Intracellular Carbonic Anhydrase Activity Sensitizes Cancer Cell pH Signaling to Dynamic Changes in CO2 Partial Pressure*

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Background: Intracellular carbonic anhydrase (CAi) activity, commonly detected in cancer, accelerates CO2/HCO3− equilibration.

Results: In cells with high CAi activity, fluctuations in pCO2 (which can arise from intermittent blood flow) evoke substantial intracellular pH oscillations that modulate downstream pathways (e.g., calcium, mTOR).

Conclusions: CAi transduces the state of perfusion to intracellular signaling.

Significance: Coupling between environment and cell behavior may influence cancer progression.

Carbonic anhydrase (CA) enzymes catalyze the chemical equilibration among CO2, HCO3− and H+. Intracellular CA (CAi) isoforms are present in certain types of cancer, and growing evidence suggests that low levels correlate with disease severity. However, their physiological role remains unclear. Cancer cell CAi activity, measured as cytoplasmic CO2 hydration rate (kf), ranged from high in colorectal HCT116 (−2 s−1), bladder RT112 and colorectal HT29, moderate in fibrosarcoma HT1080 to negligible (i.e. spontaneous kf = 0.18 s−1) in cervical HeLa and breast MDA-MB-468 cells. CAi activity in cells correlated with CAII immunoreactivity and enzymatic activity in membrane-free lysates, suggesting that soluble CAII is an important intracellular isoform. CAi catalysis was not obligatory for supporting acid extrusion by H+ efflux or HCO3− influx, nor for maintaining intracellular pH (pH$_{i}$) uniformity. However, in the absence of CAi activity, acid loading from a highly alkaline pH$_{w}$ was rate-limited by HCO3− supply from spontaneous CO2 hydration. In solid tumors, time-dependence of blood flow can result in fluctuations of CO2 partial pressure (pCO2) that disturb cytoplasmic CO2−HCO3−-H+ equilibrium. In cancer cells with high CAi activity, extracellular pCO2 fluctuations evoked faster and larger pH$_{i}$ oscillations. Functionally, these resulted in larger pH-dependent intracellular [Ca$^{2+}$] oscillations and stronger inhibition of the mTORC1 pathway reported by S6 kinase phosphorylation. In contrast, the pH$_{i}$ of cells with low CAi activity was less responsive to pCO2 fluctuations. Such low pass filtering would “buffer” cancer cell pH$_{i}$ from non-steady-state extracellular pCO2. Thus, CAi activity determines the coupling between pCO2 (a function of tumor perfusion) and pH$_{i}$ (a potent modulator of cancer cell physiology).

Due to rapid permeation of CO2 gas across cell membranes (4), carbonic buffer is present in both intra- and extracellular tissue compartments. On the time scale of cellular physiology, the spontaneous equilibration of carbonic buffer is relatively slow, as exemplified by a CO2 hydration time constant of several seconds (rate constant k$_{f}$ = 0.18 s$^{-1}$) (5). Consequently, biological processes that involve a change in the concentration of CO2, HCO3− or H+ can become rate-limited by carbonic buffer re-equilibration. This limitation has presumably driven the evolution of at least a dozen mammalian carbonic anhydrase (CA) isoforms that accelerate CO2/HCO3− equilibrium (6, 7).

The CAs are grouped as intra- (CA1) or extracellular (CA3) depending on the orientation of the catalytic site (5–8). Activity assays and immunotechniques have identified CA1 and CA3 isoforms in cancer cells (9–17). Physiologically, CA1 isoforms, such as CAIX and CAXII, facilitate CO2 and H+ diffusion across the continuous and tortuous interstitial space (18, 19).

Thus, CA$_{i}$ activity can improve the venting of acidic products of metabolism over the long diffusion distances found in inadequately perfused solid tumors, allowing their faster growth (20, 21). The role of CA$_{i}$ isoforms in cancer physiology is still debated. Down-regulation of gap junctions in cancer cells prevents the intracellular compartment from becoming syncytial (22), and this restricts the spatial range over which CA$_{i}$ activity could facilitate CO2 or H+ diffusion. Previously, it has been suggested that CA$_{i}$ activity facilitates the transport of HCO3− or H+ ions across membranes by reducing the extent to which cytoplasmic reactions slow the delivery or removal of the transported ion (23–26). To benefit from CA$_{i}$ activity, these transporter-evoked H+ or HCO3− fluxes would have to exceed the spontaneous chemical re-equilibration kinetics of carbonic buffer (27).
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Another source of disturbance to carbonic buffer equilibriums is fluctuating CO₂ partial pressure (pCO₂). Cancer cells produce large quantities of CO₂ from titration of acids (e.g., lactic) with HCO₃⁻ and decarboxylation by mitochondria and the pentose phosphate shunt (28). Ultimately, the excess CO₂ must be removed with the blood flow. In tumors, vasomotion and hemodynamic factors can produce cycles of intermittent blood flow, commonly observed with periodicities of several minutes (29–33). Unstable perfusion is the basis for acute hypoxia (32, 34–40), characterized by oxygenation-reoxygenation cycles as fast as 2/min (30) and amplitudes of tens of mmHg O₂ (30, 35, 41, 42). Episodes of inadequate blood flow produce “closed” pockets of blood, which become oxygen-depleted and accumulate CO₂ (43–45). Periodic restoration of flow returns pCO₂ and pO₂ to normal. The resulting fluctuations in pCO₂ (mirroring pO₂) are transmitted across the intracellular and extracellular compartments because CO₂ gas (unlike H⁺ or HCO₃⁻ ions) crosses membranes rapidly (4). Due to reversible intracellular CO₂ hydration, pH will respond to pCO₂ fluctuations, but the coupling between these depends critically on CA_i activity. Because cell behavior is strongly modulated by H⁺ ions (46–48), CA_i activity may act as an important transducer between blood flow and cell signaling.

