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Use of a pH-sensitive fluorescent probe for measuring intracellular pH of Caco-2 cells

Earvin Liang, Puchun Liu *, Steven Dinh

Emisphere Technologies, Inc., 765 Old Saw Mill River Road, Tarrytown, NY 10591, United States

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Abstract

This paper describes the application of a pH-sensitive fluorescent probe [2′,7′-bis(2-carboxylethyl)-5(6)-carboxyfluorescein or BCECF] to measure intracellular pH (pH_i) changes in Caco-2 cells. As a function of BCECF's ionization, the fluorescence was monitored at $\lambda_{ex} = 440$ and 503 nm, and $\lambda_{em} = 535$ nm. Time course studies were conducted with the addition of two weak acid delivery agents, one weak base delivery agent, oleic acid, or tetradecylamine. When applicable, 10 μM bovine serum albumin or 10 mM ammonium chloride was added into the cell suspension to hinder the pH gradient effect. Adding a weak acid at 2, 10, or 50 mM to the cell suspension, the pH_i dropped substantially from 7.4 to 7.1, 6.9, or 6.7, respectively. The pH_i then increased gradually over a 10-min period but did not return to its initial value. Conversely, the pH_i increased instantaneously after the addition of a weak base. When Caco-2 cells were placed in solutions with different bulk pH (7.0, 7.5, and 8.0), the lower the pH in which the cells were exposed, the larger the pH_i drop occurred with the addition of an acid. The results suggest that these weak acids or bases are transported transcellularly across Caco-2 cells.

Keywords: Intracellular pH; Caco-2 cells; pH-sensitive fluoroprobes; BCECF; Delivery agents

1. Introduction

Free fatty acids (FFA) serve as metabolic fuels and key intermediates in lipid metabolism, and perform functions in signal transduction and modulation of ion channel opening (Huang et al., 1992; Ordway et al., 1989). Movement of FFA in and out of cells is vital to these and other cellular processes. The addition of FFA to cells in vitro, e.g. pancreatic β-cells and adipocytes, produces a persistent decrease in the intracellular pH (pH_i), and the observed pH_i change can be explained by the passive diffusion of unionized fatty acid across the plasma membrane (Civelek et al., 1996). The proton pumps in the cells do not rapidly reverse the effect on pH, and the buffering capacity of the cytoplasm is not strong enough to prevent the pH change. An increase in circulating these compounds can therefore attain and sustain intracellular acidification. Pertinent physiological causes of elevated plasma fatty acids include fasting, diabetes, and certain ion channels and enzymes (Madshus, 1988). The induced pH gradients might also explain the observed in vitro effects of fatty acids on Ca^{2+} channels in myocardial cells (Huang et al., 1992).

Many oral drug delivery agents designed in our laboratories have FFA-like structures, and can be transported across cells by passive diffusion (Leone-Bay et al., 1995; Milstein et al., 1998). The objective of this study is to investigate, using a Caco-2 cell model, whether select Emisphere delivery agents are transported transcellularly by using changes in the pH_i as a marker of the transport pathway.

To accomplish this goal, the first part of this study is to develop a method for measuring the pH_i of Caco-2 cells. 2′,7′-bis(2-carboxylethyl)-5(6)-carboxyfluorescein (BCECF) is a dye that has been widely used as a fluorescent indicator for the pH_i in many cellular systems (Rink et al., 1982). The fluorescence excitation profile is pH-dependent, and its pK_a of 7.0 is ideally matched to the normal range of cytoplasmic pH. The

Abbreviations: BCECF, 2′,7′-bis(2-carboxylethyl)-5(6)-carboxyfluorescein; BCECF-AM, 2′,7′-bis(2-carboxylethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; FFA, free fatty acid; HBSS, Hanks' balanced salt solution; pH_i, intracellular pH; OA, oleic acid; TDA, tetradecylamine; WADA, weak acid delivery agent; WBDA, weak base delivery agent

* Corresponding author. Tel.: +1 914 785 4750; fax: +1 914 593 8254.
E-mail address: PLiu@Emisphere.com (P. Liu).

