Inhibition of the Proton-Coupled Folate Transporter (PCFT-SLC46A1) by Bicarbonate and Other Anions

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ABSTRACT

The proton-coupled folate transporter (PCFT) plays a key role in intestinal folate absorption, and loss-of-function mutations in the gene encoding this transporter are the molecular basis for hereditary folate malabsorption. Using a stable transfectant with high expression of PCFT, physiologic levels of bicarbonate produced potent and rapidly reversible inhibition of PCFT-mediated transport at neutral pH. Bisulfite and nitrite also inhibited PCFT function at neutral pH, whereas sulfate, nitrate, and phosphate had no impact at all. At weakly acidic pH (6.5), bisulfite and nitrite exhibited much stronger inhibition of PCFT-mediated transport,

whereas sulfate and nitrate remained noninhibitory. Inhibition by bisulfite and nitrite at pH 6.5 was associated with a marked decrease in the influx $V_{\max}$ and collapse of the transmembrane proton gradient attributed to the diffusion of the protonated forms into these cells. Monocarboxylates such as pyruvate and acetate also collapsed the pH gradient and were also inhibitory, whereas citrate and glycine neither altered the proton gradient nor inhibited PCFT-mediated transport. These observations add another dimension to the unfavorable pH environment for PCFT function in systemic tissues: the presence of high concentrations of bicarbonate.

Introduction

The proton-coupled folate transporter (PCFT) was recently cloned and its physiologic role established as the mechanism of folate absorption across the apical brush-border membrane of the proximal small intestine and transport of folates across the choroid plexus into the cerebrospinal fluid (Qiu et al., 2006; Zhao et al., 2011a). In patients with hereditary folate malabsorption, an autosomal recessive disorder, both copies of the PCFT gene harbor a loss-of-function mutation and folate levels in both the blood and the cerebrospinal fluid are markedly decreased (Diop-Bove et al., 2011). Analysis of the functional defects associated with mutations detected in subjects with hereditary folate malabsorption, along with site-directed mutagenesis, have provided insights into the residues and domains of the carrier that play a role in proton and folate substrate binding, proton coupling, and alterations in the conformational state of the protein (Zhao et al., 2011a).

PCFT has a substrate specificity that is distinct from the other major folate-specific facilitative transporter, the reduced folate carrier (RFC). The PCFT influx K_t for folic acid is in the low micromolar range at acidic pH, similar to its other preferred folate substrates, and the K_i for N^{α} -(4-amino-4-deoxypteroyl)- N^{δ} -hemipthaloyl-L-ornithine (PT523) is $>100 \mu\text{M}$ (Wang et al., 2004; Qiu et al., 2006). This is quite

opposite to the high K_i for folic acid and very low K_i for PT523 for RFC-mediated transport (Rosowsky et al., 1998; Zhao et al., 2011a). Unlike PCFT, which is a proton-coupled process that operates optimally at low pH (Qiu et al., 2006; Nakai et al., 2007; Umapathy et al., 2007; Inoue et al., 2008), RFC is an anion exchanger that operates most efficiently at pH 7.4 and is inhibited by inorganic and organic anions (Henderson and Zevely, 1983; Yang et al., 1984; Zhao et al., 2001). Although some organic anions are weak inhibitors of PCFT-mediated transport, with inhibitor constants in the sub-millimolar to millimolar range (Inoue et al., 2008), little is known about the impact of inorganic anions on PCFT-mediated transport, particularly anions that have physiologic relevance. In the current study, bicarbonate, bisulfite, and nitrite are shown to be inhibitors of PCFT-mediated transport at neutral pH using a HeLa-derived cell line that expresses very high levels of PCFT. A much stronger inhibition by nitrite and bisulfite at acidic pH is attributed largely to a collapse of the transmembrane proton gradient due to the PCFT-independent uptake of the conjugated acids of these anions. Inhibition of PCFT-mediated transport by structurally unrelated organic monocarboxylates at acidic pH was also associated indirectly with disruption of the transmembrane proton gradient by these compounds.