The aim of the present study was to measure CA_i activity in the native environment of intact cancer cells and to identify the physiological processes, relevant to cancer, that depend on CA_i activity. We describe a novel role for CA_i activity in sensitizing cancer cell pH to pCO₂ changes, thereby linking metabolism and perfusion with pH-responsive signaling cascades.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—Cancer cell lines were obtained from ATCC (colorectal HCT116), Cancer Research UK (bladder RT112, colorectal HT29, fibrosarcomal HT1080, and breast MDA-MB-468) or were a gift from Professor Silvia Pastorekova, Bratislava, Slovakia (cervical HeLa). Fibroblast cell lines were obtained from Lonza (NHDF-Ad adult dermal fibroblasts and InMyoFib intestinal myofibroblasts) or ATCC (non-tumoral Hela) (or H₁₁₀₀₀). Fibroblast cell lines were obtained from Lonza (NHDF-Ad adult dermal fibroblasts and InMyoFib intestinal myofibroblasts) or ATCC (normal colon CCD-112-Con and carcinoma-derived Hs675.T) or were a gift from Professor Silvia Pastorekova; Ref. 49); antibodies against CAI, -III (R&D Systems), -II (Novus), -VII (AbD Serotech), and -XIII (Abcam); and goat polyclonal antibody to actin (Santa Cruz Biotechnology). Kinase phosphorylation in cells cultured to high density was measured with the phospho-MAPK array profiler (R&D Systems) and mechanistic target of rapamycin (mTOR) signaling kit (Cell Signaling Technology).

Measuring Carbonic Anhydrase Activity in Lysates—Cells were lysed by repeated freeze-thaw cycles in buffer containing 140 mM potassium glutonate, 0.5 mM EGTA, 1 mM MgCl₂, 15 mM Hepes, 15 mM Mes at pH 7.8 (4 °C), and protease inhibitor. Membranes were removed by centrifugation (20 min at 15,000 rpm at 4 °C), and the supernatant was diluted to a total protein concentration between 1 and 10 mg/ml (Bradford assay). The CA-catalyzed reaction was triggered by adding 0.33 ml CO₂-saturated water to 0.67 ml of lysate in a stirred chamber at 4 °C. The time course of pH (Hamilton Biotrode) was fitted with a kinetic model (19) to obtain the CO₂ hydration rate constant kᵣ.

Superfusion—CO₂/HCO₃⁻-free solution contained 125 mM NaCl, 20 mM Hepes (pH 7.4 with NaOH), 4.5 mM KCl, 11 mM glucose, 1 mM CaCl₂, and 1 mM MgCl₂. For CO₂/HCO₃⁻-buffered solution, Hepes was replaced with 22 mM NaHCO₃, and the solution was bubbled with either 5% CO₂ (pH 7.4) or 20% (pH 6.8). For Cl⁻-free solutions, Cl⁻ was replaced with gluconate. For acetate-containing solution, 80 mM NaCl was replaced with 80 mM sodium acetate. For NH₄⁺-containing solution, 20 mM NaCl was replaced with 20 mM NH₄Cl. Cells were superfused (37 °C at 4 ml/min) in a poly-L-lysine-treated Perspex chamber (solution exchange time constant <3 s) except for fluctuating pCO₂ protocols, which were performed in 2-ml Lab-Tek chambers (Nunc) that allow a more gradual solution exchange.

Fluorescence Measurements of Intracellular pH and Calcium—Cells were imaged confocally using a Zeiss LSM 700 confocal system on an Axiovert inverted microscope. To measure pHᵢ, cells were AM-loaded for 10 min with 10 µM 5(6)-carboxy-SNARF1 (excitation, 555 nm; emission, 580 and 640 nm). The fluorescence ratio was converted to pHᵢ using a calibration curve obtained by the nigericin method (50). Calibration was performed twice a year for each cell line, and the stability of the curve was tested by the null point method (51). Membrane H⁺ (or H⁺-equivalent) flux was calculated as the rate of pHᵢ change × buffering capacity (3). Intrinsic buffering capacity was measured in separate experiments using the graded ammonium removal technique (3). CO₂/HCO₃⁻-dependent buffering was estimated using the equation for an open buffer system: 2.303 × [HCO₃⁻], Intracellular [HCO₃⁻] was calculated by best fitting a mathematical model of pHᵢ regulation to the time course of pHᵢ recovery from an acid or base load (see below). This approach derives instantaneous carbonic buffering capacity (rather than assuming that the buffer is distributed at equilibrium across the cell membrane at all times). To measure [Ca²⁺]ᵢ, cells were AM-loaded for 20 min with 50 µM Fluo3 (excitation, 488 nm; emission, >510 nm). For co-cultures, regions with distinct areas of cancer cells and fibroblasts (dis-
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tingished by cell morphology and size) were selected for imaging.

Mathematical Modeling—A mathematical model of pH control was parameterized using data for buffering capacity (β) and membrane transport fluxes of H\(^+\) and HCO\(_3^-\) ions (J\(^H\) and J\(^HCO_3^-\)) (3). The carbonic buffer equilibrium constant, K\(_{CO_2}\), was 10\(^{-6.1}\) M, and the spontaneous CO\(_2\) hydration rate constant (k\(_r\)) was 0.18 s\(^{-1}\) (the reverse rate constant, k\(_{-r}\), was k\(_r\)/K\(_{CO_2}\)). CAi activity was represented as a dimensionless scalar (ca). Surface area/volume ratio was 1/h where h is the monolayer height (10 \(\mu\)m). CO\(_2\) permeability, p, was 10\(^6\) m/s (4). pCO\(_2\) was a sinusoidal function, f\(_t\) of amplitude A, frequency \(\phi\), and baseline of 5% CO\(_2\). The equations are as follows.

\[
d[C\(_{O_2}\)]/dt = ca \times k_i \times 10^{-\phi t} \times [HCO_3^-]_i - ca \times k_i \times [CO_2]_i + p/h \times (f(t) - [CO_2]_i) \quad \text{(Eq. 1)}
\]

\[
d[HCO_3^-]/dt = ca \times k_i \times [CO_2]_i - ca \times k_i \times 10^{-\phi t} \times [HCO_3^-]_i + \mu[HCO_3^-] \quad \text{(Eq. 2)}
\]

\[
dpH/dt = -(ca \times k_i \times [CO_2]_i - ca \times k_i \times 10^{-\phi t}) \times [HCO_3^-]_i + f(t)/\beta \quad \text{(Eq. 3)}
\]

