular pH. M. Donowitz devised concept of studies, planned experiments, interpreted results, prepared the manuscript and provided funding for the study. All authors helped in manuscript preparation.

Disclosure Statement

The authors declare that no conflict of interests exists.

Results: A similar rate of intracellular alkalization by Na^+ addition in cells expressing NHE3 and by Cl^- removal in cells expressing DRA was found in mOrange2 expressing cells compared to the same cells loaded with BCECF-AM, both using the same pH calibration with K^+ /Nigericin. Using mOrange2 as the pH sensor, NHE3 basal activity was quantitated and shown to be inhibited by forskolin and stimulated by dexamethasone, and DRA was oppositely shown to be stimulated by forskolin, responses similar to results found using BCECF-AM.

Conclusion: This study demonstrates that mOrange2 protein can be an effective alternate to BCECF-AM in measuring intracellular pH (preferred setting Ex520nm, Em 563nm) as affected by NHE3 and DRA activity, with the advantage, compared to AM ester dyes, that genetic expression can provide uniform expression of the pH sensor.

Keywords

Intracellular pH; mORANGE2; NHE3; DRA

Introduction

Diarrhea related diseases remain a major cause of morbidity and mortality worldwide. Reduction of intestinal Na^+ absorption occurs in almost all diarrheal diseases. The epithelial Na^+/H^+ exchanger SLC9A3 (NHE3) is linked to the $\text{Cl}^-/\text{HCO}_3^-$ exchanger SLC26A3 (DRA) to produce the electroneutral NaCl absorptive process in the mammalian small intestine and colon. Both NHE3 and DRA are acutely regulated as part of the neutral NaCl absorptive process, although this regulation appears to be coordinated but with different characteristics. In all cells in which it has been studied, NHE3 regulation mimics the regulation of neutral NaCl absorption, being active under basal conditions and being both inhibited and stimulated in cell models by various mimics of digestive physiology. NHE3 is divided into two distinct domains: N-terminus transmembrane domain which perform Na^+/H^+ exchange, while the C-terminal cytosolic domain is regulatory and plays an important role in trafficking.

NHE3 and more recently DRA activities are usually measured indirectly by measuring the rate of alkalization of the intracellular space using the pH sensitive dye, BCECF-AM, with consideration of intracellular buffering capacity. Recent advances in fluorescent proteins have paved the way to use them to measure intracellular pH. While loading via coupling to acetoxy methyl esters in most cells has produced adequate loading of BCECF, this has been difficult in some cells, particularly epithelial cells, and loading may not be uniform. This led us to seek an alternate pH sensor for physiologic studies, which could be expressed uniformly, ideally a genetically encoded sensor. mOrange2 is a 26.8 kDa monomer protein derived from mOrange mutations at four different sites (Q64H/F99Y/E160K/G196D) that significantly increased its photo-stability ($t_{1/2}$ is 228 sec). mOrange2 has a pK_a value of 6.5 which is suitable for measuring pHs between pH 6.0 and above 7.0, which is the pH range over which both NHE3 and DRA are active.

Second messengers normally inhibit activity of NHE3, while growth factors and some hormones are stimulatory. DRA is also regulated by second messengers and altered as part of diarrheal diseases in the intestine. Specific inhibitors of both NHE3 and DRA are

available [1, 2] and a small molecule stimulator of NHE3 has also been developed that has the potential for treatment of diarrheal disease [3].

FRT (Fisher rat thyroid) cells have been used as an epithelial cell model to study CFTR and develop drugs to inhibit DRA and both stimulate and inhibit CFTR [4, 5]. We demonstrate that mOrange2 is an effective and sensitive pH sensor probe that can be genetically expressed and used to quantitate NHE3 and DRA activity in epithelial cells.

Materials and Methods

Cells and plasmids

FRT (Fischer rat thyroid) epithelial cells were grown and maintained in DMEM-F12 medium supplemented with L-Glutamine, 15 mM HEPES, 10% fetal bovine serum and 50 U/ml penicillin/streptomycin at 37°C in 5% CO₂ atmosphere. The mOrange2 construct was made by cloning synthetic mOrange2 seq. (mammalian codon optimized, sequence ID: DQ336159.1) into pCDNA3.1/Zeo (+) atggtgagca agggcgagga gaataacatg gccatcatca agggattcat ggcgtcaag gtgcatgagg agggctccgt gaacggccac gacttcgaga tcgagggcga gggcgagggc cccccctacg agggcttca gaccgtaag ctgaaggtga ccaaggggtg cccctgccc ttcgctggg acatcctgtc ccctattc acctacggt ccaaggccta cgtgaagcac cccgccgaca tccccgacta ctcaagctg tectccccg agggcttcaa gtgggagcgc gtgatgaact acgaggacgg cggcgtggtg accgtgacc aggactcctc cctgcaggac ggcgagtca tctacaaggt gaagctgccc ggcaccaact tcccctcga cggccccgtg atgcagaaga agaccatggg ctgggagggc tctccgagc ggtatgacct caggagcgtt gcctgaagg gcaagatcaa gatgaggctg aagctgaagg acggcggcca ctacacctc gagtcaaga ccactaca ggccaagaag cccgtgcagc tccccggcgc ctacatcgtc gatcatcaagt tggacatcac ctcccacaac gaggactaca ccatcgtgga acagtacgaa cgcgccgagg gccgccact caccggcggc atggcagcagc gtacaagtg a

vector (Epoch Life Science, Inc, Sugar Land, TX 77496). FRT cells were transfected with pCDNA 3.1/mOrange2(Zeo)+ vector and selected with zeocin (10 µg/ml). Single clones of FRT/mOrange2 cells were isolated microscopically (Keyence microscope) based on the brightest mOrange2 expression. Selected clone of FRT/mOrange2 cells were transfected with pCDNA3.1/HA-NHE3 plasmid with G418 as the selection marker. Using two selection markers (Zeocin and G418), high HA-NHE3 expressing single clones were selected based on immunoblot analysis.