Materials and Methods

Cells and Culture Conditions. R1-11 cells, derived from HeLa cells, lack expression of both RFC and PCFT due to a genomic deletion of the former and silencing of the latter (Zhao et al., 2004a; Diop-Bove

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ABBREVIATIONS: BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; 5-CHO-THF, 5-formyltetrahydrofolate; HBS, Hepes-buffered saline; MBS, 2-(4-morpholino)ethanesulfonic acid-buffered saline; PCFT, proton-coupled folate transporter; PT523, N^{α} -(4-amino-4-deoxypteroyl)- N^{δ} -hemipthaloyl-L-ornithine; RFC, reduced folate carrier.

et al., 2009). R1-11-RFC-6 and R1-11-PCFT-4 are stable transfectants of R1-11 cells that express RFC and PCFT, respectively, at a level similar to that of HeLa cells (Zhao et al., 2008). R1-11-PCFT-h is a stable transfectant of R1-11 cells with levels of PCFT expression much higher than that of HeLa cells (Visentin et al., 2012). Cells were grown in RPMI medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Zeocin (0.1 mg/ml) was added to the growth medium to maintain carrier expression in R1-11-RFC-6 cells and R1-11-PCFT-4 cells, while 0.3 mg/ml hygromycin was added to maintain R1-11-PCFT-h cells. Transient transfectants that express wild-type PCFT were generated by transfection of the *pcft* expression vector into R1-11 cells as described previously (Zhao et al., 2011b).

Tritiated Chemicals. [3',5',7, 9-³H(N)](6S)-5-CHO-THF (5-formyltetrahydrofolate), [3',5',7,9-³H]folic acid, [3',5',7-³H(N)]methotrexate, and generally labeled [³H]pemetrexed were obtained from Moravsek Biochemicals (Brea, CA). These compounds were purified before use and their purity monitored by high-performance liquid chromatography.

Measurement of *pcft* mRNA Levels by Quantitative Reverse-Transcription Polymerase Chain Reaction. *pcft* mRNA levels in R1-11-PCFT-h and R1-11-PCFT-4 cells were determined by real-time reverse-transcription polymerase chain reaction as previously described (Qiu et al., 2006).

Membrane Transport. Hepes-buffered saline [HBS: 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5 mM dextrose, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂; pH 7.4] was used as the incubation buffer, or as transport buffer. Mes-buffered saline [MBS: 20 mM 2-(4-morpholino)ethanesulfonic acid, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂; pH 6.5 or 5.5] was used as transport buffer at acidic pH. In preparation for experiments, the sodium salt of the various anions was added to HBS or MBS and the pH adjusted. In some preparations, sodium chloride in HBS was replaced with equimolar sodium bicarbonate. In other experiments, folate-free RPMI containing 24 mM sodium bicarbonate was used as the transport buffer when the uptake was conducted in a 5% CO₂ incubator. When folate-free RPMI medium was used as the preincubation or transport buffer in experiments performed at the bench, it was supplemented with 20 mM Hepes to stabilize the pH. Bicarbonate-free, folate-free RPMI was prepared by replacing 24 mM sodium bicarbonate with 24 mM sodium chloride. All buffers containing test anions were freshly prepared and their pH adjusted immediately before transport measurements were made. Buffers were monitored to ensure that the pH was constant over the short interval of uptake in each of the different types of experiments.

For transport measurements, cells were washed twice and incubated in the same buffer (HBS in most cases unless specified) at 37°C for 20 minutes. The incubation buffer was then aspirated and transport was initiated by the addition of 0.5 ml of prewarmed transport buffer containing a tritiated compound. Uptake was carried out at 37°C and stopped by the addition of 5 ml of ice-cold HBS. Cells were washed three times with ice-cold HBS and digested in 0.5 ml of 0.2 M NaOH at 65°C for 1 hour. Radioactivity in 0.4 ml of lysate was determined on a liquid scintillation spectrometer and normalized to protein levels obtained with the BCA Protein Assay (Pierce, Rockford, IL). In most cases, the data are expressed as a percentage of transport activity in the control buffer. Otherwise, transport is expressed in units of picomoles per milligram of protein.

Intracellular pH Measurements. R1-11 and R1-11-PCFT-h cells grown in glass-bottom dishes (MatTek, Ashland, MA) in culture media were loaded with the intracellular pH indicator 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) (10 μ M) (Molecular Probes/Invitrogen, Eugene, OR) for 45 minutes at 37°C in a humidified CO₂ incubator. Cells were then rinsed with HBS and imaged with a Nikon TE2000 (Tokyo, Japan) microscope. Changes in BCECF fluorescence intensities emitted at two excitation wavelengths (480 and 450 nm) were acquired at 1.0 Hz using filters and shutter (Lambda DG-4; Sutter Instruments, Novato, CA) driven

by a computer through Metafluor software (Molecular Devices, Downingtown, PA). Intracellular pH was determined from regions of interest placed on cells using an in vitro calibration curve obtained in the presence of nigericin (20 μ M) in a high concentration of potassium chloride (140 mM) at pH 5.3 to 7.6.