RESULTS

CA\(_i\) Activity in Cancer and Fibroblast Cell Lines—Total CA\(_i\) activity was measured in intact cells superfused continuously with physiological salt solution at body temperature. The CA\(_i\)-catalyzed reaction was triggered by switching between CO\(_2\)/HCO\(_3^-\)-free and 5% CO\(_2\)/22 nM HCO\(_3^-\)-buffered superfusates. The time constant of solution exchange (2.7 s; determined by fluorescein labeling one solution with 30 \(\mu\)M fluorescein) was adequately fast to ensure that pCO\(_2\) was manipulated rapidly. CA\(_i\)-catalyzed reaction kinetics were determined from the initial rate of pH change (reported using the fluorescent pH indicator cSNARF1 AM-loaded into cells) and intrinsic buffering capacity (52). Fig. 1A, panel i, shows an example pH\(_t\) time course recorded from colorectal HCT116 cells paired with an experiment performed in the presence of the broad spectrum CA inhibitor acetazolamide (ATZ; 100 \(\mu\)M) to determine spontaneous kinetics over a matching pH\(_t\) range. CA\(_i\) activity was expressed relative to spontaneous reaction kinetics (Fig. 1A, panel ii). Experiments were also performed on bladder RT112, colorectal HT29, fibrosarcoma HT1080, cervical HeLa, and breast MDA-MB-468 cells as well as dermal NHDF-Ad, colonic CCD-112-CoN fibroblasts, and intestinal InMyoFib myofibroblasts. CA\(_i\) activity ranged from ~9-fold above the spontaneous rate in HCT116 cells to very low in HeLa, MDA-MB-468, and fibroblast/fibroblast-related cells. Thus, the CO\(_2\) hydration capacity of cancer cell cytoplasm spanned over an order of magnitude in range and was cell line-dependent.

Soluble cytoplasmic CA (CA\(_s\)) isoforms (e.g., CAI, -II, -III, -VI, and -XII) are plausible contributors to CA\(_i\) activity. This was tested in cell lysates after removing membranes by centrifugation. Lysates were injected with CO\(_2\)-saturated water, and the CO\(_2\) hydration constant was estimated from the time course of medium pH at 4 °C (Fig. 1B, panel i). CA\(_s\) activity was quantified in terms of the CO\(_2\) hydration rate constant relative to the measurement in the presence of ATZ (100 \(\mu\)M). Kinetic data were normalized to total protein concentration (19). Substantial CA\(_i\) catalysis was detected in lysates of cancer cells with high total CA\(_i\) activity determined in intact cells. This argues that soluble isoforms underlie at least part of intracellular CO\(_2\) hydration catalysis (Fig. 1B, panel ii). The contribution of CA\(_i\) to total CA\(_i\) activity was tested in HCT116 cells by knockdown using shRNA constructs 351, 495, 597 and 695 and compared with scrambled shRNA and wild-type cells. Construct 695 resulted in the most substantial decrease in CA\(_i\) immunoreactivity (Fig. 1C, panel i), and it reduced total CA\(_i\) activity by ~90% relative to wild-type (Fig. 1C, panel ii). To test whether the decrease in total CA\(_i\) activity is attributable to disruption of CAII alone, the specificity of shRNA constructs 695 and 597 was investigated. Constructs 695 and 597 have very low sequence homology (16–42%) with membrane-associated CAs (IV, IX, XII, and XIV) and low homology (53–68%) with soluble isoforms other than CAI (I, III, VII, and XIII). Of the soluble isoforms, HCT116 expressed only low levels of CAVII and -XIII, and immunoreactivity for CAI and -III was absent (Fig. 1C, panel iii). Constructs 695 and 597 did not alter this expression pattern, indicating that the knockdown experiment reduced CA\(_i\) activity by targeting CAII selectively. The findings indicate that CAII is a principal contributor to CA\(_i\) activity in HCT116 cells.

Across the tested cancer, fibroblast, and fibroblast-related cells, CA\(_i\) immunoreactivity (Fig. 1D) correlated with in situ CA\(_i\) activity (Pearson’s correlation coefficient \(r^2 = 0.83\)). CA\(_i\) expression was notably absent in fibroblast and fibroblast-related cells, including cancer-derived Hs675.T cells. In cancer cells, CA\(_i\) expression correlated strongly (\(r^2 = 0.97\)) with CA\(_s\) activity measured in membrane-free lysates. As expected from active site topology, the expression of membrane-tethered CAIX did not correlate with CA\(_i\) activity. Hypoxia plays an important role in cancer biology by regulating gene expression through mechanisms that include hypoxia-inducible factor. To investigate the effect of long term hypoxia on CA\(_i\) expression, Western blot analysis was performed on cells incubated for 48 h with the hypoxia-inducible factor-stabilizing drug dimethyl oxalylglycine (3, 53). This treatment did not affect CA\(_i\) expression but produced the expected induction of CAIX in RT112, HT1080, MDA-MB-468, and HeLa (54) and to a lesser degree in NHDF-Ad and CCD-112-CoN fibroblasts.

Role of CA\(_i\) Activity in pH\(_t\) Regulation—The supply of H\(^+\) or HCO\(_3^-\) ions for H\(^+\)\(_t\)-regulatory transport across membranes may be rate-limited by the CA\(_i\)-catalyzed reaction. This was investigated in RT112, MDA-MB-468, and HCT116 cells. RT112 cells have naturally high CA\(_i\) activity and can produce large HCO\(_3^-\) fluxes at both low and high pH\(_t\) (3). Acid extrusion was evoked at low pH\(_t\) by means of an “ammonium prepulse” solution maneuver performed on cSNARF1-loaded cells (Fig. 2A, panel i). An “acetate prepulse” was performed to raise pH\(_t\). Because abrupt base loading of cytoplasm upon acetate removal drives carbonic buffer out-of-equilibrium, cells were superfused briefly in Cl\(^-\)-free medium to allow buffer re-equilibration (55). Subsequent re-exposure to Cl\(^-\)-containing superfusates activated acid loading transporters (Fig. 2A, panel ii).
H⁺/H⁺-equivalent flux was calculated as the product of the rate of pH change and buffering capacity (the carbonic buffer component was estimated using a mathematical model to predict out-of-equilibrium behavior during dynamic pH regulation). pHr-regulatory fluxes, shown in Fig. 2A, panel iii, were symmetrical around the resting pHr. To establish the contribution of Na⁺/H⁺ exchange (NHE) to pHr regulation, measurements were repeated in the presence of 30 μM 5-(N,N-dimethyl)amiloride (DMA). Acid loading was DMA-insensitive, whereas acid extrusion flux was reduced only modestly, confirming a minor role for NHE in pHr regulation in RT112 cells (Fig. 2A, panel iii, inset). Replacing superfusate carbonic buffer with Heps reduced flux substantially, indicating that pHr regulation relies principally on HCO₃⁻ transport. The effect of CA₁
activity on acid/base membrane transport was inferred from the effect of 100 μM ATZ (in the presence of carbonic buffer). Although ATZ targets both intra- and extracellular CAs, the latter isoforms would not be exerting a net catalytic effect because superfusion presents cells with pre-equilibrated buffer at all times (19). ATZ did not affect resting pH or acid extrusion flux, but it limited acid-loading fluxes to no greater than 14 mM/min. This flux is equal to the maximal rate of HCO₃⁻ delivery by CO₂ hydration under spontaneous reaction kinetics (κᵣ × [CO₂] = 14 mM/min). Thus, acid loading by means of HCO₃⁻ export can be rate-limited by CAᵢ activity, but this requires large fluxes evoked by substantially raised pHᵢ.