Microscopic imaging of mOrange2 to measure NHE3 activity

FRT cells transiently or stably expressing mOrange2 and HA-NHE3 were seeded into glass bottom dishes (FluoroDishes, WPI, Inc) (1×10^5 cells/well) and grown for 4–5 days. To examine the pH sensing ability of mOrange2, cells were incubated with 20 mM NH₄Cl (108 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 25 mM glucose, 20 mM HEPES, and 40 NH₄Cl, pH 7.4) at 37°C for 15–20 min and washed three times with TMA⁺ solution (130 mM tetramethylammonium chloride, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 25 mM glucose, and 20 mM HEPES, pH 7.4). Adding NH₄Cl causes intracellular alkalization and its removal causes rapid intracellular acidification which was used for initiating pH recovery studies [6]. The cells in glass bottom dishes covered with 100 µl TMA solution were studied on the microscope. Live images

of mOrange2 fluorescence were obtained at Ex_{514nm} and $Em_{545-614nm}$ every second with an Olympus FV3000RS confocal microscope (20x/0.75na objective) using the resonance mode at 37°C with cells in an OkoLab stage top plus transparent shroud incubator. After baseline fluorescence was established, 900 μ l Na^+ solution (138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4 , 25 mM glucose, and 20 mM HEPES, pH 7.4) was added. A time series was acquired with 512×512 pixels, 16 line average, 300×300 - μ m field of view for 300 time points at ~1 s per time point. Addition of Na^+ activates NHE3 activity and the intensity of mOrange2 fluorescence increased with intracellular alkalization.

NHE3 activity measured in a computerized fluorometer

Na^+/H^+ exchange activity was determined in the FRT, FRT/HA-NHE3, or FRT/mOrange2/HA-NHE3 cells grown on glass cover slides for 3–5 days. Cells without mOrange2 were loaded with BCECF-AM (10 μ M), as described [7]. Cells were incubated in 20 mM NH_4Cl for 15–20 min at 37°C. The slides were mounted in a cuvette that allowed apical and basolateral perfusion, placed in a fluorometer (Horiba-Photon Technology, Lawrenceville, NJ), and perfused with TMA⁺ solution to rapidly remove Na^+ in addition to NH_4^+ for intracellular acidification. After 2–3 min, the apical TMA⁺ medium was replaced with Na^+ medium perfused at 1ml/min. In studies of regulation of transport, cells were pretreated with forskolin (10 μ M, 20 min pretreatment) or dexamethasone (1 μ M, 5 hours pretreatment). HOE-694 (10 μ M) was used in the TMA⁺ and Na^+ solutions to inhibit endogenous NHE1 activity. In cell loaded with BCECF-AM, changes in pH_i were monitored by recording emission alternating between 440 and 490 nm with excitation at 530nm. In cells with mOrange2, pH_i was monitored by recording the emission signal at $Em_{564\pm 3nm}$ after excitation at $545\pm 3nm$. The emission fluorescence was calibrated to pH_i with the high K^+ /nigericin method on each coverslip, as described previously [8, 9]. Initial rates of Na^+ -dependent intracellular alkalization were calculated for a given pH_i over the first 1 min (within the linear phase) of Na^+ exposure and expressed as pH/ t . Calculation used Origin 8.0 software (OriginLab, Northampton, MA). Means \pm SE were determined from at least three experiments. Since excitation and emission spectra of mOrange2 have significant overlap, the best excitation wave lengths were empirically determined at which emission most directly correlated with intracellular pHs between 6.0 to 8.0.

Measuring Cl^-/HCO_3^- exchange activity

Cl^-/HCO_3^- exchange activity was measured fluorometrically using mOrange2 or, as previously described, with BCECF-AM [10]. Measurements were in mOrange2 expressing cells or cells without mOrange2 but loaded with BCECF-AM (10 μ M) in Na^+ solution (138 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 1 mmol/L NaH_2PO_4 , 10 mmol/L glucose, 20 mmol/L HEPES, pH 7.4) for 20–30 minutes at 37°C and mounted in a fluorometer. Cells were perfused with Cl^- solution (110 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 10 mmol/L glucose, 25 mmol/L $NaHCO_3$, 1 mmol/L amiloride, 5 mmol/L HEPES, 95% O_2 /5% CO_2) or Cl^- -free solution (110 mmol/L Na-gluconate, 5 mmol/L K-gluconate, 5 mmol/L Ca-gluconate, 1 mmol/L Mg-gluconate, 10 mmol/L glucose, 25 mmol/L $NaHCO_3$, 1 mmol/L amiloride, 5 mmol/L HEPES, 95% O_2 /5% CO_2) with a flow rate of 1 mL/min. The switch between Cl^- solution and Cl^-

-free solution causes HCO_3^- uptake across the cell membrane performed by $\text{Cl}^-/\text{HCO}_3^-$ exchanger(s), and the resulting change in pH_i was recorded. Multiple rounds of removing/replenishing extracellular Cl^- were performed to measure the $\text{Cl}^-/\text{HCO}_3^-$ exchange activity under basal conditions as a time control as well as in the presence of forskolin (10 μM). The cells were exposed to forskolin for at 4–5 minutes before their effects on $\text{Cl}^-/\text{HCO}_3^-$ exchange activity was determined. At the end of each experiment, pH_i was calibrated using the same K^+ /nigericin clamp solutions as used for the Na^+/H^+ exchange determinations. The rate of initial alkalization after the switch from Cl^- solution to Cl^- -free solution over the first 1 min (within the linear phase) was calculated using Origin 8.0 software (OriginLab, Northampton, MA).