The intracellular pH of R1-11 or R1-11-PCFT-h cells in HBS (pH 7.4) served as baseline for all measurements. For most experiments, HBS was rapidly replaced with MBS or MBS containing 5 mM sodium sulfate, 5 mM sodium bisulfite, 10 mM sodium nitrate, 10 mM sodium nitrite, 15 mM sodium acetate, 15 mM sodium pyruvate, 15 mM sodium citrate, or 15 mM glycine, mimicking buffer changes in the transport experiments described above. For each measurement, fluorescence signals were recorded for an initial reduction in the intracellular pH, stabilization of the intracellular pH, and recovery of the intracellular pH upon return of the buffer to HBS (pH 7.4). Studies were conducted at room temperature. Multiple measurements were conducted in cells in each dish; cells in at least three separate dishes were studied for each experimental condition. In some experiments, intracellular pH was monitored when HBS (pH 7.4) was changed to HBS containing 15 mM sodium bicarbonate, 15 mM sodium bisulfate, or 15 mM sodium nitrite at the same pH.

Statistical Analysis. Statistical analyses were performed by the two-tailed *t* test or one-way analysis of variance using the GraphPad Prism software (GraphPad Software, La Jolla, CA).

Results

The Impact of HBS or RPMI on Transport of [³H]5-CHO-THF Mediated by PCFT or RFC. R1-11-PCFT-h cells that express very high levels of PCFT were used to study PCFT-mediated transport at physiologic pH. Influx of 0.5 μ M [³H]5-CHO-THF, at pH 5.5 over 1 minute, in PCFT-h cells (99 ± 14 pmol/mg protein/min) was 68-fold greater than that in R1-11-PCFT-4 cells (1.45 ± 0.5 pmol/mg protein) that express a similar PCFT level as wild-type HeLa cells based upon three independent experiments (Zhao et al., 2008). The relative *pcft* mRNA level in R1-11-PCFT-h cells was 137-fold $\pm$ 16-fold greater than that of PCFT-4 cells based on three independent real-time polymerase chain reaction analyses.

Net uptake of [³H]5-CHO-THF over 30 minutes was assessed in R1-11-PCFT-h, R1-11-RFC-6, and R1-11 cells under three conditions: 1) Cells were preincubated with HBS at pH 7.4 followed by net uptake in the same buffer. 2) Cells were preincubated in folate-free RPMI growth medium (pH 7.4) followed by uptake in the same medium in an atmosphere of 5% CO₂. 3) Cells were preincubated in folate-free, serum-free RPMI medium (pH 7.4) followed by uptake in the same medium in an atmosphere of 5% CO₂. As indicated in Fig. 1, net uptake of 5-CHO-THF in R1-11-PCFT-h cells was 3 times greater in HBS than in RPMI growth medium, whereas net uptake in R1-11-RFC-6 cells was the same in the two buffers. Uptake in the transfection-recipient R1-11 cells, which lack RFC and PCFT, was negligible under all conditions, indicating that there was no detectable 5-CHO-THF transport mediated by passive diffusion under these conditions. Hence, inhibition of 5-CHO-THF transport in the growth medium was specific for PCFT. Uptake of 5-CHO-THF in serum-free RPMI medium was similar to that in RPMI growth medium, indicating that the serum and antibiotics do not contribute to the difference in transport observed between HBS and RPMI medium.

Inhibition of PCFT Function by Bicarbonate at Neutral pH. Although HBS and RPMI medium have many differences in their composition, one outstanding difference at a high molar concentration is the presence of 24 mM sodium

Fig. 1. A comparison of the net uptake of [^3H]5-CHO-THF in HBS, folate-free RPMI growth medium, and serum-free, folate-free RPMI medium. R1-11-PCFT-h, R1-11-RFC-6, and R1-11 cells were preincubated in the same transport buffer or medium for 20 minutes before transport was initiated. Transport in HBS (pH 7.4) was assessed in a 37°C water bath, while transport in folate-free RPMI growth medium (pH 7.4) or serum-free, folate-free medium (pH 7.4) was conducted in an atmosphere of 5% CO_2 at 37°C. Cells were exposed to transport buffers containing 0.5 μM [^3H]5-CHO-THF for 30 minutes. *** $P < 0.001$ vs. HBS group. Data are the mean $\pm$ S.E.M. from three independent experiments.

bicarbonate in the latter. Hence, the effect of bicarbonate on 5-CHO-THF influx mediated by PCFT was assessed. As indicated in Fig. 2A, 5-CHO-THF influx in R11-PCFT-h cells was inhibited by 65% when cells were exposed to HBS containing 15 mM NaHCO_3 . The ordinate intercepts of the uptake slopes of control and bicarbonate-treated cells were identical, and the rates of uptake were constant over this interval, indicating that the inhibitory effect of the bicarbonate was essentially instantaneous and reflected suppression of the unidirectional flux of 5-CHO-THF into the cells. Suppression of 5-CHO-THF influx was also confirmed in transient transfectants of R1-11 cells that express PCFT, excluding the possibility that this observation is unique to the R1-11-PCFT-h stable transfectant (unpublished data). Influx of other tritiated folates or antifolates (folic acid, pemetrexed, or methotrexate) in R1-11-PCFT-h cells was also reduced by bicarbonate, indicating that the inhibitory effect was not specific for any particular PCFT substrate (unpublished data). Figure 2B illustrates the relationship between the increasing bicarbonate concentration and inhibition of PCFT influx; the IC_{50} was 5 mM and the IC_{90} 24 mM. Hence, at physiologic concentrations, bicarbonate produced a marked suppression of PCFT-mediated transport.