Intracellular H⁺ ions diffuse considerably slower than expected from their low atomic weight because of extensive binding to larger buffer molecules, including immobile proteins (56, 57). NHE activity could plausibly be rate-limited by a slow

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**FIGURE 2. Role of CAᵢ activity in membrane H⁺/HCO₃⁻ transport and cytoplasmic H⁺ diffusion.** A, 20 mM ammonium (panel i) and 80 mM acetate (panel ii) prepulse performed on RT112 cells (5% CO₂/22 mM HCO₃⁻ superfusate). Experiment repeated in 100 μM ATZ (dotted line). Following acetate removal, cells were stabilized in Cl⁻-free superfusate to allow for HCO₃⁻ re-equilibration. Panel iii, acid-extrusion and acid-loading fluxes (n > 20). Dashed line, limit of spontaneous production of HCO₃⁻. Inset, effect of 30 μM DMA. B, 20 mM ammonium prepulse performed on MDA-MB-468 cells in the absence (panel i) or presence (panel ii) of 5% CO₂/22 mM HCO₃⁻. Cytoplasmic pH was measured in concentric layers of thickness ~1.5 μm near surface membrane (black trace) and at cell core (gray trace). Mean of 10 cells (error bars not shown). C, panel i, 20 mM ammonium prepulse performed on HCT116 cells in the absence of CO₂/HCO₃⁻. Panel ii, intrinsic buffering capacity (measured by graded ammonium removal) in control HCT116 cells and cells cultured with 6 mM carnosine for 48 h (n > 20). Panel iii, NHE flux (n > 20) in control (CO₂/HCO₃⁻-free), in carnosine-loaded cells (CO₂/HCO₃⁻-free), and in the presence of 5% CO₂/22 mM HCO₃⁻ (determined by subtracting the DMA-sensitive component of flux). Panel iv, H⁺-equivalent flux in CO₂/HCO₃⁻ buffer measured in CAII knockdown HCT116 cells (construct 695) and compared with scrambled (sham). Experiments were repeated in DMA to inhibit the NHE component of acid extrusion (n > 15). Error bars represent S.E.
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diffusive supply of H+ ions and therefore require CAi activity for delivering H+ ions from CO2 hydration. As shown in non-cancerous cells, CAi activity can facilitate H+ diffusion by improving the reaction kinetics of CO2/HCO3−, a mobile buffer (58, 59). If the intrinsic H+ ion diffusivity in cytoplasm were sufficiently restricted, a large acid/base flux at the surface membrane would be expected to produce measurable pH5 non-uniformity in the absence of carbonic buffer. This was tested in MDA-MB-468 cells, which produce very high NHE fluxes at low pH5 (3). Cells were trypsinized to produce spherical cells with the smallest possible surface area-to-volume ratio, i.e. longest average surface-to-core diffusion distance. The mean radius of MDA-MB-468 cells was 7.11 ± 0.12 μm, which is typical of most cancer cells. NHE is functional in the presence and absence of carbonic buffer; therefore it is possible to investigate the effect of removing CO2/HCO3− on spatial H+ dynamics in cytoplasm. pH5 uniformity during rapid acid extrusion was assessed by imaging pH5 confocally near the surface membrane and at the core of the cell. The pH5 time courses shown in Fig. 2B, panel i, indicate that even at maximal acid extrusion rates, pH5 remained uniform in the absence of carbonic buffer. The addition of carbonic buffer did not affect the degree of pH5 uniformity (Fig. 2B, panel ii). Thus, carbonic buffer and hence CAi activity are not required for ensuring adequate H+ diffusion in MDA-MB-468 cells. These findings are indicative of good diffusive coupling by intrinsic H+ buffers.

Measurements of pH5 gradients in bulk cytoplasm may not identify diffusion barriers in the immediate environment of the transport protein. An example of such a barrier is slow H+ transfer from immobile buffers to the transporter proteins (60, 61). Given that CO2 is a highly penetrating source of H+ ions (26), a role for CAi activity in overcoming these localized H+ barriers is conceivable. To test for possible submembrane barriers, acid extrusion was measured in HCT116 cells, which have naturally high CAi activity and the capacity to produce large NHE fluxes (3). If CAi activity were important for delivering H+ ions to NHE protein, then removal of carbonic buffer would slow overall flux to a baseline set by the intrinsic capacity of the cytoplasm to supply the transporter with H+ ions. Loading cytoplasm with exogenous mobile buffers, such as imidazoles, would then be expected to rescue acid extrusion (60). Fig. 2C, panel i, shows the time course of pH5 recovery from low pH5 in the absence of CO2/HCO3−. This eliminates CO2-sourced delivery of H+ ions and would expose any submembrane diffusional barriers. Experiments were repeated on cells loaded with the imidazole-containing mobile buffer carnosine (6 mM for 48 h). Intrinsic buffering capacity, measured by the “graded ammonium removal” technique (62), was higher in carnosine-incubated cells, consistent with the loading of cytoplasm with an exogenous buffer of pK 6.7 (similar to that of carnosine) and concentration of 12 mM (Fig. 2C, panel ii). After accounting for the additional buffering capacity, flux in carnosine-loaded cells was no different from control (Fig. 2C, panel iii). Further experiments were performed on control HCT116 cells superfused with carbonic buffer. Because HCO3−-dependent mechanisms (e.g. Na−–HCO3− co-transport) are activated in the presence of carbonic buffer, NHE-mediated flux was calculated by subtracting the DMA-sensitive component from the total flux.

Neither CO2/HCO3− nor carnosine increased NHE flux (Fig. 2C, panel iii), arguing that these additional mobile buffers are not required for delivering H+ ions to NHE. Thus, CAi catalysis is unlikely to accelerate transport by providing an additional route of H+ delivery. An earlier study had suggested that imidazole groups projecting from the CAII molecule deliver H+ ions to H+ transporters without involving the catalytic process (60). Because large acid extrusion fluxes could be produced by CAII-negative MDA-MB-468 cells, an acatalytic effect of CAII is unlikely to be an absolute requirement for fast NHE activity. This was explored further in CAII knockdown HCT116 cells (construct 695; Fig. 1C, panel i). Acid extrusion in carbonic buffer, which includes NHE and HCO3−-dependent components, was not different in CAII-negative cells compared with scrambled controls (Fig. 2C, panel iv). The HCO3−-dependent flux component, measured by inhibiting NHE with DMA, was also unaffected by CAII knockdown (Fig. 2C, panel iv).