Immunoblot analysis

FRT/mOrange2/HA-NHE3 stable cells or FLAG-DRA transfected cells were rinsed 3 times with phosphate-buffered saline and harvested in phosphate-buffered saline by scraping. Cell pellets were collected in 1.5-ml Eppendorf tubes by centrifugation at 5000 rpm for 5 minutes. Cells were mixed with HEPES lysis buffer containing protease inhibitors (20 mM HEPES, pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4 and 1% Triton X-100) and homogenized by passing them through a 1-ml syringe/26-gauge needle (20X) and rotated for ~30 minutes at 4°C to maximize solubilization. After removal of insoluble cell debris by centrifugation (10,000 rpm \times 10 min), the protein concentrations were measured with the Bio-Rad protein assay, Bradford dye-binding method. Total lysate protein 30 μg , was incubated with Laemmli sample buffer at 37°C for 15 minutes, separated by 10% SDS-PAGE, and transferred onto a nitrocellulose membrane. The blot was blocked with 5% nonfat milk, probed with primary antibodies against HA (mouse monoclonal, Biolegend, Cat# MMS-101P, 1:1000 dilution) or FLAG (mouse monoclonal, Sigma-Aldrich, Cat# F1804, 1:2000 dilution), glyceraldehyde-3-phosphate dehydrogenase (mouse monoclonal, Sigma-Aldrich, Cat# G8795, 1:5000 dilution) overnight at 4°C, followed by IR-dye secondary antibody against mouse IgG (1:10,000) for 1 hour at room temperature. Protein bands were visualized using an Odyssey CLx system and quantitated with Image Studio software (LI-COR Biosciences, Lincoln, NE).

Results

Endogenous and exogenous Na^+/H^+ exchange activity in FRT cells

FRT cells were chosen to establish a cell model for the NHE3 activity assay using mOrange2 as a pH sensor probe, based on their previous use for transport assays and drug development [4, 5]. Endogenous Na^+/H^+ exchange activity was quantitated in wild type FRT cells using BCECF-AM dye. FRT cells had a large amount of Na^+/H^+ exchange activity (0.45 pH/min) which was nearly entirely abolished in the presence of 10 μM HOE-694 (Fig. 1A), consistent with this representing NHE1. FRT cells with HA-NHE3 expression had a higher Na^+/H^+ exchange activity (0.69 \pm 0.08 pH/min). In the presence of 10 μM HOE-694, Na^+/H^+ exchange activity was reduced to ~half (0.34 \pm 0.03 pH/min) (Fig. 1B, C). These experiments indicate that FRT cells have endogenous Na^+/H^+ exchange activity, most probably due to the presence of NHE1, which was inhibited by 10 μM HOE-694. Exogenous expression of HA-NHE3 represents the difference between total

Na⁺/H⁺ exchange and that after HOE-694 exposure and the results indicate this was a modestly overexpressing model.

Expression and testing mOrange2 as pH sensor protein

The pH sensing ability of mOrange2 as a genetically modified pH sensor protein was evaluated in the FRT/HA-NHE3 cells. FRT/HA-NHE3 cells grown in the glass bottom dish were transiently transfected with pCDNA/mOrange2 plasmid. After serum starvation and NH₄Cl/TMA acidification, live cell imaging was begun with Na⁺ media addition. A rapid Na⁺ dependent change in fluorescent intensity of mOrange2 was observed due to intracellular alkalinization by Na⁺/H⁺ exchange activity (Fig. 2A). A time series acquired with 512 × 512 pixels for 300 time points at ~1 s per time point in Olympus FV30000RS confocal microscope was quantitated by MetaMorph. Maximum mOrange2 intensity was observed within 3–4 min of Na⁺ addition (Fig. 2B). This observation confirms that mOrange2 senses intracellular pH change in FRT/HA-NHE3/m-Orange2 cells.

Stable cell lines of FRT/HA-NHE3/mOrange2

Several single clones of FRT/HA-NHE3/mOrange2 were manually selected that had the highest percent (>95%) of mOrange2 positive cells and high, uniform expression of HA-NHE3. Expression of HA-tagged NHE3 was examined in FRT/mOrange2 cells by western blot which is shown in Fig. 3A. Two clones #6 and #17 were examined microscopically to evaluate the change of mOrange2 intensity with intracellular alkalinization by NHE3. NHE1 expression in cells not expressing NHE3 is shown in the upper panel of Fig. 3B, while clones with high NHE3 expression and with NHE1 inhibited by 10 μM amiloride are shown in the other two panels. NHE3 clones #6 and #17 were studied further in the presence of 10 μM amiloride (Fig. 3C). Maximum alkalization with Na⁺ exposure, was observed within 2 to 3 minutes. We then tested the effect of forskolin on NHE3 activity indicated by a change in mOrange2 intensity microscopically. Forskolin treatment reduced the intensity of mOrange2 (~45%) compared to untreated cells (Fig. 3C shows triplicate traces of cells from a single experiment). This result indicates that using mOrange2 as pH sensor protein can reproducibly detect Na⁺/H⁺ exchange activity in live cells under basal and inhibited conditions microscopically.