Some characteristics of PCFT inhibition by bicarbonate were examined (Fig. 2C). The first set of experiments was conducted using HBS buffer. Similar to what was observed in Fig. 2A, exposure of cells to bicarbonate resulted in inhibition of PCFT-mediated 5-CHO-THF influx to the same extent whether bicarbonate was present or absent in the preincubation buffer. The small decrease in influx when cells were preincubated with bicarbonate followed by transfer into bicarbonate-free HBS was not significant ($P = 0.11$). In experiments with RPMI medium using a similar design, there was comparable suppression of transport activity by bicarbonate whether or not bicarbonate was present in the preincubation

buffer. Likewise, influx was comparable in the absence of bicarbonate irrespective of whether or not bicarbonate was present in the preincubation buffer. Hence, the marked suppression of 5-CHO-THF influx was due primarily to the extracellular presence of bicarbonate.

The nature of bicarbonate inhibition of PCFT function was further assessed by measuring the kinetics of PCFT-mediated 5-CHO-THF influx in the presence or the absence of 15 mM bicarbonate at pH 7.4. Figure 3 represents the average of four independent experiments. The influx V_{max} in the presence of bicarbonate, derived from the Lineweaver-Burk plot (378 ± 62 pmol/mg protein/2 min), was decreased by approximately half ($P = 0.0049$) as compared with the influx V_{max} in the absence of bicarbonate (742 ± 68 pmol/mg protein/2 min). The influx K_t in the presence of bicarbonate (60 ± 12 μM) was increased by a factor 2.4 ($P = 0.044$) as compared with the value in the absence of bicarbonate (25 ± 4 μM). These data suggest that inhibition of PCFT function by bicarbonate is neither solely competitive nor noncompetitive.

Effects of Other Inorganic Anions on PCFT Activity.

The effects of other inorganic anions on PCFT function were assessed at pH 7.4. In these experiments, HBS was the preincubation buffer and HBS containing 15 mM of sulfate, bisulfite, nitrate, nitrite, or phosphate was the transport buffer. As indicated in Fig. 4A, there was no significant reduction in PCFT-mediated 5-CHO-THF influx when sulfate, nitrate, or phosphate was present in the transport buffer. 5-CHO-THF influx in Hepes sucrose buffer that does not contain sodium and chloride was also not different from that in HBS, indicating that neither of these ions is required for, nor influence, PCFT function. In contrast, both bisulfite and nitrite inhibited 5-CHO-THF influx ($P < 0.001$). The extent of inhibition by bisulfite (60%) was significantly greater ($P < 0.01$) than inhibition by nitrite (35%). Likewise, the difference in inhibitory effects between bisulfite and sulfate or between nitrate and nitrite was significant ($P < 0.001$).

As indicated in Fig. 4B, both bisulfite and sulfate had small (~25%) but significant ($P < 0.001$) inhibitory effects on RFC-mediated 5-CHO-THF influx at pH 7.4 as compared with transport in HBS (control). The inhibitory effects of nitrate and nitrite were even smaller (~15%) but still significant ($P < 0.01$). These results are consistent with prior observations on the inhibitory effect of anions on RFC-mediated transport (Henderson and Zevely, 1983; Yang et al., 1984). However, no significant difference was observed between bisulfite and sulfate or between nitrate and nitrite, in contrast to what was observed for PCFT-mediated 5-CHO-THF influx.

The effects of these inorganic anions on PCFT function were also assessed at the more favorable pH of 6.5 for this transporter. As indicated in Fig. 4C, the inhibitory effects of bisulfite and nitrate were now much greater, 88 and 80%, respectively. Sulfate, phosphate, and nitrate were not inhibitory at all at this pH. Hence, inhibition becomes more selective and potent for bisulfite and nitrite at this weakly acidic pH.

Impact of Bisulfite and Nitrite on [^3H]5-CHO-THF Influx Kinetics Mediated by PCFT at pH 6.5. Inhibition of 5-CHO-THF influx by both bisulfite and nitrite was concentration-dependent at pH 6.5 (Fig. 5A). Significant (35 and 25%, respectively) inhibition could be detected for both anions even at a concentration of 1.2 mM. The IC_{50} for bisulfite and nitrate at this pH was ~2 and ~4 mM, respectively.

