Intracellular pH (pH$_i$), a major modulator of cell function, is regulated by acid/base transport across membranes. Excess intracellular H$^+$ ions (e.g. produced by respiration) are extruded by transporters such as Na$^+/H^+$ exchange, or neutralized by HCO$_3^-$ taken up by carriers such as Na$^+$/HCO$_3^-$ cotransport. Using fluorescence pH$_i$ imaging, we show that cancer-derived cell lines (colorectal HCT116 and HT29, breast MDA-MB-468, pancreatic MiaPaca2, and cervical HeLa) extrude acid by H$^+$ efflux and HCO$_3^-$ influx, largely sensitive to dimethylamiloride and 4,4’-diisothiocyanato stilbene-2,2’-disulfonate (DIDS), respectively. The magnitude of HCO$_3^-$ influx was comparable among the cell lines and may represent a constitutive element of tumor pH$_i$ regulation. In contrast, H$^+$ efflux varied considerably (MDA-MB-468 > HCT116 > HT29 > MiaPaca2 > HeLa). When HCO$_3^-$ flux was pharmacologically inhibited, acid extrusion in multicellular HT29 and HCT116 spheroids (~10,000 cells) was highly non-uniform and produced low pH$_i$ at the core. With depth, acid extrusion became relatively more DIDS-sensitive because the low extracellular pH at the spheroid core inhibits H$^+$ flux more than HCO$_3^-$ flux. HCO$_3^-$ influx inhibition also decelerated HCT116 spheroid growth. In the absence of CO$_2$/HCO$_3^-$, acid extrusion by H$^+$ flux in HCT116 and MDA-MB-468 spheroids became highly non-uniform and inadequate at the core. This is because H$^+$ transporters require extracellular mobile pH buffers, such as CO$_2$/HCO$_3^-$, to overcome low H$^+$ ion mobility and chaperone H$^+$ ions away from cells. CO$_2$/HCO$_3^-$ exerts a dual effect: as substrate for membrane-bound HCO$_3^-$ transporters and as a mobile buffer for facilitating extracellular diffusion of H$^+$ ions extruded from cells. These processes can be augmented by carbonic anhydrase activity. We conclude that CO$_2$/HCO$_3^-$ is important for maintaining uniformly alkaline pH$_i$, in small, non-vascularized tumor growths and may be important for cancer disease progression.

Intracellular pH (pH$_i$) is a permissive facilitator of growth and development in normal tissue and in tumors (1–3). Specialized proteins carry excess acid or base across membranes in a bid to maintain optimal pH$_i$. A major source of pH$_i$ disturbance is cellular respiration, which loads cells with CO$_2$ or lactic acid. If uncompensated, these respiratory products ionize and acidify the intracellular milieu. Elevated metabolism in tumors predisposes cells to significant acid loading (4, 5), thus placing demand on mechanisms that regulate pH$_i$. The ability of tumors to maintain an alkaline pH$_i$ (6) has been proposed as necessary for cancer progression (7). It is therefore of interest to study pH$_i$ regulation in cancer, notably with respect to the characteristic features of tumor biology such as up-regulated glycolysis, acidic extracellular milieu, and aberrant vasculature (4, 5, 8).

Proteins that extrude H$^+$ ions have been the most intensively studied components of pH$_i$ regulation in cancer. Na$^+/H^+$ exchangers (9–12) and V-type ATPase H$^+$ ion pumps (13, 14) are expressed in tumors and, at least under some conditions, have been linked to tumorigenesis through their role in pH$_i$ regulation (1, 15, 16). Acid extrusion can also be produced by membrane transporters that load cells with HCO$_3^-$ (or CO$_3^{2-}$) ions (17). These HCO$_3^-$ transport proteins include electroneutral or 1:2 electronegative Na$^+$/HCO$_3^-$ cotransporters (NBC) (18–20) and Na$^+$-dependent Cl$^-$/HCO$_3^-$ exchangers (21–23). Titration of intracellular acid with HCO$_3^-$ produces CO$_2$, a soluble gas that exits through membranes passively to complete the acid extrusion process. For this component of pH$_i$ regulation to function, cells must be supplied with CO$_2$/HCO$_3^-$, which in vivo is the principal extracellular pH buffer. Previous work on cancer cells has shown that HCO$_3^-$ transport can alkalinize pH$_i$ at normal and acidic extracellular pH (pH$_e$) (24) and contribute to pH$_i$ recovery from acid loads (23, 25). However, a more complete characterization of HCO$_3^-$ versus H$^+$ fluxes, in a range of cancer-derived cell lines at different values of pH$_e$ and pH$_i$, has not been undertaken. Moreover, many studies of pH$_i$ regulation have been carried out in the absence of CO$_2$/HCO$_3^-$ buffer, i.e. under conditions where HCO$_3^-$ transport is blocked.

For complete pH$_i$ regulation at tissue level, the activity of membrane-bound acid extruders must be complemented with adequate diffusion of their transport solutes across the extracellular space. Accordingly, the rate of membrane HCO$_3^-$ transport could be limited by diffusion of HCO$_3^-$ toward cells and CO$_2$ diffusion in the opposite direction. Similarly, the activity of membrane-bound H$^+$ extruders must be complemented by efficient dissipation of the extracellularly deposited acid load; otherwise, extracellular acidification could slow the removal of cellular acid through a well documented inhibitory effect of extracellular H$^+$ ions on acid extruders (26, 27). In healthy tissue, plentiful blood perfusion helps to maintain the constancy...
and uniformity of extracellular pH, \([\text{HCO}_3^-]\), and \([\text{CO}_2]\). This, in turn, unifies pH regulation. In many solid tumors, however, perfusion tends to be heterogeneous, interrupted, and inadequate (5). This lengthens cell-to-capillary distances and weakens diffusive coupling across the extracellular space, particularly if this has high tortuosity (28). \(\text{H}^+\) ions are, in addition, chemically reactive, and their binding to prototatable sites on large molecules, such as proteins, can impede free \(\text{H}^+\) ion diffusion (29). It is therefore particularly important to ensure adequate mobility of extracellular \(\text{H}^+\) ions. This can be achieved by the presence of mobile (i.e. diffusible) pH buffers. The diffusion of protonated buffer away from the site of \(\text{H}^+\) ion production and the counter-flux of unprotonated buffer represent a form of facilitated \(\text{H}^+\) ion diffusion. Although mobile buffers have been shown to facilitate \(\text{H}^+\) ion diffusion inside cells (29–31), their role in facilitating extracellular \(\text{H}^+\) ion mobility has not been studied in detail. A major extracellular mobile buffer is \(\text{CO}_2/\text{HCO}_3^-\), but to evaluate its ability to facilitate \(\text{H}^+\) ion diffusion, it is necessary to study multicellular growths that resemble tissue (i.e. harbor significant diffusion distances). Studies of pH regulation have, however, typically focused on biological processes occurring at the level of individual cells only.