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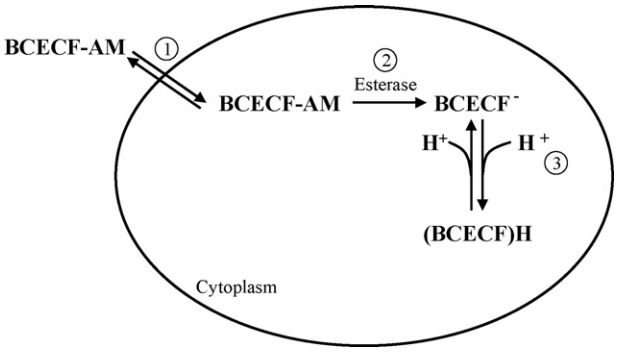

Fig. 1. Diagram of partition of BCECF-AM into cells (step 1), digestion of BCECF-AM by esterase into BCECF in cytoplasm (step 2), and ionization of BCECF based upon surrounding proton (step 3).

nonfluorescent BCECF acetoxymethyl ester (BCECF-AM), which is relatively lipophilic and membrane permeable, can be converted into the fluorescent pH-sensitive BCECF inside the cells via the action of intracellular esterases (Fig. 1). Due to the increased hydrophilicity, BCECF would then be trapped inside the viable cells and exhibited fluorescence activities as a function of its surrounding pH (pH_i).

After establishing and validating the pH-sensitive BCECF fluorescent probe method, experiments were designed to evaluate changes of the pH_i in Caco-2 cells when in contact with select model compounds: weak acid delivery agents (WADA1 and WADA2), oleic acid (OA), weak base delivery agent (WBDA), and tetradecylamine (TDA) (Fig. 2). The effects of inhibitors and bulk pH were also evaluated.

2. Materials and methods

2.1. Materials

BCECF and BCECF-AM were purchased from Molecular Probes (Eugene, OR). Bovine serum albumin (BSA) fraction V was purchased from Boehringer Mannheim (Indianapolis, IN). OA, TDA, and NH_4Cl were purchased from Aldrich (Milwaukee, WI).

WADA1, WADA2, and WBDA were synthesized and purified at Emisphere Technologies, Inc. (Tarrytown, NY). Dulbecco's modified Eagle medium (DMEM), Hanks' balanced salt solution (HBSS), fetal bovine serum, non-essential amino acids, penicillin, streptomycin, and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY).

2.2. Methods

2.2.1. Cell culture

Caco-2 cells, originating from a human colorectal carcinoma, were obtained from American Type Culture Collection (Rockville, MD), and grown at 37 °C in an atmosphere of 5% CO_2 in DMEM growth medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, penicillin (100 Unit/ml), and streptomycin (100 μg/ml). Confluent cell monolayers were subcultured every 7 days by treatment with 0.25% trypsin containing 1 mM EDTA. Caco-2 cells were seeded at a density of 80,000 cells/cm^2 in petri dishes (Liang et al., 2000). The cells were then allowed to grow for additional 3