Fig. 2. Characteristics of inhibition of [³H]5-CHO-THF influx by sodium bicarbonate in R1-11-PCFT-h cells. (A) Inhibition of [³H]5-CHO-THF influx by 15 mM sodium bicarbonate. Cells were preincubated in HBS before exposure to 5-CHO-THF-containing HBS in the presence or absence of sodium bicarbonate at a pH of 7.4. (B) Concentration-dependent inhibition by sodium bicarbonate. Cells were preincubated in HBS (lacking bicarbonate) for 20 minutes before exposure to HBS that contained a spectrum of bicarbonate concentrations with the same concentration of 5-CHO-THF. The buffers with different concentrations of sodium bicarbonate were prepared by mixing HBS and HBS containing 24 mM sodium bicarbonate proportionally. (C) The effect of sodium bicarbonate in the preincubation buffer, or in the transport buffer, on 5-CHO-THF influx. The “HBS + HCO₃⁻” buffer was prepared by replacing 24 mM NaCl in HBS with 24 mM NaHCO₃. The “RPMI-HCO₃⁻” medium (folate-free) was prepared by adding additional 24 mM NaCl to NaHCO₃⁻ free RPMI (folate-free). Because these experiments were performed at the bench, 20 mM Hepes was added to the folate-free, and NaHCO₃⁻ and folate-free, RPMI medium to stabilize the pH. For each bar, the first and second buffers (media) indicate

Fig. 3. The effect of 15 mM NaHCO₃ on [³H]5-CHO-THF influx kinetics in R1-11-PCFT-h cells. Cells were preincubated in HBS before exposure to the transport buffer containing a spectrum of [³H]5-CHO-THF concentrations all at pH 7.4. In the control group, the transport buffer was HBS; in the experimental group, the transport buffer was HBS containing 15 mM NaHCO₃. Influx of 5-CHO-THF was determined over 2 minutes. Both the Lineweaver-Burk and influx as a function of concentration (inset) plots are shown. The data are the average ± S.E.M. of four independent experiments.

The kinetics of 5-CHO-THF influx were determined in the presence or absence of 10 mM nitrite (Fig. 5B) or 5 mM bisulfite (Fig. 5C). The kinetic parameters were derived from best fits to the Michaelis-Menten equation. In the presence of 10 mM nitrite, the influx $V_{\max}$ was decreased by 85% from 856 ± 248 pmol/mg protein/min to 130 ± 35 pmol/mg protein/min ($P = 0.039$) as compared with this parameter in the absence of nitrite. The small increase in the influx K_t from 4.72 ± 0.50 μM in the absence of nitrite to 7.6 ± 1.2 μM in the presence of nitrite was not significant ($P = 0.076$). In the presence of 5 mM bisulfite, the influx $V_{\max}$ was decreased by 79% from 744 ± 160 pmol/mg protein/min to 159 ± 33 pmol/mg protein/min ($P = 0.022$). The small increase in the influx K_t from 4.33 ± 0.73 μM in the absence of bisulfite to 5.20 ± 1.93 μM in the presence of bisulfite was not significant ($P = 0.33$). Therefore, both anions appear to be noncompetitive inhibitors of PCFT-mediated 5-CHO-THF influx.

Effects of Bisulfite and Nitrite on the Transmembrane pH Gradient. In the experiments conducted at pH 6.5, cells that were preincubated in neutral HBS (pH 7.4) were exposed to MBS (pH 6.5) buffer and [³H]5-CHO-THF simultaneously. Therefore, a pH gradient was established at the initiation of transport. Because PCFT function is highly dependent on the transcellular pH gradient, the effects of bisulfite and nitrite on the intracellular pH were assessed. Both anions are dissociated from weak acids and could reduce the intracellular pH if the protonated forms enter the cells.

Intracellular pH was monitored in R-11-PCFT-h cells at room temperature using the pH-sensitive dye BCECF. As indicated in Fig. 6, there was a negligible change in intracellular pH when cells were exposed to sulfate or nitrate, anions that do not alter transport function at pH 6.5. On the other

the preincubation and transport buffers, respectively. For each panel, 5-CHO-THF (0.5 μM) influx was measured at pH 7.4 for 3 minutes; the data are the mean ± S.E.M. from three independent experiments.