Many cancer types have recently been linked with the expression of membrane-tethered, extracellular-facing carbonic anhydrase (CA) isoforms IX and XII (32, 33). The catalytic activity of these CAs helps to maintain equilibrium between extracellular \(\text{CO}_2, \text{HCO}_3^-\), and \(\text{H}^+\) ions (34). The presence of these CAs in cancer tissue may be indicative of an important role for \(\text{CO}_2/\text{HCO}_3^-\) reactions in tumor pH regulation. In isolated cells, CA activity has been shown to accelerate the activity of membrane acid/base transporters (35–37) (“transport metabolon”). Within respiring spheroids, CAIX facilitates CO2 removal from constituent cells (28, 38). It remains to be seen whether CA activity can support a role for \(\text{CO}_2/\text{HCO}_3^-\) in facilitating extracellular \(\text{H}^+\) ion diffusion.

In the present work, we study the role of \(\text{CO}_2/\text{HCO}_3^-\) in pH regulation in a number of cancer-derived cell lines, prepared as single cells or as multicellular tissue-like growths (spheroids). We investigate the importance of \(\text{CO}_2/\text{HCO}_3^-\) as (i) a supply of substrate for acid-extruding \(\text{HCO}_3^-\) transporters and (ii) a mobile buffer supporting acid extrusion by facilitating extracellular \(\text{H}^+\) ion diffusion. We show that \(\text{HCO}_3^-\) transport is an important component of pH regulation in all cell lines studied and, in that, a number of cases, it can produce an acid extrusion flux that exceeds the capacity of \(\text{H}^+\) transporters. In spheroids, we show that extracellular \(\text{CO}_2/\text{HCO}_3^-\) supports the activity of \(\text{H}^+\) extruders by shuttling acid away from cells and minimizing the degree of extracellular acidification, which might otherwise inhibit membrane transport. Finally, we show that the effectiveness of \(\text{CO}_2/\text{HCO}_3^-\) as a mobile buffer is improved in the presence of CA activity. Our findings highlight an indispensable role of \(\text{CO}_2/\text{HCO}_3^-\) in tumor biology.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Spheroid Culturing**—Human colorectal carcinoma lines HCT116 and HT29 and the breast cancer cell line MDA-MB-468 were kind gifts from Professor Adrian Harris (Oxford, UK). The cervical carcinoma cell line HeLa was a kind gift from Professor Silvia Pastorekova (Bratislava, Slovakia). The pancreatic cell line MiaPaca2 was a kind gift from Professor Holger Kalthoff (Kiel, Germany). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing NaHCO3, in an atmosphere of 5% \(\text{CO}_2\) for 48–72 h until 70–90% confluence was reached. Prior to experiments, cells were resuspended in Hepes-buffered DMEM (up to 3 h), and aliquots of 200 μl were used for experiments. Spheroids were cultured using the hanging drop method (HCT116, 500 cells/20 μl; HT29, 1,000 cells/20 μl; MDA-MB-468, 5,000 cells/20 μl grown in 2.5% Matrigel) in HCO3-containing DMEM. For superfusion experiments, spheroids were grown for 2–3 days until they attained spherical symmetry and a radius of 130–200 μm (6,000–22,000 cells). To follow growth over a longer time frame, HCT116 spheroid formation and growth in hanging drops was extended to 4 days, after which spheroids were collected and transferred to non-tissue culture Petri dishes with HCO3-buffered DMEM media. This ensures that nutrient supply does not limit spheroid growth. Where desired, drugs were added to media before the onset of spheroid formation and throughout their growth, and compared with dimethyl sulfoxide (DMSO)-injected controls.

**Confocal Imaging of pH**—Single cells and spheroids were imaged using a Leica TCS NT confocal system and an IRBE microscope with a transparent superfusion chamber (capacity 2 ml), the base of which was pretreated with 0.01% poly-L-lysine to facilitate cell/spheroid adhesion. Solutions were heated to 37°C and delivered at a constant rate (2 ml/min). Suction was adjusted to maintain a steady-state solution volume of ~0.5 ml. To measure intracellular pH, cells were loaded with the membrane-permeant acetoxyethyl ester of carboxy SNARF-1 (10 μM), a pH fluorophore, for 3 min (28). In the case of spheroids, loading time was extended to 30 min to allow adequate dye access to the core. Excess extracellular dye was washed away by superfusion. Carboxy SNARF-1 fluorescence, excited at 514 nm, was measured ratiometrically at 580 and 640 nm. To measure extracellular pH, spheroids were superfused with solution containing the membrane-impermeant pH fluorophore fluorescein-5-(and-6)-sulfonic acid (30 μM) (28). Fluorescein-5-(and-6)-sulfonic acid fluorescence, excited at 488 nm, was measured >510 nm.

**Solutions, Drugs, and Superfusion Protocols**—Cells and spheroids were superfused with solutions buffered by \(\text{CO}_2/\text{HCO}_3^-\) or Hepes. All solutions contained 4.5 mM KCl, 11 mM glucose, 1 mM CaCl2, 1 mM MgCl2. Hepes-buffered solutions contained 1–40 mM Hepes, and the pH of the solutions was adjusted with 4 M NaOH. \(\text{CO}_2/\text{HCO}_3^-\)-buffered solutions were bubbled with either 5% \(\text{CO}_2/95\%\) air or 20% \(\text{CO}_2/80\%\) air and contained a concentration of NaHCO3 that yielded the desired pH, in accordance with the Henderson-Hasselbalch equation (\(\text{pH} = 10^{−6.15} + \log([\text{HCO}_3^-]/[\text{CO}_2])\)). For all solutions, NaCl was added to produce a total osmolality of 290–300 mossm/liter. In the case of Cl−-free solutions, all Cl− salts were substituted with gluconates. All chemicals were obtained from Sigma-Aldrich (Poole, UK). Na+/H+ exchange was inhibited with 5-(N,N-dimethyl)amiloride (DMA; Sigma-Aldrich). \(\text{HCO}_3^-\) transport was inhibited with 4,4′-diiodothiocyanostilbene
2,2'-disulfonic acid (DIDS; Sigma-Aldrich). Na⁺-HCO₃⁻ cotransport was inhibited with S0859 kindly provided by Sanofi-Aventis (Frankfurt, Germany) (39). CA activity was blocked by acetazolamide (Sigma-Aldrich). The concentrations of DMA (30 µM), DIDS (150–300 µM), and S0859 (100 µM) used do not greatly affect membrane CA activity (supplemental Fig. S1). To produce an intracellular acid load, cells/spheroids were superfused with solution containing 20 mM NH₄Cl (less 20 mM NaCl) for 6 min. During exposure to ammonium, the intracellular compartment undergoes rapid alkalinization followed by gradual acidification arising from the activity of acid loaders. Removal of extracellular ammonium deposits an intracellular acid load that is subsequently removed by acid extruders, if active. Buffering (β) was measured using the ammonium removal method (supplemental Fig. S2) (40). Briefly, cells were exposed to a series of solutions of decreasing NH₄Cl concentrations that produce stepwise changes to pHᵢ. β was estimated from the ratio of the concentration of acid deposited in the cell during NH₄⁺ withdrawal and the associated pHᵢ change.

