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Monitoring Intracellular pH Changes in Response to Osmotic Stress and Membrane Transport Activity Using 5-Chloromethylfluorescein

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ABSTRACT Intracellular free H^+ concentration (pHi) responds to numerous extracellular stimuli. The use of fluorescent indicator dyes to measure pHi is strongly influenced by the ability of target cells to retain activated dye within the cytoplasmic compartment. Here, 3 pH-sensitive indicator dyes—acetoxymethyl (AM) esters of SNARF-1 and BCECF, and the thiol-reactive 5-chloromethyfluorescein (CMFDA)—were examined for monitoring pHi. The stability of pH measurements was strongly affected by temperature, cell type, indicator dye, and use of transport inhibitors to prevent dye export. Cellular retention of CMFDA, which forms covalent complexes, was sufficient to permit monitoring of transient pHi changes over extended time periods in a multi-well plate assay format. In human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) cells, increasing osmotic pressure caused a significant rise in pHi. In contrast, activation of native or transfected β-adrenergic, cholinergic, and d and m opioid receptors did not measurably affect pHi in HEK293 cells. Decreases in pHi were observed in CHO cells expressing the human H^+/peptide transporter PEPT1 upon addition of dipeptide substrates. The use of CMFDA in multi-well formats should facilitate study of osmotic and transport activity and screening for drugs that affect pHi.

KEYWORDS: intracellular pH, fluorescent pH-sensitive, dyes, Chinese hamster ovary cells, human embryonic, kidney cells, human colon adrenocarcinoma cells, hypertonic stress.

INTRODUCTION Changes in intracellular pH (pHi) and intracellular Ca^{2+} (Ca^{2+} i) concentrations play a major role in cellular processes. Several reports have demonstrated that pHi and Ca^{2+} i are interdependent in various cell types (ie, changes in one system affect the activity of the second). [1-6] A previous report from our laboratory described a rapid and sensitive assay to measure intracellular calcium from multi-well plates using a fluorometer equipped with on-line injectors. [7] Similarly, Grant and Acosta [2] have described a ratiometric assay of pHi, using BCECF fluorescent dye and a multi-well plate reader. However, their assay was performed at room temperature rather than 37°C, required a ratiometric approach with 2 emission wavelengths measured, and did not permit rapid analysis of pH changes in the millisecond range.

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The purpose of this study was to develop a rapid fluorimetric assay using the multi-well plate format to measure intracellular pH change in live cells and examine pHi changes induced by various effectors. The principal advantages of using pH-sensitive dyes over other methods (eg, distribution of weak acids or bases, ^{31}P-NMR spectroscopy, ion-sensitive microelectrodes, pH-sensitive Green fluorescent protein [GFP] mutants) are sensitivity, applicability to many cell lines, and continuous monitoring of rapid kinetic pH changes. However, the main disadvantage is often substantial dye leakage from the cells, especially at 37°C. Since we have observed that intracellular calcium responses can dramatically differ at 20°C and 37°C (unpublished data), one aim of this study was to find a dye providing a stable signal to allow pHi measurements at physiological temperature in several different cell lines. Furthermore, use of a plate reader with dual injectors (control and experimental samples) and the capability to take measurements in the millisecond scale would further enhance the utility of the assay. Last, this approach also enhances the accuracy of single-wavelength measurements, thereby reducing the time required for repeat measurements.

In the present investigation, we have tested the pH-sensitive dyes SNARF-1/AM, BCECF/AM, and CellTracker Green CMFDA as possible candidates to measure intracellular pH in Chinese hamster ovary (CHO), human embryonic kidney (HEK293), Caco-2 cells with respect to stability of cellular fluorescence. The possibility of detecting transient changes in pHi induced by ammonium chloride and propionate was examined. Furthermore, pHi changes generated by hypertonic stress, intestinal dipeptide transport by hPEPT1, and activation of G-protein coupled receptor were investigated.

Our laboratory studies the H^+-coupled peptide transporter, hPEPT1, because of its essential role in the absorption of dipeptides, tripeptides, and peptide-like drugs such as cephalosporins. [8] Previous studies have demonstrated that transport of Gly-Sar is associated with a pHi decrease in PEPT1 cRNA-injected oocytes. [9] Therefore, the stably transfected cell line, CHO/hPEPT1 [10] might be useful for rapid screening of drug substrates by measuring pHi given that transport is associated with the movement of protons across the plasma membrane and intracellular acidification.