Emission scan for mOrange2 fluorescence sensitivity to intracellular pH of FRT/HA-NHE3/mOrange2 cells with PTI system

Excitation and emission spectra of mOrange2 have significant overlap. Therefore, we determined the excitation wavelength at which there was lowest overlap on emission spectra of mOrange2 fluorescence while still providing a detectable signal at different intracellular pHs. Excitation at 520±3 nm had very little overlap with mOrange 2 emission. The sensitivity of mOrange2 to different intracellular pHs was determined. FRT/HA-NHE3/mOrange2 cells grown on glass cover slip were perfused with K-clamp solution at indicated pHs and emission spectra were collected between 545–580 nm (maximum 564 nm) at 520nm excitation. As shown in Fig. 4A, mOrange2 is very sensitive at intracellular pH range between 6.0 to 8.0. mOrange2 is insensitive at pH 5.5 and less sensitive at pH above 8.0. A pH curve of mOrange2 fluorescence (emission 563 nm) in FRT/HA-NHE3/mOrange2 cells

is shown in Fig.4B. mOrange2 fluorescence intensity is linear between pH 6.0 and pH 8.0, with the estimated K_d of ~ 6.7 pH.

NHE3 activity quantitation using mOrange2 as pH sensor protein using a computerized fluorometer

Whether mOrange2 could allow quantitation of NHE3 activity using dual excitation and single wave length emission in a fluorometer was determined in FRT/HA-NHE3/mOrange2 cells. Cells were grown on glass slides for 4–5 days, serum starved for ~ 2 hours and then bathed in 25 mM NH_4Cl for 15 min. Cells were then perfused with TMA to acidify the intracellular pH, which after reaching a constant intensity, was then exposed to Na^+ solution which caused rapid alkalization. Fig. 5A shows the intensity of emission at 565 ± 3 nm with 545 ± 3 nm excitation under basal conditions in FRT cells expressing mOrange2 without or with HA-NHE3 stably expressed, with all studies done in the presence of 10 μM HOE-694. Results were similar with excitation 520 ± 3 nm. Cells expressing HA-NHE3 showed a rapid increase of mOrange2 intensity representing intracellular alkalization with Na^+ perfusion. Cells without HA-NHE3 had negligible change in mOrange2 intensity with Na^+ perfusion.

We then tested the effect of 10 μM forskolin exposed for 20 min before Na^+ addition on NHE3 activity in the same FRT/mOrange2/HA-NHE3 cells. Forskolin treatment inhibited NHE3 activity by $\sim 47\%$ compared to basal activity as shown in Fig. 5B.

Stimulation of NHE3 by Dexamethasone

To determine if NHE3 stimulation could be quantitated with mOrange2, we used short-term exposure to dexamethasone that is known to stimulate NHE3 activity, without altering the amount of NHE3 protein over this time [11, 12]. Treatment of cells with 1 μM dexamethasone for 5 hours stimulated NHE3 activity in FRT/HA-NHE3/mOrange2 cells in the presence of 10 μM HOE-694. Dexamethasone treatment stimulated NHE3 activity by 41% and 48% stimulation in Clone # 6 and # 17 respectively (Fig. 6). These results indicate that NHE3 activity under basal, inhibited and stimulated conditions can be quantitated with mOrange2, producing quantitative results similar to those found using BCECF-AM [11, 12].

DRA activity quantitation using mOrange2 as pH sensor protein using a computerized fluorometer

DRA activity was quantitated in FRT/HA-NHE3/mOrange2 cells under basal and forskolin treated conditions. Since FRT cells do not endogenously express significant amounts of DRA, FRT/HA-NHE3/mOrange2 cells were transiently transfected with the pcDNA/FLAG-hDRA plasmid. Expression of FLAG-DRA and HA-NHE3 in FRT cells evaluated by immunoblot is shown in Fig. 7A. DRA activity was measured in the fluorometer using mOrange2. Traces of intracellular alkalization driven by HCO_3^- entry after apical Cl^- removal under basal and forskolin conditions are shown in Fig. 7B and calculated DRA activity is shown in Fig. 7C. DRA activity was stimulated 50% by forskolin treatment compared to basal activity. We then examined DRA activity in the FLAG-DRA transfected cells loaded with BCECF-AM dye. Basal and forskolin stimulated DRA activities are as shown in Fig. 7D and 7E. DRA activities measured using BCECF and mOrange2 were

similar. These observations indicate that both NHE3 and DRA activities can be measured in live cells using mOrange2 as a pH sensor.

Discussion

We have established a convenient assay to measure intracellular pH change due to NHE3 or DRA activity in epithelial cells using a genetically modified protein, mOrange2 as a pH sensor probe which can be uniformly expressed in the cells studied. Recent development and description of its properties [13–15] encouraged us to investigate whether it could be used to measure NHE3 and DRA activity in live cells. mOrange2 is a fluorescent protein, derived from mOrange after 4 single mutations by which photo-stability is increased significantly [13]. The pKa value of mOrange2 is 6.5, which is within the pH range that both NHE3 and DRA are active. There are multiple positive properties of mOrange2 to serve as a pH indicator in live cells; these include uniform labeling of cells, resistance of bleaching, and, since it is a protein that is expressed intracellularly, dye leaking as occurs with AM dyes does not occur. In addition, it can be targeted to specific subcellular locations by including association sequences.

In FRT cells, using mOrange2 as pH sensor, Na⁺/H⁺ exchanger induced intracellular alkalization was seen microscopically by changes in mOrange2 fluorescence; and changes in the fluorescence intensity occurred with both inhibition and stimulation by forskolin and dexamethasone, respectively. Observations made in the current studies support that mOrange2 can be used to measure pHi in a 96 well format, which could be used for drug screening or other large-scale studies. Recommended settings, excitation 520 nm, emission 563 nm.