**RESULTS**

**Measuring the HCO₃⁻ Dependence and pHᵢ/pH Sensitivity of Acid Extrusion from Isolated Cells—** Transmembrane extrusion of acid was first measured in single cells, using the time course of pHᵢ recovery from an intracellular acid load. Low pHᵢ was attained by an ammonium prepulse solution maneuver. Fig. 1A shows averaged pHᵢ time courses measured in carboxy SNARF-1-loaded HCT116 cells. Continuous superfusion ensured that the composition of the extracellular bathing medium was controlled. On removal of ammonium, pHᵢ decreased to a level that...
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stimulated acid extrusion. Recovery of pH\textsubscript{i} in the absence of physiological CO\textsubscript{2}/HCO\textsubscript{3} buffer (replaced with Hepes) was mediated by HCO\textsubscript{3}\textsuperscript{−}-independent transport only. When pH\textsubscript{e} was set to 7.4 (the pH of normal blood plasma), pH\textsubscript{i} recovery was complete within 8 min. This was blocked by 30 μM DMA, indicating that Na\textsuperscript{+}/H\textsuperscript{+} exchange (NHE) underlies the observed acid extrusion (27). In 5% CO\textsubscript{2}/22 mM HCO\textsubscript{3}−-buffered superfusates (Fig. 1B), pH\textsubscript{i} recovery was accelerated and only partially blocked by DMA. The residual recovery was inhibited by DIDS, indicating that HCO\textsubscript{3}\textsuperscript{−} transport contributes to pH\textsubscript{i} regulation.

Acid extrusion from cells was quantified in terms of net H\textsuperscript{+} equivalent flux (J\textsubscript{H\textsuperscript{+}}), calculated as the product of the rate of pH\textsubscript{i} change and intracellular buffering capacity (J\textsubscript{H\textsuperscript{+}} = −dpH\textsubscript{i}/dt × β). This algorithm takes into account the vast concentration of H\textsuperscript{+} ions held on buffers. Buffering capacity consists of a component due to intracellular CO\textsubscript{2}/HCO\textsubscript{3}− (β\textsubscript{carb}) and an “intrinsically” component (β\textsubscript{int}) derived from membrane-impermeant intracellular buffers. β\textsubscript{int} was measured in the absence of CO\textsubscript{2}/HCO\textsubscript{3}− (supplemental Fig. S2, A and B) (40). Measurements in CO\textsubscript{2}/HCO\textsubscript{3}−-buffered superfusates yield the sum of β\textsubscript{int} plus β\textsubscript{carb} (supplemental Fig. S2, C and D). In 5% CO\textsubscript{2}, measured β was equal to β\textsubscript{int} plus an estimate of β\textsubscript{carb} derived from the Henderson-Hasselbalch equation (β\textsubscript{carb} = 2.303×[HCO\textsubscript{3}\textsuperscript{−}]×([H\textsuperscript{+}]\textsubscript{e}/[H\textsuperscript{+}]\textsubscript{i})). The DMA-sensitive component of J\textsubscript{H\textsuperscript{+}} measured in 5% CO\textsubscript{2}/22 mM HCO\textsubscript{3}− was equal to the total J\textsubscript{H\textsuperscript{+}} measured in the absence of CO\textsubscript{2}/HCO\textsubscript{3}− (supplemental Fig. S4A). NHE activity in superfused single cells does not, therefore, require CO\textsubscript{2}/HCO\textsubscript{3}− buffer. The DMA-insensitive component of J\textsubscript{H\textsuperscript{+}} was inhibited by the broad spectrum HCO\textsubscript{3}− transport blocker DIDS or the Na\textsuperscript{+}−H\textsuperscript{+} cotransporter blocker S0859 (supplemental Fig. S4B).

Acid extrusion in the absence of CO\textsubscript{2}/HCO\textsubscript{3}− was reduced rapidly and reversibly at pH\textsubscript{e} of 6.8 (the typical pH\textsubscript{e} of tumors (2, 41)), as expected from the inhibitory effect of extracellular H\textsuperscript{+} ions on NHE (27) (Fig. 1C). Likewise, acid extrusion in the presence of CO\textsubscript{2}/HCO\textsubscript{3}− buffer was also reduced at low pH\textsubscript{e} attained by dropping solution [HCO\textsubscript{3}\textsuperscript{−}] to 5.5 mM (a “metabolic acidosis”; Fig. 1D). Fig. 1E, panels i and ii, plot J\textsubscript{H\textsuperscript{+}} as a function of pH\textsubscript{e} and pH\textsubscript{i} measured in CO\textsubscript{2}/HCO\textsubscript{3}−-free and CO\textsubscript{2}/HCO\textsubscript{3}−-containing superfusates, respectively. For experiments in CO\textsubscript{2}/HCO\textsubscript{3}−, pH\textsubscript{i} was varied by changing [HCO\textsubscript{3}\textsuperscript{−}] of HCO\textsubscript{3}\textsuperscript{−} and H\textsuperscript{+} fluxes increased as pH\textsubscript{e} was reduced or as pH\textsubscript{i} was raised. The decrease in J\textsubscript{H\textsuperscript{+}} at low pH\textsubscript{i} could be due to greater acid loading by transporters such as Cl\textsuperscript{−}/HCO\textsubscript{3}− exchange (42), rather than reduced activity of acid extruders. This was tested experimentally using solutions in which Cl\textsuperscript{−} salts were substituted with membrane-impermeant gluconates. Two sequential ammonium prepulses were performed to ensure that Cl\textsuperscript{−} had leaked out of cells. The process of pH\textsubscript{i} recovery from an imposed acid load is referred to as net acid extrusion.

The metabolic acidosis attained by lowering solution [HCO\textsubscript{3}\textsuperscript{−}] may have reduced HCO\textsubscript{3}− flux because of rate-limiting substrate concentrations. To test this, recovery of pH\textsubscript{i} was recorded in solutions that produce respiratory acidosis. Solution pH of 6.8 was attained by raising CO\textsubscript{2} partial pressure 4-fold at constant [HCO\textsubscript{3}\textsuperscript{−}] (supplemental Fig. S5A). This produced the same inhibitory effect on HCO\textsubscript{3}− flux as metabolic acidosis, suggesting that the activity of HCO\textsubscript{3}− transporters is instructed by extracellular [H\textsuperscript{+}] rather than HCO\textsubscript{3}− availability.