This report describes a rapid fluorimetric assay of pHi using a multi-well plate reader format. The procedure can be readily scaled up for rapid throughput screening of a large number of tissue samples.

MATERIALS AND METHODS

Tissue culture and stable cell lines

Human embryonic kidney cells (HEK293; ATCC, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) F12/H16 containing 10% fetal bovine serum (Sigma Chemicals, St Louis, MO), 50 µg/mL streptomycin, and 50 U/mL penicillin. Stable cell lines of HEK293 cells were prepared using cDNA containing the murine µ-opiate receptor, MOR (pRcCMV/MOR) as described elsewhere. [11] Stable HEK293 clonal cell lines were maintained in a medium supplemented with 200 µg/mL of G418 (Bethesda Research Laboratories, Bethesda, MD). CHO (ATCC) were cultured in Ham's F12 medium containing 10% fetal bovine serum, 50 µg/mL streptomycin, and 50 U/mL penicillin. Stable cell lines of CHO cells were prepared by transfecting pCDNA3-hPEPT1, as described elsewhere. [10] Stable CHO clonal cell lines displaying the highest transport of ^{3}H-Gly-Sar (CHO-PEPT1) were cultured in a medium supplemented with 200 µg/mL of G418 for up to 2 months, after which time transport began to diminish. Stably transfected cell lines of CHO and HEK293 cells were obtained by co-transfection with pSG5 (Strategene, La Jolla, CA), containing an insert for the full-length coding sequence of the human M1 receptor, and with pRSVneo (Strategene), as described elsewhere. [12] Human colon carcinoma Caco-2 cells (ATCC) were maintained in Dulbecco's modified Eagle's (DME) H-21 containing 10% fetal bovine serum, 1% nonessential amino acids, 50 µg/mL streptomycin, and 50 U/mL penicillin. All cell lines were cultured in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C.

Instrumentation

A FLUOstar 97 fluorometer multi-well plate reader (BMG LabTechnologies, Inc, Durham, NC) was used to measure intracellular pH. Gain was calibrated to ensure consistency between wells and set to 80% of resting baseline intensity. Excitation and emission were Exc 485, Em 590 nm for carboxy SNARF-1; Exc 500, Em 538 nm for BCECF; and Exc 485, Em 538 nm for CMFDA. Excitation and emission bandwidths were 20 nm and 25 nm, respectively.

Measurements of intracellular pH

HEK293, CHO, and Caco-2 cells were prepared for intracellular pH as follows. Cells were grown to 90% confluence, harvested with trypsin, and immediately quenched with culture medium containing 10% fetal bovine serum, pelleted, and then rinsed once. Pelleted cells were resuspended in fresh medium, allowed to recover under 5% CO_2 at 37°C for 1 hour, rinsed twice with bicarbonate-free Krebs-Hepes buffer (130 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11.7 mM D-glucose, 1.3 mM $CaCl_2$, 10 mM HEPES, pH 7.4) and then loaded with the dye. Dye loading was performed as follows: HEK293, CHO, and Caco-2 cells were incubated with 5 µM 5-(and-6)-carboxy SNARF-1/AM (Molecular Probes, Eugene, OR) for 30 minutes at room temperature in bicarbonate-free Krebs-Hepes buffer (pH 7.4) containing 1% (wt/vol) Pluronic F-127 (Sigma Chemicals, St Louis, MO). HEK293 and CHO cells were loaded with 1 µM 2-,7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF/AM) (Molecular Probes) for 45 minutes at room temperature in buffer, while Caco-2 cells were incubated with 5 µM BCECF/AM for 45 minutes at 37°C. HEK293, CHO, and Caco-2 cells were loaded with 5 µM CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) (Molecular Probes) for 45 minutes at 37°C in buffer. Where indicated, 250 µM (±)-sulfinpyrazone, 10 µM indomethacin, or 10 µM procainamide (Sigma Chemicals) were added to the loading incubation. After loading, cells were rinsed twice with buffer and resuspended in fresh medium, allowed to recover under 5% CO_2 at 37°C for 1 hour (HEK293 and Caco-2 cells) or overnight (CHO cells).

After dye loading with CMFDA and recovery time, the cells were rinsed 3 times with bicarbonate-free Krebs-Hepes buffer (pH 7.4 or 6.0 as indicated), resuspended to a final concentration of 2.5 x 10^6 cells/mL and held at 4°C. The cells were then diluted and distributed evenly (approximately 35 000 cells/well) into an opaque white 96-well plate. Buffer alone or buffer containing test compounds were injected sequentially into separate wells and the fluorescence intensity recorded at 20-second intervals. Five baseline readings were taken at 20-second intervals prior to each injection.