Fig. 4. Effects of inorganic anions on $[^3\text{H}]5\text{-CHO-THF}$ influx. (A) Impact of inorganic anions (15 mM) on PCFT-mediated $[^3\text{H}]5\text{-CHO-THF}$ influx at pH 7.4. R1-11-PCFT-h cells were preincubated in HBS (pH 7.4) for 20 minutes before 5-CHO-THF ($0.5\ \mu\text{M}$) influx was determined over 3 minutes in HBS containing the different anions. HEPES-sucrose is a transport buffer in which all cations or anions were isomotically replaced with sucrose. (B) Effects of sulfate, bisulfite, nitrate, and nitrite (all at 15 mM) on RFC-mediated 5-CHO-THF influx conducted at pH 7.4. 5-CHO-THF ($0.5\ \mu\text{M}$) influx was determined over 3 minutes in R1-11-RFC-6 cells that express RFC but not PCFT under the same conditions as in PCFT-h cells. (C) 5-CHO-THF ($0.5\ \mu\text{M}$) influx at pH 6.5. PCFT-h cells were preincubated in HBS (pH 7.4). Influx was measured in MBS (pH 6.5) over 1 minute in the presence or absence of anions (15 mM). ** $P < 0.01$; *** $P < 0.001$ vs. HBS group. For all panels, influx is expressed as percentage of activity in control buffer (HBS or MBS). Data are the mean $\pm$ S.E.M. from three independent experiments.

hand, intracellular pH fell rapidly to values < 6 when cells were exposed to either 10 mM nitrite or 5 mM bisulfite. When the buffer was changed back to HBS alone, the pH returned to

Fig. 5. Impact of bisulfite and nitrite on $[^3\text{H}]5\text{-CHO-THF}$ influx and influx kinetics at pH 6.5. (A) Concentration dependence of inhibition of $[^3\text{H}]5\text{-CHO-THF}$ influx by nitrite or bisulfite. PCFT-h cells were preincubated in HBS (pH 7.4) for 20 minutes before 5-CHO-THF ($0.5\ \mu\text{M}$) influx was measured over 1 minute in MBS (pH 6.5) in the absence or presence of various concentrations of bisulfite or nitrite. MBS transport buffers (pH 6.5) with different concentrations of nitrite or bisulfite were prepared by mixing MBS (pH 6.5) proportionately with MBS containing 24 mM nitrite or bisulfite (pH 6.5), respectively. Influx is expressed as percentages of activity in MBS. (B) Effect of 10 mM nitrite on 5-CHO-THF influx kinetics at pH 6.5 in R1-11-PCFT-h cells. (C) Effect of 5 mM bisulfite on 5-CHO-THF influx kinetics at pH 6.5 in R1-11-PCFT-h cells. For all panels, data are the mean $\pm$ S.E.M. from three independent experiments. The lines are computed as best fits to the Michaelis-Menten equation.

near baseline in the case of nitrite and increased, but to a lower level relative to the baseline, in the case of bisulfite. Comparable reductions in intracellular pH were also observed under these conditions in R-11 cells that do not express PCFT, indicating that the effects of nitrite and bisulfite on intracellular pH are not dependent on the presence of, or transport mediated by, PCFT (unpublished data).

Measurements of intracellular pH were also extended to conditions in which the incubation buffer was changed from HBS (pH 7.4) to the same buffer (pH 7.4) containing additional 15 mM bisulfite, 15 mM nitrite, or 15 mM bicarbonate, reflecting the conditions in which PCFT function was assessed at neutral pH. There was no significant alteration in intracellular pH under these conditions (unpublished data). Therefore, nitrite and bisulfite reduced the intracellular pH only when an inward proton gradient ($\text{pH}_{\text{intracellular}} > \text{pH}_{\text{extracellular}}$) was present.

Effects of Monocarboxylates on PCFT-Mediated Influx and Intracellular pH. The finding that marked inhibition of PCFT function by nitrite and bisulfite at acidic pH was associated with rapid reduction of intracellular pH prompted examination as to whether monocarboxylates, in general, decrease intracellular pH and thus reduce PCFT function at acidic pH. For this purpose, acetate and pyruvate were chosen as monocarboxylates and citrate as a tricarboxylate. Glycine was also chosen because it exists in solution as a zwitterion. As indicated in Fig. 7A, at a concentration of 15 mM,

Fig. 6. Effects of bisulfite and nitrite on intracellular pH. R1-11-PCFT-h cells growing in RPMI were loaded with the fluorescent dye BCECF and then washed with HBS (pH 7.4) several times. The intracellular pH under this condition served as the baseline for subsequent measurements. At the first arrow, MBS (pH 6.5) or MBS at this pH that contained nitrate (10 mM), nitrite (10 mM), sulfate (5 mM), or bisulfite (5 mM) replaced HBS. Intracellular pH was monitored until it stabilized. At the second arrow, the incubation buffer was returned to HBS (pH 7.4) and the intracellular pH was monitored until it restabilized. Each recording shown in the panel represents average values obtained from ~50 individual cells on a glass-bottom dish. The data are representative values from three separate dishes.