NHE3 activity measured in FRT/HA-NHE3 cells using the fluorometer and either BCECF or mOrange2 revealed similar rates of pH/min, a surrogate for rates of Na⁺/H⁺ exchange activity. Using mOrange2, NHE3 activity was inhibited 30 to 50% with forskolin treatment compared to untreated cells and stimulated 40–50% by dexamethasone treatment. These were results similar to those reported previously in studies using BCECF [9–11]. Similarly, the measurements of DRA driven Cl⁻/HCO₃⁻ exchange rates measured in the same cells using BCECF and mOrange2 produced very similar basal activity measurements as well as extent of stimulation by forskolin treatment. These results strongly support the usefulness of mOrange2 for study of regulation of pHi in living cells and for measuring transport rates over the pH range delineated by its pKa.

There are multiple reports of mOrange2 use to measure pHi in the live cells. Li et al. [14] developed an assay with a pH-sensitive fluorescent IRAP-mOrange2 probe to measure insulin-stimulated GLUT4 translocation from GLUT4 storage vesicles (GSVs) to the plasma membrane. Egashira et al. [15] measured re-acidification kinetics during synaptic vesicle (SV) recycling in cultured hippocampal neurons using a mOrange2-based probe. This report is the initial documentation of its usefulness in quantitating NHE3 and DRA activity and its use in epithelial cells.

In summary, we have established mOrange2 as an efficient pH-sensitive fluorescent probe to measure NHE3 and DRA activity in epithelial cells, which would be useful in the study of NHE3 and DRA regulation in individual experiments and high throughput studies.

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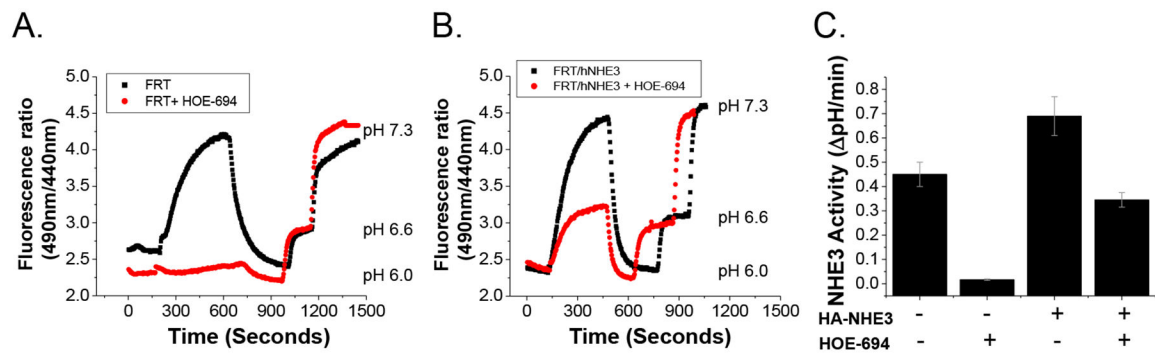
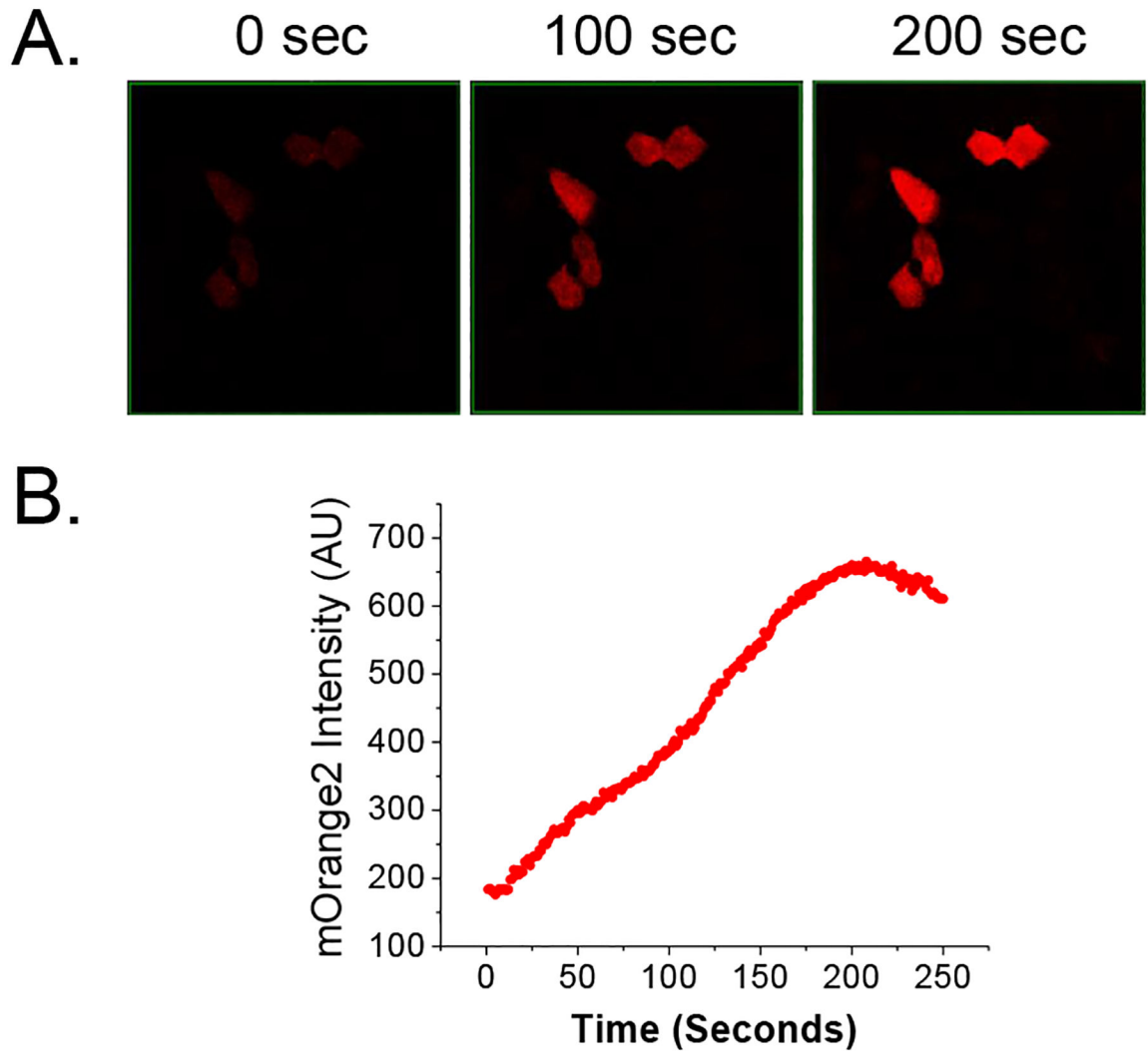
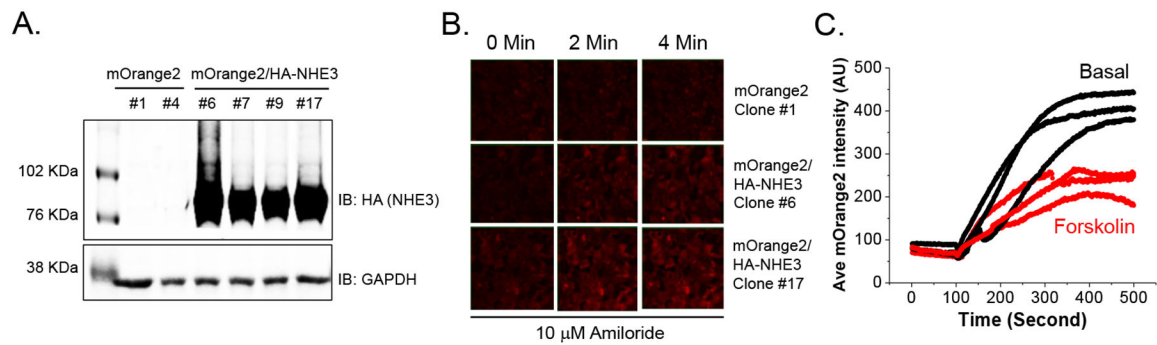