Role of CAi Activity during Fluctuations in pCO2—To explore the effects of CAi activity during pCO2 fluctuations associated with intermittent blood flow, superfusion of confluent monolayers (grown in slow exchange chambers) was alternated between 5% CO2/22 mM HCO3− (pH 7.4; representing normal arterial blood plasma) and 20% CO2/22 mM HCO3− (pH 6.8; representing respiratory acidosis) with a periodicity of 4 min. By labeling one solution with fluorescein (30 μM) in separate experiments, the solution mixing interval was estimated to be ~15 s, which allows for a relatively slow transition between 5 and 20% CO2. Fig. 3A shows the time course of extracellular [H+] in the superfusion chamber reported by fluorescein (an indicator of solution pH) added to both solutions at 30 μM. The response of intracellular [H+] to pCO2 fluctuations was recorded in cSNARF1-loaded monolayers of cells with high CAi activity (HCT116 and HT29; Fig. 3, B and C), low CAi activity (HeLa and MDA-MB-468; Fig. 3, D and E), and CAII knockdown HCT116 (CAII-KD). The measured [H+] fluctuations were quantified in terms of root mean square in Fig. 3F. Experiments were repeated in the presence of ATZ (100 μM) to inhibit CA i activity. The temporal behavior of [H+] was ATZ-sensitive in HCT116 and HT29 cells but not in HeLa, MDA-MB-468, or CAII knockdown HCT116 cells (Fig. 3F). These responses did not correlate with normoxic CAIX expression (high in MDA-MB-468 and HT29 and low in HeLa and HCT116), and therefore cannot be attributed to the inhibition of exofacial CA isoforms. Thus, in cells with high CAi activity, [H+] fluctuations were 30% faster and over a larger range, demonstrating a tighter coupling between pCO2 and cytoplasmic [H+] fluctuations.

The relationship between pCO2 and [H+], was explored further using a mathematical model parameterized using data for CAi activity and buffering capacity in HCT116 cells (3). Smooth transitions between normal and raised pCO2 were simulated with a sinusoidal wave (Fig. 3G) over a range of periodicity and amplitude. Fig. 3H shows the predicted effect of 10-fold CAi activity on the size of [H+] fluctuations. [H+] fluctuations were amplified 30% for periodicities of 4 min and at least doubled for periodicities of <2 min. The relative effect of CAi activity on [H+] dynamics was essentially independent of pCO2 amplitude. pH-regulatory H+ and HCO3− membrane transport is expected to curtail the extent of [H+] changes, but mathe-
mathematical modeling (not shown) predicts this to be a minor effect because the magnitude of membrane H⁺/HCO₃⁻/H₁¹₀₀₁⁻ transport is considerably smaller than that of CO₂ flux, particularly for short pCO₂ wave periodicities.

In summary, CA₉ activity sensitizes cancer cell pH to fluctuations in pCO₂. In contrast, cytoplasm with low CA₉ activity behaves as a low pass filter that dampens [H⁺] changes. Essentially all biological processes are pH-sensitive; therefore the effect that CA₉ activity has on [H⁺] dynamics during pCO₂ fluctuations is potentially of major functional importance. This was explored by measuring Ca²⁺/H₁¹₀₀₁⁻ signaling and kinase activity as examples of intracellular signaling.

**Ca²⁺ Signaling**—The behavior of the signaling ion Ca²⁺ was measured in Fluo3-loaded HCT116 cells during oscillations of pCO₂ between 5 and 20% (4-min periodicity). A significant increase in Fluo3 fluorescence was observed upon returning pCO₂ from 20 to 5% (Fig. 4A). Fluo3 fluorescence is modestly pH-sensitive, and to assess the magnitude of this artifact, HCT116 cells superfused in Ca²⁺/H₁¹₀₀₁⁻-free and CO₂/HCO₃⁻/H₁¹₀₀₂⁻-free media were exposed to 40 mM acetate for 4 min to evoke a pHᵢ change comparable to that caused by the pCO₂ fluctuations. Fluo3 fluorescence did not change substantially during this protocol (Fig. 4B), arguing that the responses shown in Fig. 4A report a genuine rise of cytoplasmic [Ca²⁺] ([Ca²⁺]ᵢ). The mechanism of this response was explored using inhibitor drugs and Ca²⁺/H₁¹₀₀₁⁻ buffers (Fig. 4C). The Ca²⁺ response was dampened in cells AM-loaded with 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid (BAPTA; 100 µM), adding to the evidence that Fluo3 reports [Ca²⁺]ᵢ changes rather than pHᵢ. Responses were considerably greater when intracellular Ca²⁺ stores were depleted by pretreatment with 10 µM thapsigargin (sarco/endoplasmic reticulum Ca²⁺-ATPase inhibitor). In the

**FIGURE 3. Effect of CA₉ activity on cytoplasmic pH dynamics during fluctuating pCO₂.** pCO₂ was changed between 5 and 20%. A, extracellular pH reported using fluorescein (30 µM). B, intracellular [H⁺] measured in cSNARF1-loaded HCT116 monolayers with naturally high CA₉ activity. The protocol was repeated in 100 µM ATZ. The rate of change, d[H⁺]/dt, is plotted below the [H⁺] time course. An average of >20 cells was analyzed. Experiments were also performed on HT29 monolayers with naturally high CA₉ activity (C), HeLa (D), and MDA-MB-468 monolayers with naturally low CA₉ activity (E). F, intracellular [H⁺] changes quantified as root mean square (RMS). *, p < 0.05, relative to control. G, simulated sinusoidal waveform of pCO₂ and its effect on intracellular [H⁺]. H, model simulation of the effect of CA activity (10-fold acceleration of hydration) on peak-to-nadir [H⁺], amplitude (percentage increase). Error bars represent S.E.
absence of extracellular Ca²⁺ (nominally Ca²⁺-free; residual Ca²⁺ buffered with 1 mM EGTA), the Fluo3 response was abolished, arguing that Ca²⁺ influx is required. Collectively, these data suggest that cytoplasmic alkalinization evokes Ca²⁺ influx through store-operated channels, such as Orai (64). In support of this, 2-aminoethoxydiphenyl borate (100 µM) blocked the Ca²⁺ response. The CO₂-evoked [Ca²⁺]i response was dampened in the presence of 100 µM ATZ, illustrating a role for CAi activity in accentuating a cellular response to changing pCO₂. Experiments on co-cultures of HCT116 cells with fibroblasts of low CAi activity (NHDF-Ad, CCD-112-CoN, InMyoFib; Fig. 4, D–F) confirmed that CAi activity can influence the Ca²⁺ response to pCO₂ fluctuations in a cell-dependent manner.