Acid Extrusion by HCO\textsubscript{3}− Transport in Cancer-derived Cell Lines at Physiological and Acidic pH\textsubscript{e}—In HCT116 cells superfused with 5% CO\textsubscript{2}/22 mM HCO\textsubscript{3}− buffer, DIDS-sensitive HCO\textsubscript{3}− transport accounted for approximately one-third of total acid extrusion, whereas most of the remainder was due to DMA-sensitive H\textsuperscript{+} transport (supplemental Fig. S4B). Acid extrusion was studied in four more cancer-derived cell lines: colorectal HT29, breast MDA-MB-468, pancreatic MiaPaca2, and cervical HeLa. Buffering capacity data for these cell lines are shown in supplemental Fig. S3. Membrane H\textsuperscript{+} flux was calculated from pH\textsubscript{i} recovery time courses measured in Heps buffer titrated to 7.4 (Fig. 2A) or 6.8 (Fig. 2B). The additional HCO\textsubscript{3}− flux was estimated from pH\textsubscript{i} recovery time courses in 5% CO\textsubscript{2}/HCO\textsubscript{3}− buffer, with solution pH adjusted by varying [HCO\textsubscript{3}\textsuperscript{−}]. At pH\textsubscript{e} = 7.4, the magnitude of HCO\textsubscript{3}− flux was similar in all cell lines tested. When compared at a common pH\textsubscript{e} of 6.7 (representing a modest intracellular acid load), HCO\textsubscript{3}− flux clustered at ~3.7 mM/min (from 3.0 mM/min in HeLa to ~4.3 mM/min in MDA-MB-468). In contrast, H\textsuperscript{+} flux varied considerably, increasing in the order HeLa < MiaPaca2 < HT29 < HCT116 < MDA-MB-468, from 0.7 mM/min to 7.0 mM/min at pH\textsubscript{e} = 6.7 (Fig. 2A). The mechanism of acid extrusion varied from predominantly HCO\textsubscript{3}− flux in HeLa, MiaPaca2, and HT29 to mainly H\textsuperscript{+} flux in HCT116 and MDA-MB-468 cells. Nonetheless, HCO\textsubscript{3}− transport remained a significant component of pH\textsubscript{i} regulation in all cell lines studied.

Extracellular acidification reduced HCO\textsubscript{3}− and H\textsuperscript{+} fluxes (Fig. 2B). The pH\textsubscript{e} sensitivity of these fluxes, probed at a common pH\textsubscript{e} of 6.7, is shown in Fig. 2, C and D. Over the pH\textsubscript{e} range studied, HCO\textsubscript{3}− flux was most pH\textsubscript{e}-sensitive in MiaPaca2 cells and least pH\textsubscript{e}-sensitive in HT29 cells. In contrast, HCT116 cells had the most pH\textsubscript{e}-sensitive H\textsuperscript{+} flux. Consequently, HCO\textsubscript{3}− flux in HCT116 cells became greater than H\textsuperscript{+} flux over the pH\textsubscript{e} range 7.05–6.4. The pH\textsubscript{e} sensitivity of acid extrusion is important in the context of solid tumors, which are known to develop gradients of pH\textsubscript{e} (2, 41) and hence are likely to show a depth dependence of transport phenotype. To address this, pH\textsubscript{e} regulation was studied further in spheroids.

Spatial pH\textsubscript{e} Regulation in Spheroids by Membrane HCO\textsubscript{3}− Flux—The ability of HCO\textsubscript{3}− transport to regulate pH\textsubscript{e} in tissue-like structures was first investigated in spheroids composed of HT29 cells, a cell line in which acid extrusion relies principally on HCO\textsubscript{3}− flux (Fig. 2). The intracellular compartment of carboxy SNARF-1-loaded spheroids was acidified by means of an ammonium prepulse, and the subsequent pH\textsubscript{e} recovery was monitored in 10 regions of interest (ROIs), defined as concentric rings within the boundary of the spheroid. To improve sig-
nal-to-noise ratio, the four innermost ROIs were averaged (core). In CO₂/HCO₃⁻-buffered superfusates, pHᵢ recovery was complete within 8 min and proceeded fairly uniformly across the spheroid (Fig. 3A). In the presence of 150 μM DIDS, pHᵢ recovery was considerably slower and less uniform (Fig. 3B). The spheroid core remained acidic even after 12 min of pHᵢ recovery. Ammonium prepulse maneuvers in CO₂/HCO₃⁻ buffer were repeated on HCT116 spheroids. Recovery of pHᵢ from an acid load was fast and spatially coordinated, although a sizeable standing pHᵢ non-uniformity of 0.17 units persisted at steady state (2.5-fold greater than in HT29 spheroids). DIDS (300 μM) slowed pHᵢ recovery by 23% at periphery and 50% at core (Fig. 3D). The depth-dependent inhibitory effect of DIDS could be explained in terms of the pHₑ gradient. With depth, pHₑ is expected to fall (28, 43), and this, according to data in Fig. 2, would increase the share of HCO₃⁻ flux in overall acid extrusion in HT29 and HCT116 cells. Based on pHᵢ sensitivity, HCO₃⁻ flux is expected to produce more uniform pHᵢ recovery than H⁺ flux. In support of this, acid extrusion in HCT116 spheroids was more uniform in the presence of DMA (30 μM; Fig. 3E) than in DIDS (Fig. 3D). To summarize, HCO₃⁻ transport in HT29 and HCT116 spheroids is important for spatially coordinated pHᵢ recovery from intracellular acid loads, particularly in deeper tissue regions.