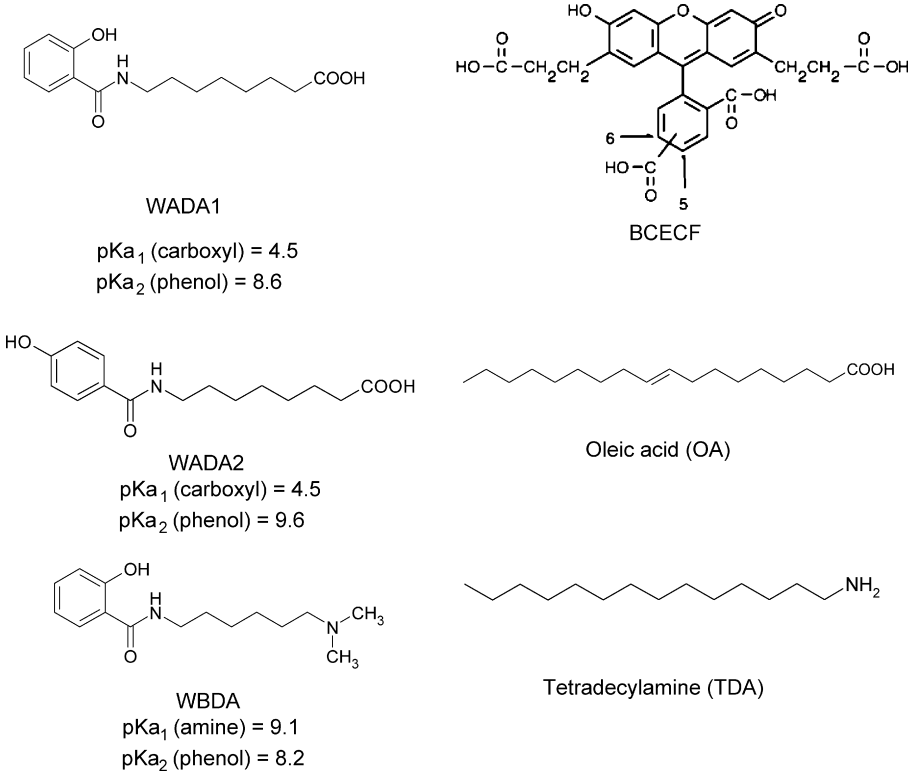

Fig. 2. Chemical structures of select model acidic and basic compounds and 2′,7′-bis(2-carboxylethyl)-5(6)-carboxyfluorescein (BCECF).

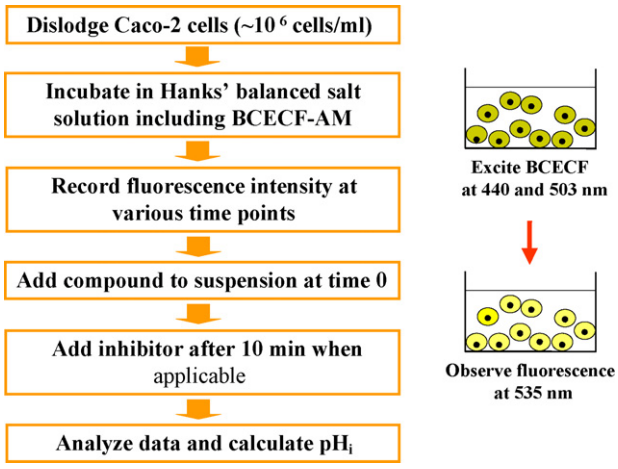

Fig. 3. Procedures to conduct the pH_i measurement studies.

days to a subconfluent state before being used. Cells of passage numbers 18–40 were used throughout.

2.2.2. Intracellular pH (pH_i) measurement of Caco-2 cells

Caco-2 cells were lifted from the petri dishes using trypsin-EDTA and incubated in HBSS consisting of 1.3 mM $CaCl_2$, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 137 mM NaCl, 0.34 mM Na_2HPO_4, 5.5 mM D-glucose, and 4.2 mM $NaHCO_3$ at 37 °C (Fig. 3). Model compounds at various concentrations were added into the cell suspension. Unless otherwise specified, the pH of the HBSS containing the cells and test materials was maintained at a pH of 7.4 ± 0.1. When applicable, at the end of a 10-min experiment, potential inhibitors were added to the cell suspension for an additional 5 min. The fluorescence intensities were recorded at λ_{ex} of 440 and 503 nm and λ_{em} of 535 nm with a Perkin-Elmer Luminescence Spectrophotometer LS-55 (Norwalk, CT) at various time points. The pH_i was then calculated based on the following equation (Graber et al., 1986):

$$pH_i = pK_a + \log\frac{R - R_{min}}{R_{max} - R} + \log\frac{F_a}{F_b}$$

where the pK_a of BCECF is 7.0 (Rink et al., 1982), R represents the fluorescence ratio at unknown pH, R_{min} and R_{max} denotes fluorescence ratio at acid extreme of 6.0 and base extreme of 8.0, respectively, and F_a and F_b are the fluorescence activity at λ_{ex} = 440 nm at acid and base extremes, respectively.

3. Results and discussion

3.1. Method validation

Before conducting the pH_i measurements, the viability of the Caco-2 cells during the course of the study was determined using Trypan Blue. Caco-2 cells were found to be viable (>98%) for up to 1 h (data not shown) when exposed to solutions containing up to 80 mM of delivery agent. In addition, the bulk pH remained constant throughout the experiment as measured by the pH meter at the end of each experiment. This indicated that the pH_i changes were a direct result of the model compounds. Moreover, it was shown that although model compounds could affect the fluorescent activity of BCECF, they did not alter the fluorescence ratios of R, R_{min}, and R_{max} used in calculating pH_i. As such, the measured pH remained reliable at delivery agent concentrations up to 80 mM.