acetate and pyruvate reduced PCFT function at pH 6.5 by 75 and 67%, respectively. In contrast, citrate and glycine did not have any impact on PCFT-mediated 5-CHO-THF influx. Similarly, acetate and pyruvate resulted in a rapid reduction in intracellular pH, a change that was fully reversed when MBS (pH 6.5) containing acetate or pyruvate was replaced with HBS (pH 7.4) (Fig. 7B). Consistent with the lack of any effect on PCFT transport function, citrate and glycine had no impact at all on intracellular pH under similar conditions.

Discussion

PCFT is essential to folate homeostasis by virtue of its critical role in the intestinal absorption of folates. Because of its acidic pH optimum, PCFT has not been considered to be

important to the delivery of folates to systemic tissues save for its proposed role in folate transport across the basolateral membrane of the choroid plexus (Zhao et al., 2009, 2011a; Diop-Bove et al., 2011). The data in this paper add another dimension to the unfavorable milieu of the extracellular compartment of systemic tissues: the high levels of bicarbonate. Hence, the rates of transport mediated by PCFT at neutral pH measured in HBS substantially overestimate folate transport as it occurs in the presence of physiologic levels of bicarbonate. However, as the pH drops, the bicarbonate level drops as well, so that at the acidic pH of the microenvironment of the intestinal epithelium, not only is the proton gradient high but the bicarbonate level is substantially reduced, favoring PCFT-mediated absorption. A similar scenario also exists in solid tumors where the interstitial pH is acidic, the extent to which depends on the tumor type and size (Wike-Hooley et al., 1984; Helmlinger et al., 1997; Raghunand et al., 1999). In this case, PCFT-mediated antifolate uptake into tumor cells will be much more rapid than occurs in normal tissues. This is the rationale for the development of antifolate compounds with high affinity for PCFT but very low affinity for RFC as a way of minimizing toxicity to normal tissues mediated by the latter transporter (Kugel Desmoulin et al., 2010, 2011; Wang et al., 2011). It is also important to point out that cells that express only PCFT are sensitive to pemetrexed in cell culture containing the physiologic level of bicarbonate, so residual transport is sufficient to sustain pharmacological activity (Zhao et al., 2004b).

Both nitrite and bisulfite inhibit PCFT transport function, but this is not the case for nitrate and sulfate. Although both nitrite and bisulfite can react chemically with folates (Vonderschmitt et al., 1967; Reed and Archer, 1979), this cannot account for their inhibitory effects in the current study. Hence, 5-CHO-THF influx mediated by RFC at pH 7.4 was not selectively inhibited by nitrite as compared with nitrate, or by nitrite as compared with bisulfite, as would occur if they resulted in the chemical breakdown of 5-CHO-THF. Both bisulfite and nitrite have cytotoxic properties. Nitrite reacts with certain amines to form carcinogen nitrosamines or highly reactive peroxynitrite (Chow and Hong, 2002), while bisulfite alters 2,3-diphosphoglycerate levels in erythrocytes due to its reducing potential (Parker, 1969). However, the impact of these two anions on PCFT-mediated transport, particularly on reduction of intracellular pH, is not related to their cytotoxic potential due to the very short interval of exposures in these experiments. Indeed, the reduction in intracellular pH induced by nitrite was rapidly reversible. It is unclear why the effect of bisulfite on the intracellular pH reduction could only be partially reversed.

The much greater inhibition of PCFT-mediated transport by nitrite and bisulfite at pH 6.5 than at pH 7.4 was associated with cellular acidification and a reduction of the transmembrane proton gradient by these two anions. In these experiments, an inward pH gradient was present at the initiation of transport. However, inclusion of 10 mM nitrite or 5 mM bisulfite in the transport buffer reduced the intracellular pH below 6.5 within 1 minute, rapidly abolishing the pH gradient. These data are consistent with the observations that elimination of the transmembrane proton gradient by ionophores, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone or nigericin in combination with KCl, markedly reduces PCFT activity (Qiu et al., 2006; Nakai et al., 2007). Previous studies