At the end of each experiment, an in situ calibration procedure with nigericin, a H^+/K^+ exchanger, was used to relate the fluorescence intensities at 485 nm to pH value. [13,14] This K^+/H^+ exchanger ionophore sets $[K^+]o = [K^+]i$ and pHo = pHi by exposing the cells to different pH buffers in a depolarizing high K^+ buffer (140 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11.7 mM D-glucose, 1.3 mM $CaCl_2$, 10 mM HEPES, pH 6.0 to pH 8.0, in the presence of 20 µM nigericin). To correct for small variations in cell density and instability of illumination intensity between the calibration and the experiments, the

CMFDA concentration was measured by permeabilizing the cells at the end of each experiment with 0.1% Triton X-100, and adjusting the pH to 11 with KOH.

Determination of dye efflux

To quantify dye leakage, the contributions from leaked and intracellular dye must be separated. Cells loaded with the dye according to procedures described for pH measurements were washed 3 times and resuspended in bicarbonate-free Krebs-Hepes buffer (pH 7.4 or pH 6.0 as indicated) to approximately 250 000 cells/mL. Cell suspension aliquots (200 µL) were divided into microcentrifuge tubes and incubated at 20°C or 37°C for various periods of time. At different time intervals, cells were spun down for 30 seconds. This centrifugation time was short compared with the time scale in which leakage occurs. Then, 150 µL of the supernatant was combined with 50 µL of 80% ethanol-water (vol/vol). Excess supernatant was removed from the cell pellet, and uptake of the dye by the cells was assessed by lysing the cells with 50 µL of 80% ethanol-water (vol/vol). Then the lysate was resuspended in 150 µL of bicarbonate-free Krebs-Hepes buffer (pH 7.4 or pH 6.0 as indicated). The fluorescence intensity of 150-µL aliquots of each supernatant and resuspended cell lysate solutions was measured. Fluorescence intensity of supernatant solutions reflects dye leakage from the cells, and the combined fluorescence intensity from both supernatant and cell lysate was used to define total amount of dye. Fluorescence intensity of controls (no dye) was subtracted from each fraction, and leakage is reported as the ratio of fluorescence intensity (supernatant/total) versus time.

Data Analysis

All data are presented as means ± SD for the indicated number of observations (n). All comparisons were made using the Student *t* -test . $P < .05$ was considered statistically significant.

Table 1. Comparison of SNARF-1, BCECF, and CMFDA leakage

Cell types	SNARF-1		BCECF		CMFDA	
	20°C	37°C	20°C	37°C	20°C	37°C
CHO cells	100	100	68 ± 1	92 ± 1	20 ± 1	38 ± 1
HEK293 cells	39 ± 1	80 ± 1	24 ± 1	100	15 ± 1	35 ± 4
Caco-2 cells	57 ± 1	80 ± 1	50 ± 4	79 ± 3	33 ± 2	52 ± 7

Dye leakage was measured as described in Material and Methods and expressed as percentage of the total after 2 hours at 20°C or 37°C. Experiments were performed in a bicarbonate-free buffer at pH 7.4. Data are expressed as means ± SD (n = 3).

RESULTS

Evaluation of dye leakage

To avoid photodamage, light scatter, and sample autofluorescence, often encountered with high excitation energy dyes, 2 visible light-excitable fluorescent pH indicators, carboxy SNARF-1/AM and BCECF/AM were chosen first. Dye efflux from CHO cells, HEK293 cells, and Caco-2 cells was tested at 20°C and 37°C. As shown in **Table 1** , the carboxy SNARF-1 efflux after 2 hours was large, even at 20°C. Addition of the anion transport inhibitor, sulfinpyrazone, had little effect on the performance of this dye in CHO cells. The efflux of BCECF also exceeded 75% after 2 hours at 37°C but was slightly slower at 20°C.

Previous studies demonstrated functional expression of the efflux pumps, multidrug-resistance-related protein (MRP) and P-glycoprotein (P-gp) in Caco-2 cells, [15] and P-gp in CHO cells. [16] To block efflux, indomethacin (MRP inhibitor), procainamide (P-gp inhibitor), and sulfinpyrazone (anion transport inhibitor) were investigated. While these inhibitors had little effect in CHO cells, the efflux of BCECF was significantly decreased in Caco-2 cells by indomethacin. The largest efflux inhibition was obtained with sulfinpyrazone in HEK293 cells (**Figure 1**). Unfortunately, with carboxy SNARF-1/AM and BCECF/AM, retention at 37°C was still insufficient, even in the presence of inhibitors, for the purpose of this plate-reader assay.