Two pre-incubation times (15 min versus 3 min) prior to the addition of model acidic compound WADA1 at 10 mM, were shown to exhibit comparable pH_i profiles (Fig. 4A). This sug-

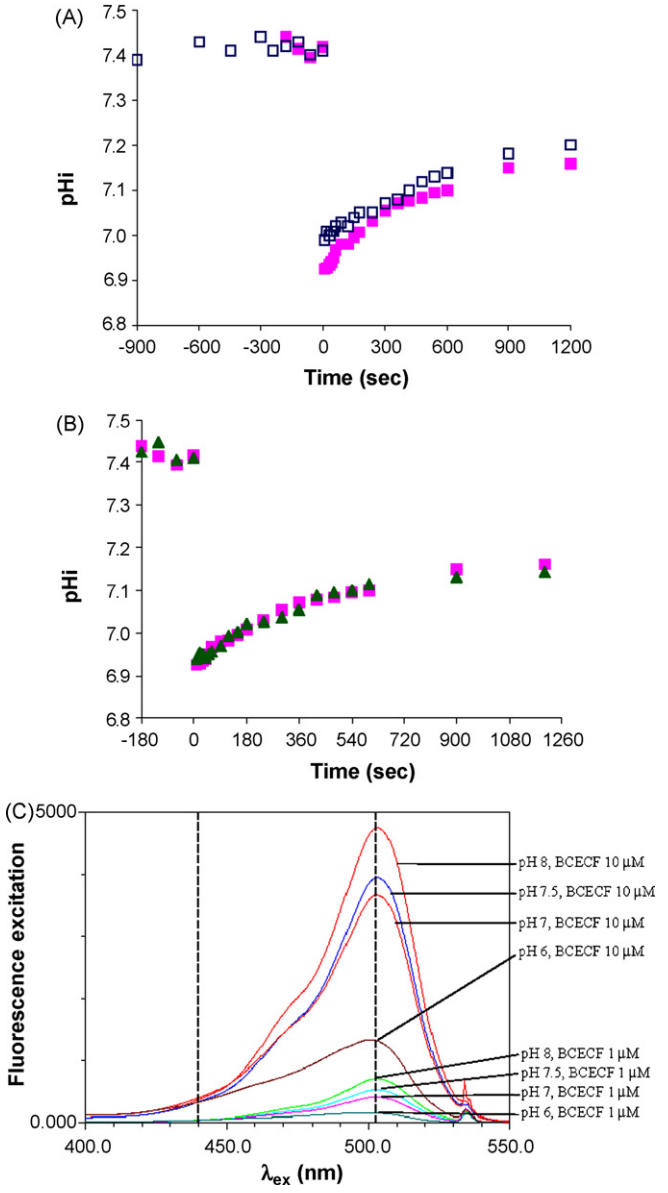

Fig. 4. Effects of (A) pre-incubation time on the pH_i of Caco-2 cells before the addition of 10 mM of WADA1 at time 0 while maintaining the BCECF-AM concentration at 10 µM: 3 (■) and 15 min (□), (B) BCECF-AM concentration on the pH_i of Caco-2 cells while maintaining pre-incubation time for 3 min before the addition of 10 mM of WADA1 at time 0: 1 µM (▲) and 10 µM (■), and (C) the pH sensitivity of BCECF at 10 and 1 µM: 6, 7, 7.5, and 8. The spectra of the fluorescence intensity at the range of excitation wavelength between 400 and 550 nm were recorded after 15 min as the emission wavelength was fixed at 535 nm.

gested that BCECF at 10 μM had reached equilibrium in and out of the cells (step 1, Fig. 1) and hence would not be a factor in affecting the pH_i profile. With the presence of BCECF-AM at up to 10 μM, acidic compound WADA1 at 10 mM induced similar pH_i profiles (Fig. 4B). The esterase was consumed by BCECF-AM present in 10^6 cell/ml Caco-2 cell suspension (step 2, Fig. 1). This indicated that the use of the fluorescent probe BCECF-AM had been saturated in this concentration range, and would not be the rate-limiting step in determining the pH_i profile. Hence, 3 min of pre-incubation and 10 μM of BCECF-AM were chosen for subsequent studies.