The findings from colorectal cancer-derived spheroids show that HCO₃⁻ transport can play an important role in tissue pHᵢ regulation. To explore the effects of acid extrusion by H⁺ transport and HCO₃⁻ transport on spheroid growth, the NHE inhibitor DMA or the NBC inhibitor S0859 was added to HCO₃⁻-buffered culture media incubated at 5% CO₂ (Fig. 3F). S0859 was used instead of DIDS to avoid nonspecific effects associated with stilbenes during exposures lasting several days. In the absence of NHE activity, spheroid growth ceased at a radius of 150 μm. In the absence of NBC activity, spheroid growth was reduced by 20–30%. The activity of NHE alone was not able to compensate for the absence of HCO₃⁻ transport in S0859. These results indicate that in HCT116 spheroids, pHᵢ-regulating fluxes via H⁺ and HCO₃⁻ transporters are both important

**FIGURE 2.** Magnitude and pHᵢ/pHₑ dependence of membrane H⁺ and HCO₃⁻ fluxes in five cancer-derived lines. A, pH dependence of H⁺ flux (light gray) and HCO₃⁻ flux (dark gray), measured from pHᵢ recovery time courses at pHₑ = 7.4, in the presence (filled circles) and absence (open circles) of CO₂/HCO₃⁻ buffer (mean of >30 cells). B, experiments repeated at pHₑ = 6.8 (pHₑ of CO₂/HCO₃⁻-buffered solutions adjusted by varying [HCO₃⁻]). Mean cell radii (in μm): HeLa, 6.53 ± 0.07; MiaPaca2, 7.09 ± 0.08; HT29, 7.14 ± 0.11; HCT116, 6.55 ± 0.14; MDA-MB-468 7.11 ± 0.12. Error bars in A and B indicate S.E. C, pHᵢ sensitivity of H⁺ flux probed at pHᵢ = 6.7. D, pHᵢ sensitivity of HCO₃⁻ flux probed at pHᵢ = 6.7.
for tissue growth. This correlates with the additive ability of these transporters to alkalinize pH.

Facilitation of Membrane H\(^+\) Flux by CO\(_2\)/HCO\(_3\)^\(-\) Buffer—H\(^+\) transport was capable of producing significant, albeit non-uniform pH\(_i\) recovery in HCT116 spheroids (Fig. 3D). Acid extrusion by membrane H\(^+\)/H\(_1\) flux was studied further in spheroids superfused with CO\(_2\)/HCO\(_3\)^\(-\) buffer (Fig. 4A). In the first set of experiments, pH\(_i\) recovery in 40 mM Hepes was similar to that measured in CO\(_2\)/HCO\(_3\)^\(-\) buffer in the presence of DIDS (Fig. 3D). This confirmed that DIDS-insensitive acid extrusion was active in Hepes-buffered superfusates. The addition of 30 \(\mu\)M DMA (supplemental Fig. S6) blocked pH\(_i\) recovery, identifying NHE as the major acid extruder. Hepes is not a physiological buffer; therefore further experiments were performed at lower Hepes concentrations: 10 mM (supplemental Fig. S7A) and 1 mM (Fig. 4B). As the concentration of buffer was reduced, pH\(_i\) recovery was slowed. This effect was most striking at the core, where diffusive coupling with the bulk superfusate was weakest. Further experiments were performed on spheroids cultured from MDA-MB-468

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**FIGURE 3.** HCO\(_3\)^\(-\) transport is important for uniform acid extrusion across HT29 and HCT116 spheroids. A, 20 mM ammonium (Amm) prepulse performed on HT29 spheroid (mean radius 162.3 ± 10.8 \(\mu\)m). Bulk superfusate (pH = 7.4) was buffered by 5% CO\(_2\)/22 mM HCO\(_3\)^\(-\). Dark traces: pH\(_i\) recovery at spheroid periphery (outer ROI) and core (mean of inner four ROIs). Gray traces: remaining ROIs. Inset: pH\(_i\) map (bar = 100 \(\mu\)m). End point pH\(_i\) gradient = 0.071 ± 0.023. B, experiment repeated in the presence of 150 \(\mu\)M DIDS to block HCO\(_3\)^\(-\) transport (mean spheroid radius = 182.6 ± 15.3 \(\mu\)m). Inset: pH\(_i\) map (bar = 100 \(\mu\)m). End point pH\(_i\) gradient = 0.180 ± 0.037. C, ammonium prepulse performed on HCT116 spheroid (mean radius 139.3 ± 6.2 \(\mu\)m). Bulk superfusate was buffered by 5% CO\(_2\)/22 mM HCO\(_3\)^\(-\) (pH = 7.4). End point pH\(_i\) gradient = 0.175 ± 0.049. D, experiment repeated in CO\(_2\)/HCO\(_3\)^\(-\) buffer in the presence of 300 \(\mu\)M DIDS to block HCO\(_3\)^\(-\) flux (mean spheroid radius = 129.7 ± 6.9 \(\mu\)m). Total flux inhibition at pH\(_i\) = 6.85 was 23% at the periphery and 50% at the core. End point pH\(_i\) gradient = 0.243 ± 0.059. E, experiment repeated in the presence of 30 \(\mu\)M DMA to block the major H\(^+\)/Na\(^+\) exchange (mean spheroid radius = 141.8 ± 5.9 \(\mu\)m). Total flux inhibition at pH\(_i\) = 6.85 was 60% at the periphery and 50% at the core. End point pH\(_i\) gradient = 0.089 ± 0.067. F, HCT116 spheroid radius measured over 7 days in HCO\(_3\)^\(-\)-containing media incubated in 5% CO\(_2\). Growth curves were repeated in the presence of 30 \(\mu\)M DMA or 100 \(\mu\)M S0859 (HCO\(_3\)^\(-\) transport inhibitor) included before the onset of spheroid formation. Specimen images of day 7 spheroids are shown on the right. Error bars in all panels indicate S.E.
Adequate extracellular buffering is therefore necessary to support membrane H⁺ transport. At 25 mM Hepes, pHᵢ recovery was fast and fairly uniform (Fig. 4C). At 1 mM Hepes, pHᵢ recovery became significantly non-uniform and incomplete at the spheroid core, even after 12 min (Fig. 4D). By extrapolation, acid extrusion from the core of HCT116 and MDA-MB-468 spheroids would be expected to cease at 0 mM Hepes.

Adequate extracellular buffering is therefore necessary to support membrane H⁺ transport. Blood contains CO₂/HCO₃⁻ that can provide pH buffering in lieu of Hepes. As shown in Fig. 3D, DIDS-insensitive H⁺ transport in HCT116 spheroids was operational in CO₂/HCO₃⁻-buffered superfusates. Similarly, CO₂/HCO₃⁻ was able to substitute for Hepes in supporting membrane H⁺ transport in MDA-MB-468 spheroids (Fig. 4E).

These findings confirm the important role of CO₂/HCO₃⁻ buffer in facilitating acid extrusion from cells. To determine whether CA activity facilitates CO₂/HCO₃⁻ buffering, acid extrusion was studied in spheroids grown from MDA-MB-468 cells, a cell line showing significant hypoxic induction of CAIX (44). Inhibition of CA activity with acetazolamide reduced the rate of pHᵢ recovery by 15% at the spheroid periphery and 36% at the core, and yielded 50% greater pHᵢ non-uniformity (Fig. 4, E and F). These data suggest that the full potential of CO₂/HCO₃⁻ as a buffer requires CA activity.