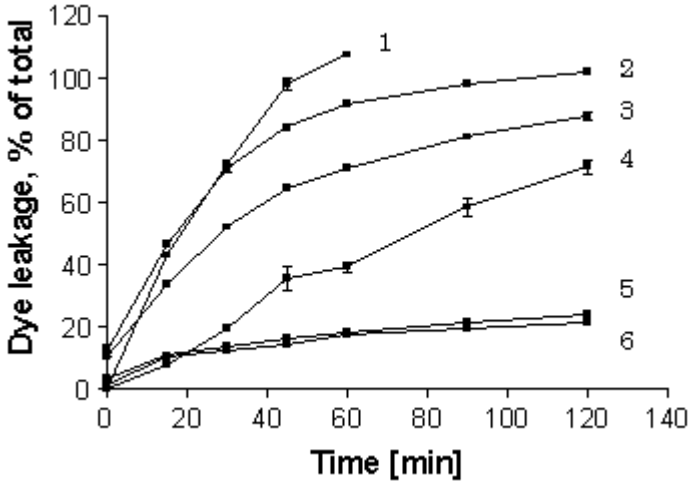

Figure 1. Effect of inhibitors on BCECF leakage in HEK293 cells. Rate of leakage of BCECF, expressed as percentage of the total, from HEK293 cells at pH 7.4 and 37°C, in absence of inhibitor (1), in presence of 10 µM procainamide (2), 10 µM indomethacin (3), 250 µM sulfinpyrazone (4) and at 20°C, in absence of inhibitor (5) and with 250 µM sulfinpyrazone (6). Data are expressed as means ± SD (n = 3).

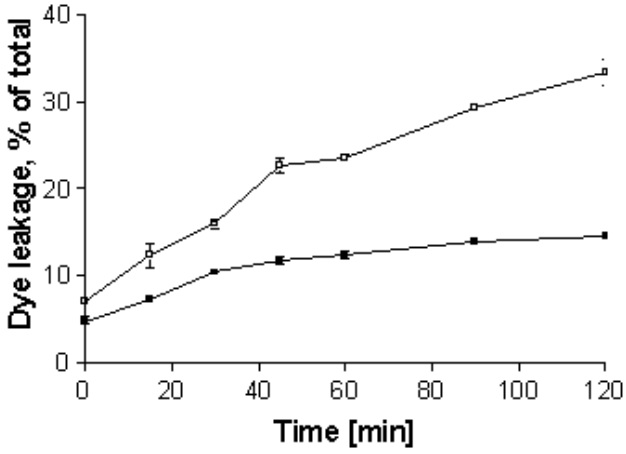

Figure 2. CMFDA leakage in HEK293 cells. Rate of leakage of CMFDA, expressed as percentage of the total, from HEK293 cells at 20°C (solid symbol) and 37°C (open symbol) in bicarbonate-free buffer pH 7.4 in absence of inhibitors. Data are expressed as means ± SD (n = 3).

To overcome the dye-leakage problem, trapping the indicator dye by conjugation to intracellular constituents was attempted. CellTracker Green CMFDA, which possesses a weakly thiol-reactive chloromethyl moiety, reacts with intracellular thiols, primarily glutathione, transforming the probe into a cell membrane impermeant fluorescent dye-thioether adduct, which cannot penetrate cell membranes. The retention of this dye over 2 hours was significantly improved for all the cell lines under study (see Table 1.). After dye loading, the cells must be pelleted and resuspended in fresh medium to ensure complete reaction of the probe. The rate of this reaction, depending on the amount of glutathione S-transferase, can vary between cell types; therefore 2 incubation time periods in fresh medium were tested (ie, 30 minutes and 12 hours). In HEK293 and Caco-2 cells, no differences were found, but in CHO cells, the longer incubation time strongly decreased the rate of dye leakage from 56% ± 3% to 20% ± 1% after 2 hours at 20°C.