Kinase Activity—Many kinase-operated signaling pathways are modulated by pH (65) and are expected to respond to changes in pCO₂. This was tested in HCT116 cells by measuring the phosphorylation state of the major mitogen-activated kinases, extracellular signal-regulated kinases, c-Jun N-terminal kinases, and p38 isoforms. pCO₂ was held constant at 5 or 20% or oscillated between 5 and 20% CO₂ for six cycles (4-min periodicity) in the presence or absence of ATZ to manipulate the dynamics of the pHi response (note that at the end of pCO₂ oscillations cells were returned to 5% CO₂ for 2 min). After 26 min of superfusion, lysates were tested for kinase activity read-outs to identify pH-sensitive and pH-insensitive kinases over the pH range studied. Among the kinases investigated, phosphorylation of ERK1 (Thr-202/Tyr-204) and ERK2 (Thr-185/Tyr-187) was unaffected by pCO₂ changes (Fig. 5A). In contrast, kinases, such as JNK1 and JNK2, were dephosphorylated (Thr-183/Tyr-185) by stably raised pCO₂. Transient exposure to 20% CO₂ (i.e. oscillating pCO₂) was inadequate to reduce phosphorylation, indicating that a sustained fall in pHi is nec-

**FIGURE 4. Effect of CAi-mediated CO₂-pH coupling on Ca²⁺ signaling.** A, intracellular Ca²⁺ (Fluo3 F/F₀ ratio) during 5–20% CO₂ oscillations (cycle length of 4 min). B, transient pH change evoked by acetate (in the absence of extracellular Ca²⁺ and absence of CO₂/HCO₃⁻) did not evoke a Fluo3 response. C, Fluo3 response recorded once consistent cyclic behavior had been attained. The response was reduced in 100 µM ATZ or in cells AM-loaded with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) (Ca²⁺ buffer; 100 µM), abolished by removing extracellular Ca²⁺ (replaced with EGTA Ca²⁺ buffer) or in 100 µM 2-aminoethoxydiphenyl borate (2-APB), and augmented in 10 µM thapsigargin (TG)-pretreated cells (endoplasmic reticulum depletion). Data are the means of n=50 cells. HCT116 cancer cells co-cultured with either CCD112-Con fibroblasts (D), InMyoFib myofibroblasts (E), or NHDF-Ad fibroblasts (F). The Ca²⁺ response was larger and ATZ-sensitive in HCT116 cancer cells. Data are the means of n=50 cells. Error bars represent S.E.
necessary for eliciting a change in JNK1/2 activity (Fig. 5B). Stably decreased pH also reduced the activity of mTOR complex 1 (mTORC1), reported as S6 kinase (S6K) phosphorylation at Thr-389 (65–67). Unlike the other kinases tested, mTORC1 signaling was dependent on CAi activity under fluctuating pCO2 protocols (higher S6K phosphorylation in the presence of ATZ; Fig. 5C; panel ii), arguing that the dynamics of pH must be influencing the mTORC1 pathway. A single, transient (2-min) exposure to 20% CO2 was sufficient for observing an effect of CAi activity on mTORC1 (higher S6K phosphorylation in the presence of ATZ; Fig. 5D). The mTOR inhibitor rapamycin (10 μM) produced a substantial decrease in S6K phosphorylation (Fig. 5E), confirming the validity of using S6K as an mTORC1 readout. At constant (5%) pCO2, ATZ did not affect mTORC1 activity (Fig. 5E), indicating that the ATZ sensitivity of S6K phosphorylation measured under oscillating pCO2 is not an off-target effect of the CA inhibitor. Consistent with this finding, ATZ had no effect on S6K phosphorylation in CAII knockdown HCT116 (Fig. 5F) and wild-type MDA-MB-468 (Fig. 5G), i.e. cells with low CAi activity. In summary, mTORC1 signaling responds to sharp pCO2 changes that are

FIGURE 5. Effects of CAi-mediated CO2-pH coupling on kinase phosphorylation. A, Phosphorylation state of ERK1 (Thr-202/Tyr-204) and ERK2 (Thr-185/Tyr-187) was unaffected by changing pCO2 either tonically (5% or 20% for 30 min) or dynamically (six cycles of 5–20% fluctuations; cycle length of 4 min); densitometric analysis (n = 3). Time-averaged intracellular [H+] was measured in separate experiments. B, JNK1/2 were dephosphorylated (Thr-183/Tyr-185) by stably raised pCO2 but unaffected by oscillating pCO2 (n = 3). C, panel i, Western blot for S6 kinase phosphorylation (PThr-389) (readout of mTORC1 signaling) under 5% CO2, 20% CO2, and fluctuating pCO2 (six cycles; 5–20%) in the presence or absence of ATZ (100 μM). Panel ii, pCO2 fluctuations reduced S6 kinase phosphorylation but only under full CAi activity. S6K activity did not correlate with time-averaged [H+] (n = 3). D, panels i and ii, analysis for one cycle of pCO2 oscillation in HCT116 cells. E, at 5% CO2, mTOR activity was unaffected by ATZ but strongly inhibited by rapamycin (10 μM). F, six cycles of pCO2 fluctuations in CAII knockdown (KD) HCT116 cells (construct 695). G, six cycles of pCO2 fluctuations in MDA-MB-468 cells (low CAi activity). Error bars represent S.E. **p < 0.01.
attainable with high CAi activity. These proof-of-principle experiments demonstrate that CAi activity is able to modulate the coupling between extracellular pCO₂ (a function of blood flow) and intracellular signaling.