Fig. 1.

Endogenous and exogenous Na^+/H^+ activity in FRT cells. Na^+/H^+ exchange activity measured in FRT cells (A) and in FRT/HA-NHE3 cells (B) in the absence (squares) and presence of 10 μM HOE-694 (circles) using a computerized fluorometer with BCECF-AM as the pH indicator dye. NHE3 activity was measured from the initial rates of Na^+ -dependent intracellular alkalization. Calculated NHE3 activities are shown in bar graph (C).

**Fig. 2.**

Testing mOrange2 as pH sensor protein in FRT cells. FRT/HA-NHE3 cells grown on the glass bottom dishes were transfected with the pCDNA/mOrange2 plasmid. NH_4Cl and TMA treated cells were monitored in an Olympus FV3000RS confocal microscope to observe Na^+/H^+ exchanger driven intracellular alkalization as detected by mOrange2 as pH sensor. A time series was acquired with 512×512 pixels, $300 \times 300\text{-}\mu\text{m}$ field of view for 300 time points at ~ 1 s per time point. Addition of Na^+ solution stimulated NHE3 activity and intensity of mOrange2 fluorescence increased with intracellular alkalization. Still images of mOrange2 intensity at indicated time points are shown (A). Intensity of mOrange2 fluorescence was quantitated by Meta-Morph and plotted (B).

**Fig. 3.**

Single clones of FRT cells stably expressing HA-NHE3 and mOrange2 were isolated and studied after expansion. After several rounds of screening, a few single clones of FRT/mOrange2/HA-NHE3 were selected for study of NHE3 activity. HA-NHE3 expression in FRT/mOrange2 cells was verified by western blot analysis using HA monoclonal antibody and GAPDH as a loading control (A). Microscopically, NHE3 driven intracellular alkalization was visible with mOrange2 in Clone #6, #17 in the presence of 10 μ M amiloride but was not visible in Clone #1 which does not express NHE3 and was then used as a negative control. Confocal still images taken at different time points are shown in (B). In a mixed clone of FRT/mOrange2/HA-NHE3 cells, the effect of 10 μ M forskolin on NHE3 driven intracellular alkalization was examined microscopically. Forskolin treatment of cells for 15 minutes significantly decreased mOrange2 fluorescence intensity compared with untreated cells. Microscopic data of mOrange2 fluorescence intensity over time of three sets of cells for basal and Forskolin treatment from a single experiment were calculated using Metamorph and are plotted (C).