BCECF was incubated with Caco-2 cells at four different pH (6, 7, 7.5, and 8) for 15 min to evaluate the effect of pH on its fluorescence intensity. For a given concentration of BCECF, such as 10 or 1 μM, the fluorescence intensity increased with increasing pH (Fig. 4C). These observations are consistent with those reported in literature (Silver, 1998) and indicate that the fluorescence intensity of BCECF is strongly determined by its surrounding intracellular pH environment (pH_i). In addition, the incubation time scale of 10–20 min would minimize if any, the effect of Caco-2 cellular metabolism for each of all compounds tested.

3.2. Concentration effect of delivery agents

Three concentrations of WADA1 at 2, 10, and 50 mM were selected to evaluate its impact on the pH_i of Caco-2 cells. The pH_i remained constant prior to the addition of WADA1, and as soon as it was loaded to the cell suspension, the pH_i dropped substantially to approximately 7.1, 6.9, and 6.7, respectively (Fig. 5A). The pH_i then increased gradually over a 20-min period but did not return to its initial value of approximately 7.5.

When WADA1 was replaced by WADA2, it induced a similar pH_i profile to that observed with WADA1 (Fig. 5B). However, the pH_i drop caused by WADA2 (approximately 0.3 and 0.2 pH units at 50 and 10 mM, respectively) was less than that produced by WADA1 (approximately 0.7 and 0.5 pH units at 50 and 10 mM, respectively). Furthermore, the effect on the pH_i was negligible when the concentration was decreased to 2 mM. These results suggest that the change in pH_i may be due to the permeability of the Caco-2 cells to the different delivery agents.

In contrast to the weak acid delivery agents, WADA1 and WADA2, the pH_i following the addition of the weak base delivery agent WBDA initially increased by approximately 0.4, 0.2, and 0.1 units with concentrations of 50, 10, and 2 mM, respectively (Fig. 5C). In each case, the pH_i initially increased then decreased gradually toward its initial value over a 20-min period, but did not return to the baseline.

The immediate decrease in pH_i after the addition of weak acid delivery agents to Caco-2 cells suggest that additional protons were transported across the lipid bilayer of the cell membrane, probably by a flip-flop mechanism (Civelek et al., 1996). The pH_i then gradually recovers as the excess protons diffuse out of the cell. Similarly, the opposite effect is seen when the cells are in contact with solutions containing weak base delivery agents.