In summary, the rate of net acid extrusion from cells within a spheroid is limited by the availability of extracellular mobile buffers. This highlights the critical role of extracellular pH regulation in maintaining metabolic homeostasis within tumor spheroids.
buffering. It is noteworthy that this rate-limiting step for pH regulation can only be studied in multicellular models, such as spheroids. In the diffusively restricted environment of tissue, extracellular acid can accumulate to levels that may modulate acid extrusion. Mobile buffers, such as Hepes and CO$_2$/HCO$_3^-$, can curtail the inhibitory effects of low pH on acid extrusion (Fig. 2) by chaperoning extracellular H$^+$ ions away from the cell surface. This process was imaged in HCT116 spheroids using the membrane-impermeant pH$_e$ dye fluorescein-5-(and-6-)sulfonic acid. Extracellular H$^+$ ion diffusion was driven by rapid switching between superfusates at pH 6.4 and 7.4 (Fig. 5, A–C). The delay of pH$_e$ change at the core of the spheroid, relative to its periphery, provided an estimate of the apparent extracellular H$^+$ ion diffusion coefficient ($D_{H^+}^{app}$). Periphery-to-core time delays were estimated from the time constants of exponentials best-fitted to the periphery and core time courses. These were then converted to $D_{H^+}^{app}$ using a diffusion model with spherical symmetry (28). $D_{H^+}^{app}$ estimates in superfusates buffered with 1, 10, and 40 mM Hepes were 137, 134, and 129 $\mu$m$^2$/s, respectively (Fig. 5, A and B; supplemental Fig. S7B). As explained under “Discussion,” the lack of correlation between $D_{H^+}^{app}$ and [Hepes] suggests that Hepes is the principal extracellular mobile buffer. In superfusates buffered with CO$_2$/HCO$_3^-$, the core delay was shorter and yielded a higher $D_{H^+}^{app}$ of 211 $\mu$m$^2$/s (Fig. 5C).

The diffusive flux of H$^+$ ions is given by the product of $D_{H^+}^{app}$ and the concentration gradient of protonated buffer. Lower concentrations of buffer would therefore tend to produce smaller diffusive H$^+$ ion fluxes and allow greater pH$_e$ displacements during extrusion of cellular acid. Changes in pH$_e$ were investigated in HCT116 spheroids subjected to the ammonium prepulse maneuver in 1 mM, 40 mM Hepes (Fig. 5, D and E) or 5% CO$_2$/22 mM HCO$_3^-$ (Fig. 5F). Exposure to ammonium produced an instantaneous and transient fall of pH$_e$ that was more pronounced under low buffering conditions (Fig. 5D). This pH$_e$ transient arises from NH$_4^+$ deprotonation driven by rapid NH$_3$ entry into cells (45). pH$_e$ transients in the opposite direction were observed on ammonium withdrawal under low buffering conditions (Fig. 5E and F).

**FIGURE 5. Extracellular pH dynamics in HCT116 spheroids.** A, spheroid (mean radius 107 $\mu$m) pH$_e$, measured at the core and periphery ROIs (using the extracellular pH dye fluorescein-5-(and-6-)sulfonic acid, included in all superfusates at 30 $\mu$M) during solution maneuvers switching bulk superfusate pH from 6.4 to 7.4. Bulk superfusate was buffered by 1 mM Hepes. Delay in pH$_e$ changes at the core and periphery of the spheroid gives an indication of the H$^+$ diffusion coefficient. B, experiment repeated in 40 mM Hepes. Mean spheroid radius = 109 $\mu$m. C, experiment repeated in 5% CO$_2$/22 mM HCO$_3^-$ instead of Hepes. Mean spheroid radius = 92 $\mu$m. D, pH$_e$ measured at the core of spheroids during 20 mM ammonium prepulse. Bulk superfusate was buffered by 1 mM Hepes. Inset: pH$_e$ map (bar = 100 $\mu$m). E, experiment repeated with 40 mM Hepes. F, experiment repeated with 5% CO$_2$/22 mM HCO$_3^-$, the transient pH$_e$ acidification on the addition of ammonium is due to NH$_4^+$ deprotonation. Extracellular acidification following ammonium removal is due to the activation of acid extruders at low pH$_e$. Error bars in D–F indicate S.E.
CO$_2$/HCO$_3^-$ in Tumor pH Regulation

**DISCUSSION**

CO$_2$/HCO$_3^-$ as a Source of Substrate for pH$_i$ Regulation

- **HCO$_3^-$ Transport**—Tumors require good pH$_i$ control to support an intensive program of growth. The large metabolic acid loads deposited by elevated tumor metabolism emphasize the need for a pH$_i$ regulatory system that remains functional even in tissue regions that are poorly perfused with blood. In all five cancer-derived cell lines tested, net acid extrusion is achieved by parallel pathways, one involving H$^+$ flux and another involving HCO$_3^-$ flux. Despite awareness of HCO$_3^-$ transporters in cancer, the latter flux has been largely ignored. H$^+$ and HCO$_3^-$ fluxes can be quantified experimentally by measuring total acid extrusion fluxes in the presence and then absence of CO$_2$/HCO$_3^-$ buffer, the substrate for HCO$_3^-$ transport. These fluxes can also be dissected pharmacologically. Acid extrusion by H$^+$ transport is largely sensitive to DMA, a Na$^+$/H$^+$ exchanger inhibitor (Fig. 1A). The broad spectrum HCO$_3^-$ transport inhibitor DIDS blocks most of the HCO$_3^-$ flux (supplemental Fig. S4B). In HCT116 cells, HCO$_3^-$ transport is sensitive to the Na$^+$/HCO$_3^-$ cotransporter inhibitor, S0859 (supplemental Fig. S4B) (39). Our preliminary data (not shown) provide evidence for NBCe1 expression in all five cell lines tested. However, S0859 is less potent on HT29 cells, indicating that the expression of different HCO$_3^-$ transporters varies among cell lines.

- The relative magnitude of H$^+$ and HCO$_3^-$ fluxes varies with cell line, pH$_i$, and pH$_e$ (Fig. 2). As expected from an effective homeostatic system for regulating pH$_i$, a rise in intracellular [H$^+$] stimulates net acid extrusion in all five cell lines tested (Fig. 2). Allosteric activation of acid extruders by intracellular H$^+$ ions is likely to underlie this effect (26, 27). In contrast, a rise in extracellular [H$^+$] inhibits acid extrusion (Fig. 2, C and D). This may represent a form of negative feedback that limits the degree of extracellular acidification. The share of the two acid extrusion mechanisms (i.e. H$^+$ versus HCO$_3^-$ flux) forms a spectrum, ranging from mostly H$^+$ flux in MDA-MB-468 cells to largely HCO$_3^-$ flux in HT29 or HeLa cells. In HT29 and HCT116 cells, unequal pH$_i$ sensitivity of the two components of acid extrusion is responsible for the increase in the share of HCO$_3^-$ transport as pH$_i$ is reduced from 7.4 to 6.8. At pH$_i$ = 7.4, H$^+$ flux varies by an order of magnitude, from very low in HeLa and MiaPaca2 to high in MDA-MB-468, and correlates with total $j^H$. Cell line-dependent variation in total $j^H$ is therefore largely due to differences in H$^+$ flux. These differences do not correlate with cell radius, ranging narrowly from 6.53 ± 0.07 μm in HeLa to 7.14 ± 0.11 μm in HT29. Instead, variation in H$^+$ flux may arise from differences in transporter expression levels at the plasma membrane. In support of this, our preliminary data (not shown) provide evidence for higher expression of the mature (110-KDa) form of NHE1 in MDA-MB-468 and HCT116 cells, as compared with HeLa cells. It remains to be investigated whether, for example, variation in H$^+$ flux also correlates with respiratory rate or invasiveness. In contrast, similar levels of HCO$_3^-$ flux are observed in all cell lines tested, suggesting that HCO$_3^-$ transport may represent an essential and conserved element of tumor pH$_i$ regulation.