**DISCUSSION**

**CAi Activity in Cancer Cells**—In this study, we quantified CAi activity in a panel of cancer cells and compared these data with results from fibroblast and fibroblast-related cells. Activity measurements were performed under physiological conditions, in the native and undiluted cytoplasmic environment of intact cells, and in the presence of relevant regulatory cytoplasmic influences (Fig. 1A). CAi activity varied considerably among cancer cell lines from essentially absent in HeLa and MDA-MB-468 cells to high in HCT116, HT29, and RT112 cells. Low CAi activity was characteristic of fibroblasts/fibroblast-related cells, arguing that CAi catalysis in solid tumors is more likely to be associated with cancer cells rather than stromal fibroblasts. Soluble (but not secreted) and cytoplasm-facing membrane-tethered CA isoforms may contribute toward the CAi activity. Cancer cells with high total CAi activity were also positive for soluble CAi activity in membrane-free cell lysates (Fig. 1B) and for CAi immunoreactivity (Fig. 1D). Genetic knockdown of CAII in HCT116 cells demonstrated that the majority of CAi activity was attributable to CAII (Fig. 1C). Additional isoforms may contribute to CAi activity in other cell lines, such as HT1080 cells that lack CAII expression and soluble CAi activity, but have measurable overall CAi. Based on data from all cells investigated, CAII expression correlated strongly with soluble CAi activity and was a good predictor of high total CAi activity.

**CAi Activity Is Not Universally Rate-limiting for pHi Regulation**—The physiological role for CAi activity in cancer is contentious although clearly distinct from that of exofacial CAs (6). Here, we explored the cellular processes that may depend on CAi activity. Previous studies have argued for a role of CAi activity in facilitating H⁺ or HCO₃⁻ transport across membranes (23–26) and H⁺ diffusion in cytoplasm (58, 59), but these interactions have not been tested robustly in cancer cells. CAi activity in the six cancer cell lines studied does not correlate with resting pHᵢ, NHE flux, or HCO₃⁻ transporter flux measured previously in these cells (3). Our present data (Fig. 2) show that the H⁺ and HCO₃⁻ fluxes produced by cancer cells over the physiological pHᵢ range are not of sufficient magnitude to require CAi catalysis, with the exception of acid loading by HCO₃⁻ export at high pHᵢ. In the absence of CAi activity, the maximal capacity of CO₂ hydration to generate HCO₃⁻ ions for extrusion is ~14 mm/min at 5% CO₂ (i.e. kₑ × [CO₂]), and this can be rate-limiting for fast HCO₃⁻-dependent acid-loading transporters as measured in RT112 cells (Fig. 2A, panel iii). However, the highly alkaline intracellular conditions that are required for producing this CAi dependence are unlikely to be typical of cancer cells. CAi-catalyzed hydration of CO₂ has recently been proposed to facilitate Na⁺-HCO₃⁻ co-transport in cardiac myocytes by supplying the transport protein with H⁺ ions for titrating HCO₃⁻ (26). However, genetic CAII knockdown (HCT116; Fig. 2C, panel iv) or pharmacological inhibition with ATZ (RT112; Fig. 2A, panel iii) did not affect HCO₃⁻-dependent acid extrusion. This difference may reflect contrasting structural and chemical properties of cardiac and cancer cytoplasm. Alternatively, CAi dependence may only be observed experimentally under high Na⁺-HCO₃⁻ co-transport fluxes, which are attainable in cardiac myocytes (under hyperkalemic stimulation) but not in cancer cells. NHE activity was able to produce rapid H⁺ extrusion in MDA-MB-468 cells that naturally have very low CAi activity. This argues that CAi-independent delivery of H⁺ ions does not limit the membrane transport process (Fig. 2B) in agreement with an earlier study on ventricular myocytes (26). In HCT116 cells (high CAi activity), intrinsic cytoplasmic buffers alone supported the same magnitude of NHE flux as measured in the presence of CO₂/HCO₃⁻ or after loading cells with the highly mobile buffer carbosine (Fig. 2C). Even in the absence of CO₂/HCO₃⁻ (hence no CAi catalysis), activation of NHE did not evoke measurable pHᵢ non-uniformity. This supports the case that diffusive H⁺ coupling across cancer cytoplasm is normally adequate with intrinsic (non-carbonic) buffers. Knockdown of CAII did not affect NHE activity in HCT116 cells (measured as the DMA-inhibitable component of flux; Fig. 2C, panel iv), arguing that neither catalysis nor the presence of CAII protein is necessary for high NHE fluxes. Collectively, these observations argue for the absence of rate-limiting barriers to H⁺ diffusion over the relatively small dimensions of cancer cells (mean radii, <10 μm). Additionally, cancer cells typically down-regulate gap junctions (22), which in other tissues allow for greatly expanded diffusion lengths. The absence of electrical coupling explains why CAi catalysis cannot facilitate CO₂ or H⁺ diffusion appreciably in tumors as this would require HCO₃⁻ ions to diffuse relatively freely between cells.

**CAi Activity Influences the Degree of Coupling between pCO₂ and pHᵢ Dynamics**—Most investigations of the role of CAi activity in pHᵢ regulation have been performed under constant pCO₂. This condition may be appropriate for well perfused tissues with stable CO₂ production and venting but is not representative of metabolically active solid tumors with intermittent blood flow. Fluctuations in tumor blood flow are the basis for time-dependent changes in pO₂ (also known as acute hypoxia) that influence tumor biology (34). During periods of inadequate capillary washout, pCO₂ rises as pO₂ is depleted, and these changes reverse upon blood reperfusion. Although direct, high resolution measurements of pCO₂ fluctuations are not available because of technical limitations, the inverse correlation between time-averaged pO₂ and pCO₂ (acidity) is well established (43–45). Our standard experimental protocol of varying pCO₂ between 5 and 20% with 4-min periodicity is based on measurements of extracellular pH, blood flow, and pO₂ in solid tumors in vivo. The pH of 20% CO₂ superfusate is 6.8, which is considered to be typical of the mean extracellular pH of most solid tumors (63, 68); pH 7.4 of 5% CO₂ superfusates is normal for blood plasma. Our choice of periodicity is in the range of fluctuation frequencies of blood flow and/or pO₂ established by Fourier analysis in tumors in vivo (30, 33, 42).

Changes in pCO₂ will have knock-on effects on [H⁺⁺], dynamics and hence the many pH-sensitive downstream processes. The coupling between pCO₂ and pHᵢ is strongly dependent on CAi as illustrated in Fig. 3 where the same experimental protocol of changing pCO₂ between 5 and 20% produced different
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[\text{H}^+]$, responses depending on CA activity. [\text{H}^+] fluctuations were slow and ATZ-insensitive in cells with very low CA activity (MDA-MB-468 and HeLa). In contrast, high CA activity in HCT116 and HT29 cells resulted in accentuated [\text{H}^+] fluctuations that were dampened in the presence of ATZ. The effect of ATZ was not due to inhibition of exofacial CAs because (i) superfusates were pre-equilibrated (obviating the need for CA catalysis) and (ii) [\text{H}^+] dynamics were ATZ-insensitive in CAIX-expressing MDA-MB-468 cells.