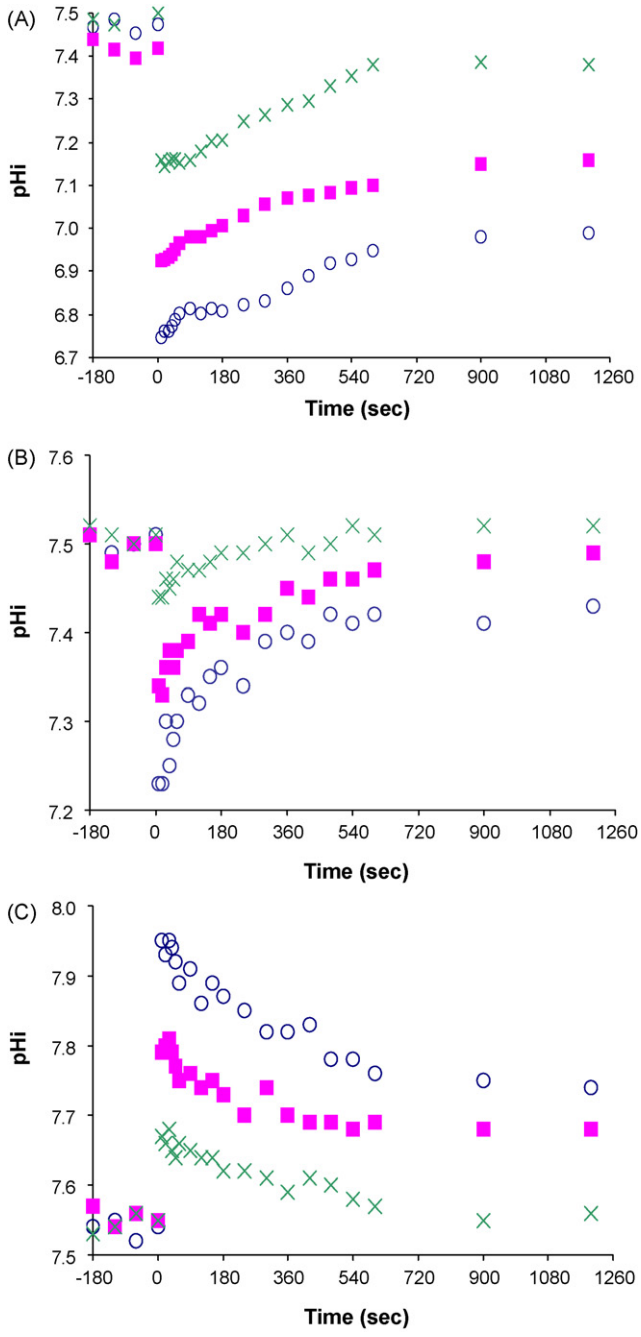

Fig. 5. Time course studies of the effect of select model acidic and basic compounds, (A) WADA1, (B) WADA2, and (C) WBDA, added at time 0 on the pH_i of Caco-2 cells at three different concentrations: 50 mM (◯), 10 mM (■), and 2 mM (×).

3.3. Use of potential inhibitors and comparison with other compounds

After the addition of WADA1, WADA2, and WBDA at 50 mM, like in previous studies, the pH_i instantaneously changed and then gradually returned toward its initial value. As soon as 10 mM of NH_4Cl was added to the cell suspension, the pH_i values observed with these three model compounds were all elevated while the bulk pH still remained constant (Fig. 6A). Instantaneous alkalinization demonstrated the integrity of the

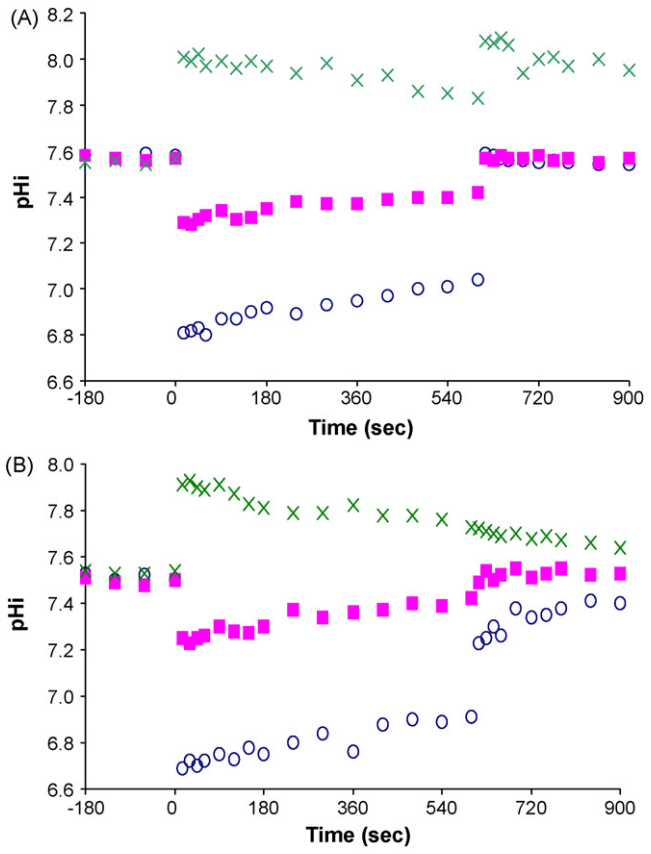

Fig. 6. Reversal effect of the inhibitors: (A) NH_4Cl at 10 mM and (B) BSA at 10 μM, on the pH_i of Caco-2 cells after the addition of 50 mM of WADA1 (◯), WADA2 (■), and WBDA (×) at time 0 for 10 min (600 s).