Furthermore, in CHO and HEK293 cell lines, the rate of leakage was found to depend on the pH of the extracellular medium, the rate decreasing with increasing external pH. For instance, for HEK293 cells suspended in pH 6.0 buffer, approximately 35% of the CMFDA was located extracellularly 2 hours after resuspension; whereas in pH 7.4, only 15% of the CMFDA was extruded from the cells. On the other hand, in Caco-2 cells, the rate of leakage in external pH 6.0 buffer was similar to that in pH 7.4 buffer (data not shown).

Knowing that the transformed dye, thought to be mostly glutathione-methylfluorescein, is a substrate of MRP, [17] indomethacin was tested for its inhibitory effect on dye efflux. However, no significant difference between control cells and indomethacin-treated cells was observed. Further, sulfinpyrazone and procainamide were also ineffective (data not shown). However, even in the absence of inhibitors, dye retention was significantly improved with CMFDA, particularly at 37°C, as shown in **Figure 2** with HEK293 cells. The amount of dye present outside the cells after 2 hours at 37°C did not allow the use of HEK293 cells for this period without further manipulation. CMFDA was well retained in cells stored at 4°C. Immediately after washing and cell resuspension, the extent of extracellular dye was 10% to 20% depending on the cell line, probably because of leakage during or after washing or lysis. No further efflux was detected within 4 hours; thus, the loaded cells can be kept at 4°C for at least 4 hours. Twenty minutes before the experiment (to allow temperature stabilization), the cells were washed, diluted, and transferred to the multi-well plate. The results show that the CellTracker Green CMFDA is a sufficiently stable fluorescent indicator for protracted pHi measurement in CHO, HEK293, and Caco-2 cells in a multi-well plate reader. Furthermore, the fluorescence response was linear in the range of pH 6 to pH 8.

Calibration of CMFDA in situ

Preliminary results indicated that the treatment time with nigericin was critical to allow complete equilibration of extracellular and intracellular H^+ concentration. As shown in **Figure 3** , the Caco-2 cells needed at least 15 minutes after the addition of 20 µM of nigericin to reach equilibration at pH 6.0. Therefore, the in situ calibration of CMFDA-loaded cells was performed in different pH buffers 20 minutes (for HEK293 and Caco-2 cells) or 30 minutes (CHO cells) after the injection of nigericin. In the 3 cell lines studied, the calibration curve with CMFDA, measured at 485 nm, yielded a linear range from pH 6 to pH 8 (r^2 =0.962). Shown in **Figure 4** is a calibration curve for CMFDA in HEK293 cells that have been exposed to buffers of pH 2 to pH 12.

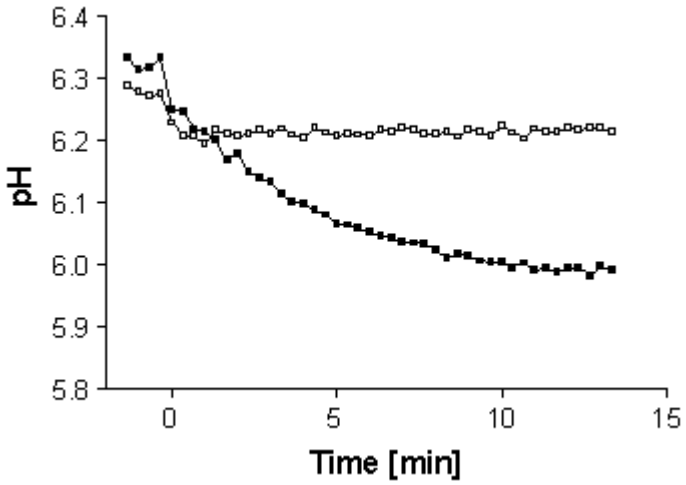

Figure 3. Kinetics of extracellular and intracellular pH equilibration with nigericin.
Representative tracing of intracellular pH changes in Caco-2 cells after injection of 20 µM nigericin (solid symbol) and buffer (open symbol), as a control. In situ calibration was performed in a high K^+ buffer (see Materials and Methods), pH 6.0 and 37°C. Traces shown are representative of 3 similar experiments.