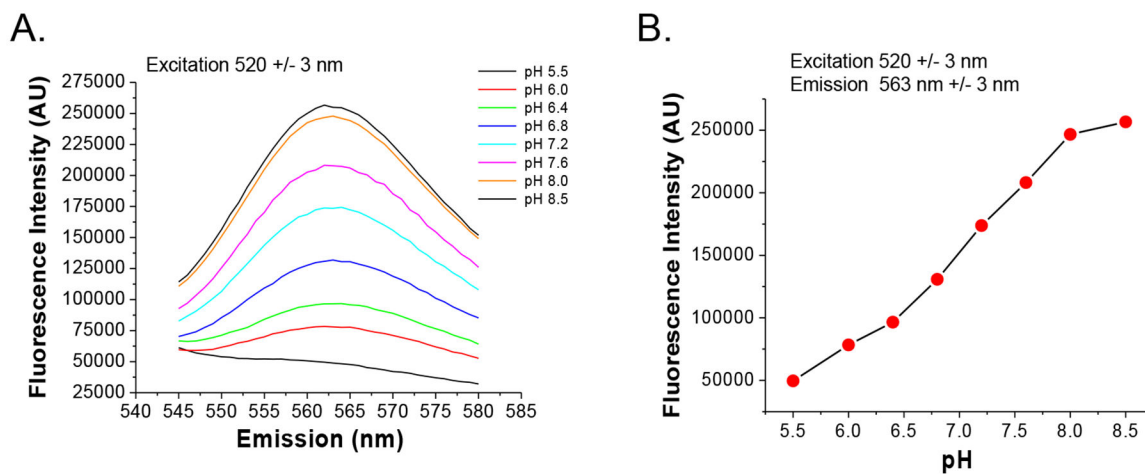


Fig. 4. Emission spectrum of mOrange2 at different intracellular pHs. FRT/mOrange2/HA-NHE3 cells were grown on glass cover slides to confluent monolayers and were perfused with high potassium/nigericin solutions of indicated pH for at least 5 min to stabilize intracellular pH. Excitation wave length was set at 520 ± 3 nm with emission at 565 ± 3 nm. Emission spectra of mOrange2 at indicated pHs (5.5 to 8.5) are shown (A). Maximum intensity of mOrange2 emission at different pHs was 563 nm. Intensity of mOrange2 emission at 563 nm was plotted against corresponding intracellular pHs.

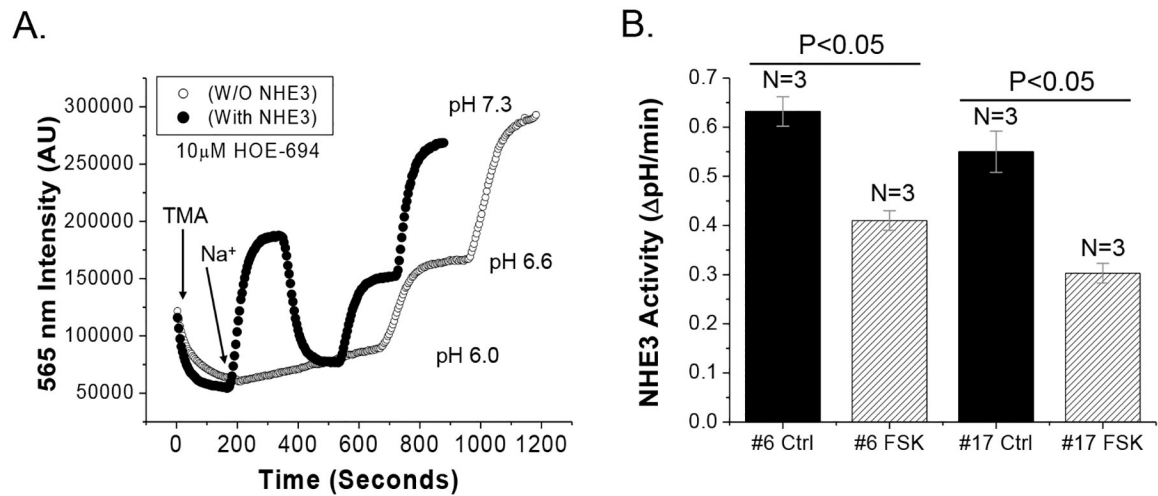


Fig. 5. Measurement of NHE3 activity in FRT/mOrange2/HA-NHE3 cells using mOrange2 as pH sensor protein in a computerized fluorometer. FRT/mOrange2/HA-NHE3 cells were grown on glass slides in confluent monolayers and NHE3 activity was measured in the presence of 10 μ M HOE-694. Traces of NHE3 activity in FRT/mOrange2 cells without (empty circle) and with HA-NHE3 expression (filled circle) are shown (A). NHE3 activity was measured from the initial rates of Na-dependent intracellular alkalization and calibrated with standards at three pHs. Calculated NHE3 activity under basal conditions and after 10 μ M forskolin treatment of clones #6 and #17 of FRT/mOrange2/HA-NHE3 cells is shown in bar graphs (B). Three experiments of each clone were averaged and mean \pm SEM and p values are shown.