Caco-2 cells, as only viable cells can internalize NH_4^+ and sustain a pH gradient.

To evaluate the direct role of select model compounds in the changes of the pH_i, 10 μM of BSA was added to the cells, and immediately, rapid restoration toward the initial pH_i was observed in the weak acid delivery agent-treated groups but not in the weak base delivery agent-treated group (Fig. 6B). This indicated that there was a strong binding of WADA1 and WADA2 to BSA. Addition of BSA to cells instantly annihilated the pH gradient, indicating complete removal of these compounds, which required rapid "flip-flop" of the chemicals from the inner to the outer leaflet of the Caco-2 cell lipid bilayers. The above results further clarified that, instead of the model compounds that influenced the proton re-distribution, it was the direct partition of the model compounds into the Caco-2 cells which concurrently brought in their counter ion proton that caused changes of the pH_i.

The flip-flop mechanism can also explain the pH_i increase after the addition of BSA. FFA-like compounds leave the outer membrane to bind to BSA and then are replenished by those moving from the inner to outer leaflet of the membrane, transporting protons from the cytoplasm to the external medium (Hamilton and Cistola, 1986; Kamp et al., 1993). In living cells, these FFA-like compounds can be rapidly metabolized into the first metabolite of acyl-CoA inside the cells. Although long chain acyl-CoA esters bind to BSA, they do not move spontaneously between the leaflets of a bilayer (Boylan and Hamilton, 1992) and are therefore not expected to be removed from the cell by BSA added to the buffer. Since the pH_i decrease caused by the influx of FFA-like compounds into the Caco-2 cells was largely reversed by BSA, it would then appear that the majority of the added long chain FFA-like compounds were not metabolized during the time period of observation.

The effect of two commercially available compounds, OA at 1 mM and TDA at 10 mM, were additionally compared with that of WADA1 at 10 mM in inducing changes in the Caco-2 cell pH_i over time. As soon as OA was added into the cell suspension, there was a slight decrease in pH_i, although not as dramatic as observed with WADA1 (Fig. 7). However, due to the very low solubility in aqueous buffer at pH 7.5, a significant portion of OA was seen to bind to the walls of the cuvette or precipitate before binding to cells (Richieri and Kleinfeld, 1989; Vorum et al., 1992). As a result, the amount of OA available was much lower than the amount added. In contrast, there was a 0.3 pH_i unit increase immediately after the addition of TDA. In all, the pH_i gradually tapered off during the time of observation, and similar to previous studies, BSA did not trigger any pH_i response to the TDA-treated group as seen with the OA-treated one. The use of these two compounds supports the role of a passive diffusion mechanism in the transport of delivery agents, e.g. WADA1, WADA2, and WBDA, as similar observations were seen by other researchers and have been well established in literatures (Civelek et al., 1996; Hamilton et al., 1994; Kamp and Hamilton, 1992).

It was of interest to note that when 100 or 200 mM WADA1 (beyond the cytotoxic concentration threshold of 80 mM) was added to the cell suspension, the pH_i dropped instantaneously (less than 5 s) by a greater extent and returned to pre-incubation pH within 20 s before the integrity of the cells were compromised (data not shown). Such an observation might be attributed to opening and closing of membrane channels or transporters induced by the delivery agents after passing a certain concentration threshold.

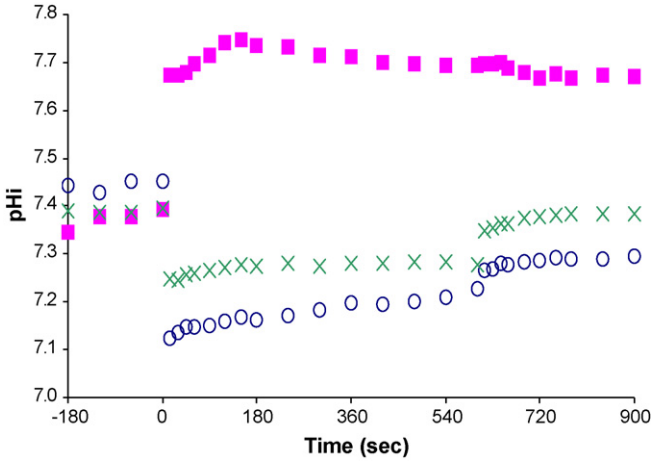

Fig. 7. Comparison of WADA1 at 10 mM (◯) to two commercially available compounds of TDA at 10 mM (■) and OA at 1 mM (×) with regard to their respective effect on the pH_i of Caco-2 cells. The reversal effect of BSA at 10 μM after the addition of select model compounds for 10 min (600 s) was demonstrated.