Effects of weak acid and weak base on pHi

Using CMFDA and the multi-well plate format, we examined whether transient changes in intracellular pH could be detected. The addition of a weak base and a weak acid served to illustrate that the expected pHi changes can be observed. Addition of ammonium chloride (20 mM) rapidly increased pHi for CHO, HEK293 (see **Figure 5**), and Caco-2 cells, because permeable NH_3 entered the cells. In the continuous presence of NH_4Cl, pHi returned progressively to the baseline value, since NH_4^+ ions are thought to penetrate the cells gradually and release H^+. The addition of a

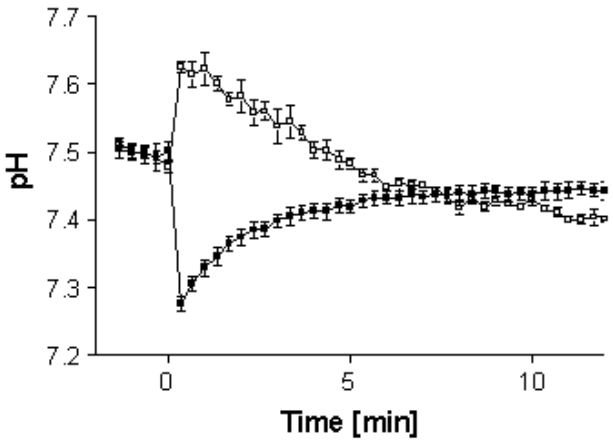

Figure 5. Effect of weak base and weak acid on pHi in HEK293 cells. Representative examples of the effect of 20 mM NH4Cl (open symbol) and 30 mM propionate (solid symbol) on pHi of HEK293 cells in bicarbonate-free buffer pH 7.4, at 37°C. After the cells were loaded with CMFDA, pHi was measured. Each point represents mean ± SD of 3 independent cell-loaded suspensions.

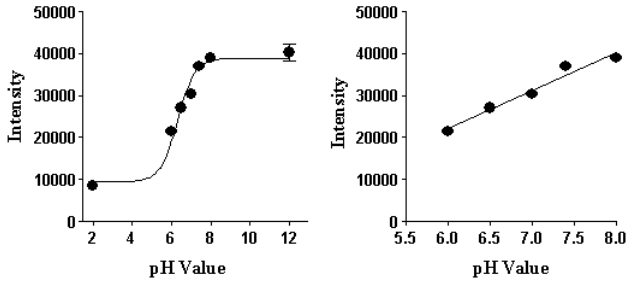

Figure 4. In situ pH calibration curve for CMFDA using extracellular and intracellular pH equilibration with nigericin. A representative calibration curve is shown for HEK293 cells. The calibration curve is linear in the range of pH 6-pH 8.

permeant weak acid, such as propionate (30 mM), caused a reversible intracellular acidification because of proton-release from the neutral weak acid after it entered the cell by nonionic diffusion. Acidification was followed by partial recovery in HEK293 cells (**Figure 5**), as well as in CHO and Caco-2 cells (data not shown), as a result of pHi-regulatory mechanisms. [18-20] As a control, the injection of buffer containing 30 mM NaCl, instead of 30 mM propionate Na^+, had no effect on pHi.

Effects of different osmolytes on pHi

In many, but not all cell types, osmotically shrunken cells can recover near-normal size by a process called regulatory volume increase. The volume recovery is associated with an increase of intracellular Na^+, apparently mediated by the activation of the Na^+/H^+ exchanger, [21] and, therefore, with an increase of pHi.
To investigate whether the effect of hypertonic stress on intracellular pH can be detected by our assay, the medium was made hypertonic by addition of sucrose to a final concentration of 200 mM. In nominally HCO3--

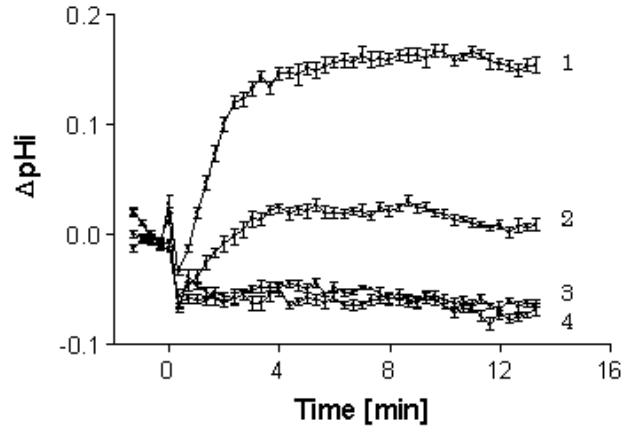

Figure 6. Effect of hyperosmolarity on pHi in HEK293 cells. Representative tracing of the relative change in pHi induced by 200 mM NaCl (1), 200 mM sucrose (2), 200 mM urea (3), and buffer (4). Experiments were performed in bicarbonate-free buffer pH 7.4, at 37°C. After the cells were loaded with CMFDA, pHi was measured. Each point represents mean ± SD of 6 independent cell-loaded suspensions.