- Based on the present results, tumors with a pH$_i$ regulation phenotype similar to HeLa or MiaPaca2 cells will rely on HCO$_3^-$ transport for acid extrusion because of their low H$^+$ flux. Colorectal HT29 and HCT116 cell lines have a greater H$^+$ flux, yet when grown as spheroids, pH$_i$ regulation at their core is driven largely by HCO$_3^-$ flux (Fig. 3). The presence of DIDS produces non-uniform acid extrusion and increases core-to-periphery pH$_i$ gradients (ΔpH$_i$), measured after 12 min of pH$_i$ recovery, by 155% in HT29 spheroids and by 40% in HCT116 spheroids (Fig. 6A). HCO$_3^-$ transport in these spheroids appears to be more capable of producing spatially unified pH$_i$ regulation, as illustrated by the 2.7-fold smaller ΔpH$_i$ measured in DMA in comparison with DIDS (Fig. 3, D and E). This arises because HCO$_3^-$ transport in HT29 and HCT116 cells is less pH$_i$-sensitive than H$^+$ transport and more likely to persist at the acidic spheroid core. The shallow pH$_i$ sensitivity of HCO$_3^-$ flux may be a favorable adaptation in tumors that develop hypoxic cores yet require uniformly alkaline pH$_i$ for coordinated growth. The deceleration of HCT116 spheroid growth in the presence of S0859 highlights the importance of HCO$_3^-$ flux in cell proliferation (Fig. 3F) and the inability of residual H$^+$ flux to compensate for this.

CO$_2$/HCO$_3^-$ as a Mobile Buffer Facilitating Extracellular Diffusion of Cell-extruded Acid—Regulation of pH$_i$ has been viewed largely as a process involving acid/base transport across membranes, treating the cell as a self-contained entity. However, regulation of pH$_i$ in tissue extends to acid/base fluxes across the extracellular space. This is illustrated by HCT116 and MDA-MB-468 spheroids, in which H$^+$ transporters cannot function efficiently without extracellular mobile buffers (Fig. 4). A number of tumors express high levels of H$^+$ transporters, akin to HCT116 and MDA-MB-468 cells. These transporters deposit H$^+$ ions into the extracellular space, where their mobility is restricted by the presence of protonatable sites on mobile buffer molecules, such as membrane-tethered proteins (29). In superfused single cells, solution flow is sufficient to wash away H$^+$ ions. However, in multicellular tissue, extracellular acidity may build up in poorly perfused spaces, thereby inhibiting further acid extrusion. In order for H$^+$ transporters to regulate pH$_i$, their activity must be complemented by facilitated H$^+$ ion diffusion across the extracellular space. Increasing the concentration of Heps, a mobile buffer, accelerates acid extrusion in HCT116 and MDA-MB-468 spheroids (Fig. 4) by buffering the ensuing fall in pH$_i$ (Fig. 5).

Physiologically, CO$_2$/HCO$_3^-$ is the principal extracellular mobile buffer without which membrane H$^+$ transporters would not function to their full potential (Fig. 6B). In effect,
CO$_2$/HCO$_3^-$ in Tumor pH Regulation

**A**

![Diagram](image)

**B**

![Diagram](image)

**FIGURE 6. Dual role of CO$_2$/HCO$_3^-$ buffer in spatial pH regulation in tumor models.** Histograms show the pH gradient from the spheroid core (upper edge of histogram bar) to its periphery (Periph, lower edge) measured after 12 min of recovery from an imposed acid load. Asterisks denote statistically significant differences as compared with control (shaded bars) at the 5% level using unpaired Student’s t tests, except for testing the effects of acetazolamide (ATZ), where paired Student’s t tests were performed. Error bars in both panels indicate S.E.M. The importance of membrane HCO$_3^-$ transport in spheroids bathed in 5% CO$_2$/22 mM HCO$_3^-$ buffer (data from Fig. 3). In HT29 and HCT116 spheroids treated with the HCO$_3^-$ transport inhibitor DIDS, core pH$_i$ was more acidic than control, and hence core-to-periphery pH$_i$ gradients were increased. These results indicate that core pH$_i$ transport is particularly important for regulating pH$_i$ at the spheroid core, thereby ensuring uniform pH$_i$ control throughout the spheroid. The diagram (lower panel) illustrates the role of HCO$_3^-$ transport in acid extrusion. The thin gray arrow represents negative feedback on net acid extrusion. B, the importance of mobile buffers in facilitating H$^+$ extrusion in HCT116 and MDA-MB-468 spheroids. Core-to-periphery pH$_i$ gradients were measured in media buffered by 5% CO$_2$/22 mM HCO$_3^-$ or Hapes (data from Figs. 3 and 4). HCT116 spheroids bathed in CO$_2$/HCO$_3^-$ were treated with DIDS to ensure that the principal process driving acid extrusion was H$^+$ efflux. pH$_i$ gradients increased as extracellular Hapes concentration was decreased. Mobile buffering provided by 5% CO$_2$/22 mM HCO$_3^-$ was equivalent to ~30 mM Hapes. In CO$_2$/HCO$_3^-$ buffer, inhibition of CA activity with acetazolamide (ATZ) slowed pH$_i$ recovery and increased pH$_i$ non-uniformity. These results indicate that CO$_2$/HCO$_3^-$, by acting as a mobile buffer, facilitates acid extrusion throughout the spheroid and helps to attain more uniform pH$_i$ control. The diagram (lower panel) illustrates the role of CO$_2$/HCO$_3^-$, the physiological extracellular mobile pH buffer, in spatial pH$_i$ regulation. The thin gray arrow represents negative feedback on net acid extrusion.