Mathematical modeling demonstrates that the effect of CA activity on [\text{H}^+] dynamics was important even with a smoother sinusoidal pCO$_2$ waveform (Fig. 3G), which is more representative of undulating blood flow. The ability of CA to influence [\text{H}^+] dynamics was predicted for a wide range of pCO$_2$ waveform amplitudes but required a periodicity of 4 min or less for physiologically meaningful effects (Fig. 3H). This frequency dependence is expected because even at spontaneous reaction rates, [\text{H}^+] is able to track slow changes in pCO$_2$. Given that fluctuations of blood flow (29–32) and pO$_2$ (30, 32, 34–41) in solid tumors have been observed with a periodicity as short as 0.5 min (i.e., two cycles/min), a range of effects on [\text{H}^+] dynamics could be achieved by regulating CA activity: from tight temporal pCO$_2$-pH coupling at high CA activity to low pass filtering with negligible CA activity. Cancer cells would be able to tune their pH responsiveness to changes in pCO$_2$ (blood flow) by regulating CA activity, e.g., through CAII expression.

Our study explored the functional significance of the CA dependence of [\text{H}^+] dynamics using Ca$^{2+}$ signaling and kinase-operated cascades as proof-of-principle examples (Fig. 6). In HCT116 cells, a transient pH$_i$ rise, evoked by reducing pCO$_2$, increased [Ca$^{2+}$]. Our evidence points to store-operated calcium entry as the mechanism triggering the [Ca$^{2+}$]$_i$ response (Fig. 4C). Recent work has demonstrated that store-operated calcium entry is reduced at low pH$_i$ because of an uncoupling between the endoplasmic reticulum Ca$^{2+}$ sensor STIM and the surface membrane Ca$^{2+}$ channel Orai (64).

Because [Ca$^{2+}$]$_i$ responds dynamically to changes in the balance between influx and efflux pathways, pH$_i$ sensitivity of Ca$^{2+}$ entry would produce the observed [Ca$^{2+}$]$_i$ fluctuations. Under CA inhibition with ATZ, the same pCO$_2$ stimulus evoked a slower and smaller [Ca$^{2+}$]$_i$ response.

Previous studies have shown that the environmental sensor mTOR, which is strongly linked with cancer (66, 69, 70), is inhibited at low pH$_i$ (65). The present work shows that the degree of mTOR inhibition also depends on CA activity when pCO$_2$ is fluctuating. In HCT116 cells, a single cycle of pCO$_2$ oscillation produced a lower readout of mTORC1 signaling (S6 kinase phosphorylation) when CA activity was intact (Fig. 5D). This inhibitory effect of CA catalysis on mTORC1 signaling was substantiated by expanding the protocol to six cycles of pCO$_2$ fluctuations (Fig. 5C). mTORC1 signaling was generally higher when pCO$_2$ oscillations were performed on HCT116 cells with pharmacologically (ATZ) or genetically (knockdown; Fig. 5F) reduced CA activity or on cells with naturally low CA activity, such as MDA-MB-468 (Fig. 5G). mTORC1 activity was not a unique function of time-averaged pH$_i$. For example, holding pCO$_2$ stably at 20% or fluctuating pCO$_2$ between 5 and 20% in the presence of ATZ produced a similar time-averaged pH$_i$, but S6 kinase phosphorylation differed by a factor of 2 (Fig. 5C, panel ii). We conclude that the dynamics of pH$_i$ changes must influence mTORC1. Akin to the notion that transient hypoxia (perfusion-limited) and stable hypoxia (diffusion-limited) have distinct biological consequences (71), there may be similar “frequency modulation” and “amplitude modulation” (72) aspects of pH$_i$ signaling in cancer. A possible explanation why some kinases (such as JNK1/2) do not respond to fluctuating pCO$_2$ is a slow binding of H$^+$ ions to modulatory sites that acts like a low pass filter. mTOR may be able to register rapid pH$_i$ fluctuations by faster H$^+$ binding kinetics. Further studies are necessary to explore these possible mechanisms.

CA-catalyzed CO$_2$ hydration will produce fluctuations in HCO$_3^-$ that parallel pH$_i$ changes, and it is plausible that HCO$_3^-$ ions interact with targets in a pH-independent manner. Removing HCO$_3^-$ ions from cytoplasm also removes the substrate (CO$_2$) for CA; therefore a simple buffer substitution experiment would not be an appropriate test to distinguish the effects of H$^+$ and HCO$_3^-$ ions. However, it is generally accepted that H$^+$ ions are more reactive than HCO$_3^-$ ions. Furthermore, earlier observations of the acid response of store-operated calcium entry (64) and mTORC1 (65) were made in the absence of CO$_2$/HCO$_3^-$, arguing for H$^+$ ions as the key regulators.

Growing evidence points to a negative correlation between the expression of CA isoforms, such as CAII, and cancer disease severity (11, 13–15). Importantly for disease progression, CAII down-regulation would help to protect cytoplasmic pH from pCO$_2$ fluctuations and bestow cancer cell pH with a greater degree of autonomy from extracellular influences, such as those arising from intermittent blood flow (73). Conversely, rapid changes in blood flow would be registered by cells with high CA activity. For example, mTOR-regulated metabolism and proliferation would be more responsive to fluctuating blood flow in cancer cells with higher CA activity, particularly in regions close to aberrant blood vessels, which experience the sharpest pCO$_2$ fluctuations. Incidentally, these tumor regions...
are also important for metastasis and nutrient sensing. In solid tumors made of cancer cells with high CAi activity surrounded by a fibroblast stroma of low CAi activity, pCO2 fluctuations would evoke distinct [H+] responses in the two cell types as illustrated by [Ca2+] responses (Fig. 4).

In conclusion, this study demonstrates a novel role for CAi activity in cancer as a transducer of pCO2 fluctuations (which arise from intermittent blood flow) into a potent cytoplasmic signal (H+ ions). Cancer cells may alter the degree of temporal coupling between pCO2 and pHi by tuning cytoplasmic CAi activity, e.g. through CAII expression (Fig. 6). This work highlights the importance of dynamic aspects of pH signaling as a modality distinct from responses to stable pH changes.

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