free buffer, the addition of sucrose caused pHi to increase 0.08 pH unit in HEK293 cells (**Figure 6**) and 0.16 pH unit in CHO cells, whereas no significant pH rise was observed in Caco-2 cells. To examine whether the pHi increase depends on the membrane permeability of the osmotic compound, 2 other osmolytes were tested. The addition of 200 mM NaCl induced a pHi increase in HEK293 cells (see **Figure 6**) and in CHO cells of 0.22 pH and 0.32 pH unit, respectively, whereas it had no effect in Caco-2 cells. Finally, the addition of a permeant osmolyte, 200 mM urea, did not induce a significant pHi change in the 3 cell lines under study.

Effects of human dipeptide transport activity on pHi

Because the hPEPT1 transporter has high transport capacity and relatively broad substrate specificity, this transporter plays an important role in nutrient and drug absorption. To examine whether the multi-well plate format can be used to screen candidate substrates for absorption via H^+-coupled transport mechanisms, pHi was measured after the addition of dipeptides.

In CHO-hPEPT1 cells, 15 minutes after the injection of 20 mM of the hydrolysis-resistant peptide Gly-Sar, a pHi decrease of 0.15 pH unit was observed, compared with cells transfected with pcDNA3 vector alone (**Figure 7**). This pH decrease was linear at least during the first 20 minutes, which is consistent with previous findings showing that the accumulation of Gly-Sar was linear for the first 30 minutes. [10] The uptake of 20 mM of the dipeptide Gly-Gly caused the same pHi decrease, as shown in **Figure 7** . However, in Caco-2 cells, which also express peptide transporters [22] as a function of cellular differentiation, the pHi changes after the injection of Gly-Sar and Gly-Gly were not significant (data not shown).

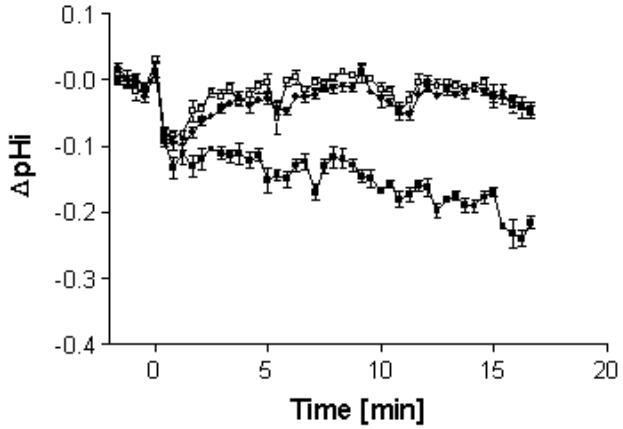

Figure 7. Effect of dipeptide transport on pHi in CHO/hPEPT1 cells. Representative tracing of the relative change in pHi induced by the addition of 20 mM Gly-Sar to CHO cells transfected with hPEPT1 (solid square) and in CHO cells transfected with pcDNA3 vector (open square). As an additional control, the response to injection of buffer only was recorded in CHO/hPEPT1 cells (solid circle). Experiments were performed in bicarbonate-free buffer pH 7.4, at 37°C. Each point represents mean ± SD of 4 independent cell-loaded suspensions.

Effects of receptor activation on pHi

Several investigators have reported that Ca^{2+} and pHi are linked (ie, changes in one affect the activity of the other. [1-4] Using a fluorescence plate-reader assay developed in our laboratory, rapid intracellular calcium increases were observed by activation of muscarinic, μ-opioid, and β-adrenergic receptors. [7] To examine whether these Ca^{2+} responses were connected to an intracellular pH change, acetylcholine 10 μM was injected into HEK293-M1 and CHO-M1 cells. In both cell lines expressing a high level of muscarinic receptors, activation by acetylcholine did not cause any significant pHi response compared with wild type cells (data not shown). The injection of morphine (10 μM) also did not produce any pHi change in m-opioid and d-opioid receptor-expressing HEK293 cells (data not shown). Similarly, stimulation of endogenously expressed β-adrenergic receptors by 10 μM isoproterenol (chosen to maximally stimulate cAMP production in HEK293 cells) had no effect. Moreover, the addition of the calcium ionophore, ionomycin 1 μM (RBI, Natick, MA), which elicited a robust Ca^{2+} increase, showed no significant pHi change in HEK293 cells.