![Equation](image)

However, as [Hapes], and hence its β, is reduced, measured $D_{H^+}^{app}$ does not change. This indicates that even at 1 mM, Hapes is the principal extracellular buffer. Tumors in situ may, however, regulate $D_{H^+}^{app}$ by secreting mobile buffers yet to be characterized.

By comparing pH$_i$ gradient data from Fig. 6B, 5% CO$_2$/22 mM HCO$_3^-$ can be interpolated to have a mobile buffer capacity equivalent to ~30 mM Hapes, i.e. higher mole-for-mole, partly because of its smaller size and hence higher diffusion coefficient (Fig. 5). The ability of CO$_2$/HCO$_3^-$ to facilitate H$^+$ ion diffusion can be limited by its inherently slow reaction kinetics, in particular CO$_2$ hydration (34). This limiting factor could be eliminated by expressing cancer-related extracellular CAs, which catalyze CO$_2$-HCO$_3^-$ interconversion. Indeed, CA inhibition with acetazolamide reduces the rate of acid extrusion in MDA-MB-468 spheroids and increases ΔpH$_i$ by 50% (Fig. 6B). CA activity can therefore accelerate membrane H$^+$ flux, most likely by increasing the effective buffering capacity provided by CO$_2$/HCO$_3^-$. Because a membrane-permeant inhibitor was used, it could be argued that inhibition of intracellular CA activity underlies the acetazolamide effect. However, the effect of CA inhibition increases with spheroid depth, suggesting that CA accelerates a process that is distance-dependent, such as extracellular diffusion (34). Any such effect would add to previously described interactions of H$^+$ transporters with intracellular CAs (35).

**Spheroids as Models of Developing Tumors**—Studies of single superfused cells cannot adequately describe pH$_i$ regulation in cancer tissue because of the multicellular nature and suboptimal perfusion of tumors. Spheroids, like growing tumors, harbor a restricted extracellular space in which cell-extruded H$^+$ ions may accumulate. In addition, spheroids can be imaged for pH$_i$ and pHe, and the composition of their extracellular space can be manipulated through the bulk superfusate (28, 38). The radii of spheroids (~150 µm) imaged in the present work mimic the diffusion distances that are characteristic of the viable rim in tumors (46), which does not develop severe hypoxia (48). Larger spheroids were not studied in order to limit the
development of hypoxia that could per se affect acid extrusion significantly (47). It is plausible that modest hypoxia, acting via gene regulation or energy supply, could have contributed to the slowing of pH$_i$ recovery at the core of spheroids. Maneuvers such as changing extracellular buffering capacity or inhibiting acid/base transport are not expected to alter O$_2$ gradients, so by pairing experiments with controls, it should be possible to account for any background effects of hypoxia.

Superfusates buffered by 5% CO$_2$/22 mM HCO$_3$ mimic blood plasma. Within the spheroid extracellular space, the composition of this buffer is likely to change in a manner that reflects the tumor microenvironment, i.e. low pH$_e$, cellular CO$_2$ production, and HCO$_3$ transport across membranes. Thus, CO$_2$/HCO$_3$-dependent buffering established within spheroids is likely to attain a magnitude that is relevant to developing tumors. The low pH$_e$ in tumors is usually associated with low HCO$_3$ concentrations. For instance, as pH$_e$ is dropped from 7.4 to 6.8 at constant CO$_2$ partial pressure, equilibrium [HCO$_3$] falls 4-fold. Such a fall in extracellular [HCO$_3$] is not rate-limiting for HCO$_3$ flux, at least in HCT116 cells, as measurements at pH$_e$ 6.8 with 22 mM and 5.5 mM HCO$_3$ yield the same acid flux (supplemental Fig. S5). With such high affinity for HCO$_3$, even low millimolar concentrations of HCO$_3$ may suffice for HCO$_3$-driven acid extrusion.

CO$_2$/HCO$_3$ and Cancer—The flux diagrams in Fig. 6 summarize the two roles of CO$_2$/HCO$_3$ in pH$_e$ regulation. These fluxes are ultimately powered by active membrane transport. However, without CO$_2$/HCO$_3$, they would cease to operate because of the lack of transporter substrate (Fig. 6A) or inadequate dissipation of extruded H$^+$ ions (Fig. 6B). The dominance of either pathway will depend on the relative magnitude of transmembrane H$^+$ and HCO$_3$ fluxes during acid extrusion. As shown in the present work, the balance between H$^+$ and HCO$_3$ fluxes varies with cell line and therefore cancer type. Moreover, the share of these fluxes may vary regionally within a solid tumor due to the action of modulators, including pH$_e$.

A notable difference in the two schemes is the relationship between extracellular H$^+$ ions and CO$_2$/HCO$_3$. Under the first scheme (Fig. 6A), cellular uptake of HCO$_3$ in exchange for CO$_2$ produces an out-of-equilibrium state that drives H$^+$ release from CO$_2$, particularly in the presence of extracellular CAs (51). In contrast, under the second scheme (Fig. 6B), CO$_2$/HCO$_3$ buffer takes up a fraction of cell-extruded H$^+$ ions. At steady state, the two schemes will converge at an acidic pH$_e$ a hallmark of cancer that is believed to exert selection pressure against normal cells and promote tumor invasiveness (7, 8). Inhibition of H$^+$ and HCO$_3$ fluxes at very low pH$_e$ would, however, serve as a feedback mechanism to prevent excessive extracellular acidification (Fig. 6). Buffering of extracellular acidity with plasma HCO$_3$ supplementation has been proposed as a novel means of combating tumors (49). The effectiveness of such treatment will have to be weighed against the beneficial effect of raised HCO$_3$ on pH$_e$ control in tumor cells.

The present work highlights the importance of CO$_2$/HCO$_3$ for coordinating pH$_e$ regulation spatially. Non-uniformity of acid extrusion will tend to produce spatially heterogeneous steady-state pH$_e$, which may lead to poor coordination of tissue growth and function. Unlike normal tissue, cancer cannot rely on blood flow to unify pH$_e$ or gap junctions to synchronize pH$_e$ because perfusion tends to be heterogeneous, interrupted, and inadequate (5) and gap junction proteins are typically absent (50). Expression of HCO$_3$ transporter isoforms, particularly of low pH$_e$ sensitivity, plus extracellular carbonic anhydrases to keep CO$_2$/HCO$_3$ buffer at equilibrium may be a compensatory means of improving spatial pH$_e$ regulation by exploiting the dual role of CO$_2$/HCO$_3$ buffer. Proteins implicated in CO$_2$/HCO$_3$ reactions and transport may improve tumor survival and may therefore be targets for therapy.

Acknowledgments—We thank Professor Adrian Harris for valuable discussions. S0859 was kindly provided by Dr. Heinz-Werner Kleeumann (Sanofi-Aventis).

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VOLUME 286 • NUMBER 16 • APRIL 22, 2011