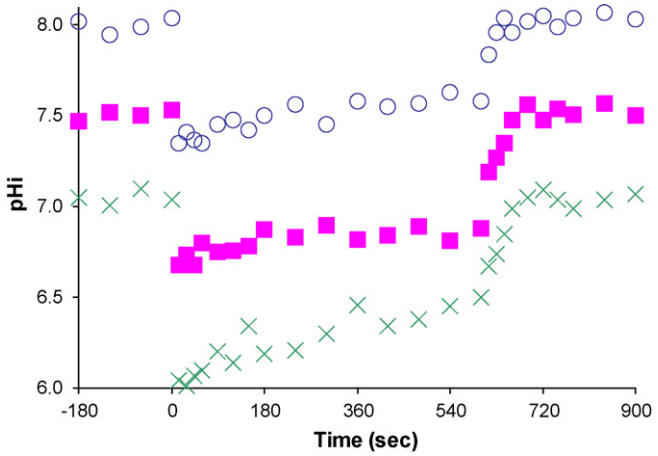

Fig. 8. Effect of the bulk pH at 8 (◯), 7.5 (■), and 7 (×) on the pH_i of Caco-2 cells with the addition of WADA1 at 50 mM. The effect of adding 10 μM BSA at time 10 min (600 s) was also demonstrated.

3.4. Effect of bulk solution pH on transport

If molecules penetrate through cells via passive diffusion, the degree of partitioning should be directly related to the pK_a and the surrounding pH, as only the unionized form of the molecule can be flip-flopped through the lipid bilayers and eventually into the cells. To assess this conjecture, Caco-2 cells were preconditioned in three different bulk pH environments (pH 7.0, 7.5, and 8.0) before the addition of 50 mM WADA1. At these three pHs, WADA1 was able to induce the pH_i decline (Fig. 8). The lower the bulk pH, the greater the degree of pH_i decrease was observed: 1.0, 0.7, and 0.5 units at bulk pH of 7, 7.5, and 8, respectively. As expected, immediately following BSA addition to the cell suspension, the effect of WADA1 at these three bulk pHs was reversed. Similar observations were seen from other model compounds (data not shown).

Variations of the bulk pH affected the pH_i in the presence of the model compounds, whether weak acid or weak base, which were in accordance with their respective pK_a. This again suggested that passive diffusion, not active transport, appeared to be mainly, if not entirely, responsible for the transport of these compounds across Caco-2 cells in vitro.

4. Conclusions

The effective use of a pH-sensitive probe, BCECF, to measure the pH_i of Caco-2 cells when exposed to solutions containing various model compounds was demonstrated. Acidification of the Caco-2 cell interior following the addition of FFA-like compounds may be explained by the movement of FFA across the cell membrane. These FFA-like compounds are relatively water insoluble but bind avidly to membranes, initially to the outer leaflet, when added to the external buffer. Upon binding to a phospholipid bilayer, the compounds become largely ionized, and the rapid movement (flip-flop) of the unionized (versus ionized) FFA-like compounds across the bilayer followed by deprotonation of the compounds in the inner leaflet causes a pH decrease inside cells. Additional evidence for this mechanism is provided by the argument that if movement of these compounds occurred via an anion transporter, an increase in pH_i in cells would be expected, as opposed to the observed pH_i decrease.

The studies reported here revealed fundamental properties of FFA-like delivery agents in phospholipid bilayers, the structural framework of most cell membranes. The fast flip-flop of unionized FFA-like compounds across phospholipid bilayers provides a simple, non-energy dependent mechanism that satisfies the physiological requirement for their rapid entry into and removal from cells. These results suggest that these weak acids or bases are transported transcellularly across Caco-2 cells.

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