DISCUSSION The purpose of this study was to evaluate the performance of 3 visible-light excitable fluorescent dyes, SNARF-1/AM, BCECF/AM, and CellTracker Green CMFDA in CHO, HEK293, and Caco-2 cells for measuring intracellular pH changes in a multi-well plate format. The major problem encountered was the rate of the dye efflux, which was found to depend on the dye, cell type, temperature, and extracellular pH. The fact that dye efflux was considerably decreased when temperature was lowered suggested the involvement of a facilitated transport. Therefore, various inhibitors were tested (ie, inhibitors of anion transporters, MRP, and P-glycoprotein. Both SNARF-1/AM and BCECF/AM, even in the presence of inhibitors, were insufficiently retained in the cells and thus unsuitable for our assay at 37ºC. If wells are read sequentially in a plate-reader format, or over prolonged time periods, stability of the pH-associated fluorescence signal is critical. The performance of the CellTracker Green CMFDA was significantly better with respect to cellular dye efflux. Therefore, this dye was selected for use in further studies with CHO, HEK293, and Caco-2 cells.

Typical intracellular pH changes caused by incubation with a weak base or weak acid were readily detectable using the multi-well plate format. These pH changes were clearly intracellular responses as they were transient. To ascertain that the injection of the buffer containing test compounds did not change the external pH, the test compound solution was injected, prior to each experiment, in buffer containing only CMFDA (no cells) and compared with the injection of the buffer alone.

In agreement with earlier studies, [23-25] the exposure of HEK293 and CHO cells to hypertonic medium induced cytoplasmic alkalization. The pHi increase (0.16 pH unit) obtained with sucrose in the multi-well plate reader in CHO cells is similar to results obtained by others in a fluorescence cuvette assay with BCECF-loaded CHO cells (0.13 pH unit). [23] Furthermore, another osmolyte with low membrane permeability, NaCl, also induced a large pHi increase, whereas permeant solutes like urea caused only negligible pHi changes. In Caco-2 cells, the osmolytes tested did not produce a significant pHi change, consistent with the finding that in Caco-2 cells the Na^+/H^+ exchanger activity was unaffected by cell shrinkage. [26]

In CHO/hPEPT1-transfected cells, dipeptides such as Gly-Sar and Gly-Gly significantly lowered pHi, demonstrating that the multi-well plate reader assay was able to detect the activation of the H^+-coupled peptide transporters. Since the stably transfected cell line, CHO/hPEPT1, exhibits enhanced transport over other experimental cell lines with native expression of hPEPT1, [10] it was possible to follow dipeptide uptake, even at pH 7.4, where dye leakage was minimal. In Caco-2 cells derived from a human colon carcinoma and widely used as a model of intestinal epithelial cells, no pHi change was observed following dipeptide injection. This was probably a result of low hPEPT1 expression. To exhibit typical intestinal epithelial transport function, the Caco-2 cells must be highly differentiated and polarized, which was not the case in this study. Furthermore, local pH gradients may dissipate rapidly by the action of Na^+/H^+ exchangers [28,29]; thus the magnitude of a pH signal averaged over the entire cell depends on multiple factors. Our results show that the multi-well plate-reader assay using the CHO/hPEPT1 cell system can serve for rapid screening of drugs transported by an H^+-coupled peptide transporter. The assay is likely to apply to other H^+-coupled transporters as well.

Previous studies have reported pHi changes following d-opioid receptor activation by morphine and naloxone, [3] Î±1-adrenergic receptors by phenylephrine, [5] and β2-adrenergic receptors by isoproterenol [27] in cardiac myocytes. We were unable to detect any response evoked by stimulation of M1-muscarinic, μ-and σ-opioid, and β-adrenergic receptors in CHO and HEK293 cells, even though changes as little as 0.02 pHi unit induced by weak acid and base or osmolytes are detectable in our assay. Either, these receptors fail to alter pHi or counter-regulation of pHi is too fast to observe net changes in the cells tested. To observe any net changes, one might need to use different cell lines or prevent pH counter-regulation.

The present study shows that changes in intracellular pH measured with the fluorescent pH-sensitive dye, CellTracker Green CMFDA, in a multi-well plate reader format were comparable to the findings of other investigators with other assay systems. We conclude that this rapid assay might facilitate screening procedures assessing the effect of growth factors, hormones, hypertonic stress, or drugs on intracellular pH homeostasis.

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