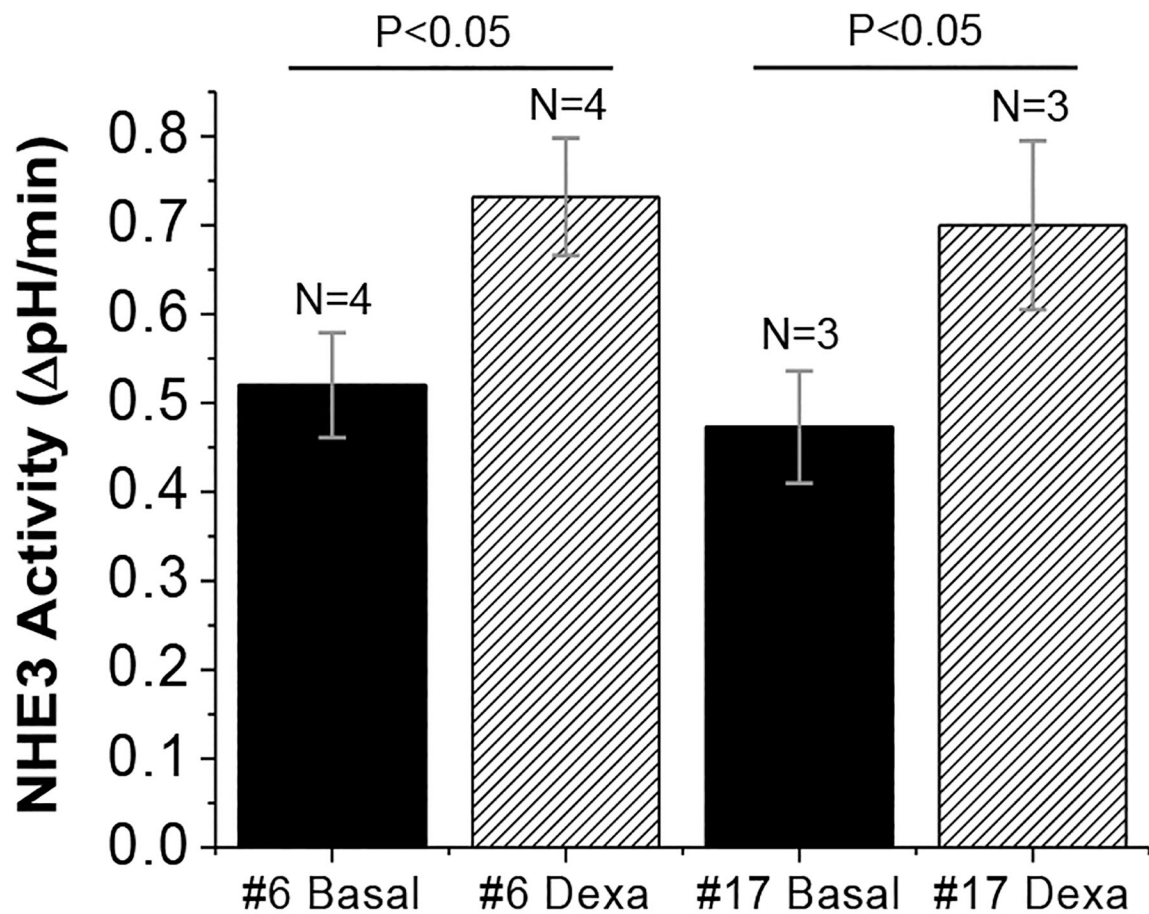


Fig. 6. Dexamethasone stimulates NHE3 activity in FRT/mOrange2/HA-NHE3 cells. NHE3 activity was measured in clones of FRT/mOrange2/HA-NHE3 cells treated with dexamethasone using mOrange2 as pH sensor probe in a computerized fluorometer. Cells treated for 5 hours with 1 μ M dexamethasone stimulated NHE3 activity in both clones #6 and #17 compared to basal activity. n, number of experiments. p values are comparison with basal NHE3 activity (paired t tests).

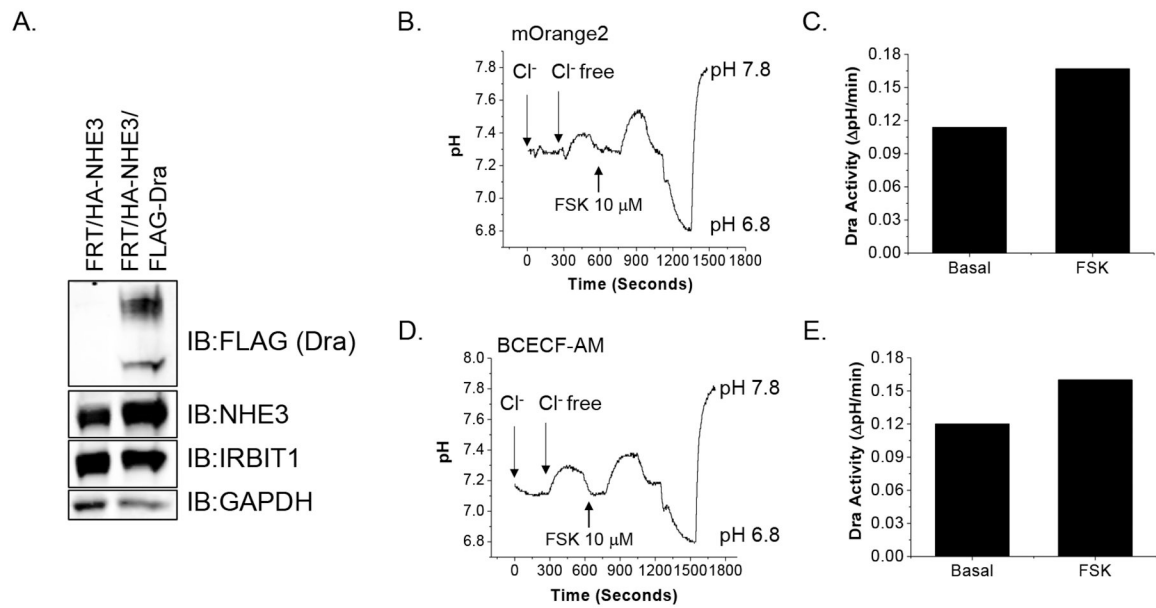


Fig. 7. DRA activity in FRT/mOrange2/HA-NHE3 cells transfected with FLAG-DRA. FRT/mOrange2/HA-NHE3 cells were grown on glass slides and transfected with pcDNA/FLAG-DRA plasmid. Expression of FLAG-DRA was verified by western blot analysis using anti-FLAG antibody (A). Traces of DRA activity under basal conditions (first peak) and after 10 μM forskolin (2nd peak) using mOrange2 as the pH sensor in a computerized fluorometer are shown (B). DRA activity under these conditions is shown in the bar graph (C). For comparison, we measured DRA activity in FLAG-DRA transfected FRT/mOrange2/HA-NHE3 cells using BCECF-AM as pH sensor dye. Traces of basal and forskolin treated DRA activity are shown in (D) and calculated DRA activity under basal and forskolin stimulated condition is shown in the bar graph (E).