Fig. 7. Effects of organic anions on PCFT-mediated [^3H]5-CHO-THF influx and the intracellular pH in R1-11-PCFT-h cells. (A) Effects of 15 mM acetate, pyruvate, citrate, or glycine on [^3H]5-CHO-THF influx at pH 6.5. Cells were preincubated in HBS (pH 7.4) for 20 minutes before influx of 5-CHO-THF (0.5 μM) was assessed in MBS (pH 6.5) in the absence or presence of organic anions over 1 minute at 37°C. Inhibition is expressed as percentage of influx in MBS. *** $P < 0.001$ vs. control (MBS). Data are the mean $\pm$ S.E.M. from three independent experiments. (B) Effects of 15 mM acetate, pyruvate, citrate, or glycine on the intracellular pH upon replacement of the extracellular buffer (HBS, pH 7.4) with MBS (pH 6.5). BCECF-loaded cells were incubated in HBS (pH 7.4) for baseline intracellular pH measurements at room temperature. At the arrow, the extracellular buffer was replaced with MBS (pH 6.5) containing 15 mM acetate, pyruvate, citrate, or glycine, following which the buffer was replaced with HBS (pH 7.4) after the pH stabilized. Each recording shown in the panel represents average values obtained from ~50 individual cells on a glass-bottom dish. The data are representative values from three separate dishes.

indicate that as the extracellular pH is increased from pH 5.5 to 7.4, the influx $V_{\max}$ decreases and the influx K_t increases, changes that are substrate-dependent (Qiu et al., 2006; Zhao et al., 2008). The inhibitory effects of nitrite and bisulfite on 5-CHO-THF influx at pH 6.5 were associated the loss of the pH gradient and were characterized by a fall in the influx $V_{\max}$ without a significant alteration in influx K_t . This is consistent with the observation that the E185A-PCFT mutation that resulted in a loss of proton-folate coupling was associated with a marked fall in the influx $V_{\max}$ but no change at all in influx K_t under conditions in which there was no change in the extracellular proton level (Unal et al., 2009b). On the other hand, changes in the affinity of PCFT for protons, as was the case for the H281A-PCFT mutant, result solely in alterations in the influx K_t (Unal et al., 2009a).

Both nitrite (10 mM) and bisulfite (5 mM) can serve as proton ionophores because they can be protonated to form their weak acids, nitrous acid or sulfurous acid, respectively, which freely diffuse across cell membranes. Based upon a pK_a of 3.3, the nitrous acid concentration is more than 3 orders of magnitude lower than the nitrite concentration at pH 6.5. The sulfurous acid concentration is more than 4 orders of magnitude lower than bisulfite at pH 6.5 due to its pK_a of 1.8. Hence, nitrous acid at a concentration of 10 μ M or sulfurous acid at a concentration of 0.5 μ M is apparently high enough to rapidly collapse the pH gradient. Nitrate and sulfate cannot be protonated in aqueous solution and thus are not proton ionophores. This also applies to molecules carrying carboxyl groups with a pK_a of $\sim$ 4.8. Hence, 15 mM acetate or pyruvate decreased the intracellular pH, whereas citrate or glycine at the same concentration had no effect.

In the case of citrate, a tricarboxylate, the possibility that its three carboxyl moieties are protonated to form a neutral, membrane-permeant molecule is nil at pH 6.5. Similarly, glycine exists like other amino acids as a zwitterion and is always charged and membrane-impermeant. These results may explain why a variety of anions including a number of monocarboxylates, such as diclofenac and indomethacin, inhibit function of the highly folate-specific PCFT (Nakai et al., 2007; Inoue et al., 2008). In general, interpretation of the nature of inhibition of proton-coupled transport should take into consideration whether this is secondary to a decrease in the transmembrane proton gradient as distinguished from a direct interaction with the carrier. This is of particular importance when studies are performed in cells that do not exist in an acidic environment under physiologic conditions and do not have sufficient compensatory mechanisms to sustain their intracellular pH.

The mechanism by which bicarbonate, nitrite, and bisulfite inhibit PCFT-mediated influx at pH 7.4 is unclear. Kinetic analysis indicates that bicarbonate inhibition of PCFT-mediated transport is not a classic competitive phenomenon. Both nitrate and sulfate cannot be protonated in aqueous solution at any pH, and neither has an inhibitory effect on PCFT at pH 7.4. One possibility is that bicarbonate, nitrite, or bisulfite can interfere with proton binding to PCFT and/or proton translocation mediated by PCFT. The former possibility might, in turn, reduce folate binding to PCFT and the latter reduce mobility of PCFT during substrate translocation. It should be pointed out that the inhibitory potential of the anions (bicarbonate > bisulfate > nitrite) does not correspond to the respective pK_a of their conjugated acids (6.5, 1.8, and 3.3,

respectively). Interestingly, phosphate that has a pK_{a1} of 2.1 did not inhibit PCFT-mediated influx at either pH 7.4 or 6.5.

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Authorship Contributions

Participated in research design: Zhao, Suadicani, Goldman.

Conducted experiments: Zhao, Visentin, Suadicani.

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Wrote or contributed to the writing of the manuscript: Zhao, Suadicani, Goldman.

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