Effects of metabolic acidosis on intracellular pH responses in multiple cell types

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Salameh AI, Ruffin VA, Boron WF. Effects of metabolic acidosis on intracellular pH responses in multiple cell types. Am J Physiol Regul Integr Comp Physiol 307: R1413–R1427, 2014. First published September 10, 2014; doi:10.1152/ajpregu.00154.2014.—Metabolic acidosis (MAc), a decrease in extracellular pH (pHo) caused by a decrease in [HCO3−]o, at a fixed [CO2]o, is a common clinical condition and causes intracellular pH (pHi) to fall. Although previous work has suggested that MAc-induced decreases in pHi (ΔpHi) differ among cell types, what is not clear is the extent to which these differences are the result of the wide variety of methodologies employed by various investigators. In the present study, we evaluated the effects of two sequential MAc challenges (MAc1 and MAc2) on pHi, in 10 cell types/lines: primary-cultured hippocampal (HCN) neurons and astrocytes (HCA), primary-cultured medullary raphe (MRN) neurons, and astrocytes (MRAs), CT26 colon cancer, the C2C12 skeletal muscles, primary-cultured bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC), Ink4a/Arf-null melanocytes, and XB-2 keratinocytes. We monitor pHi using ratiometric fluorescein while imposing MAc: lowering (pHo) from 7.4 to 7.2 by decreasing [HCO3−]o from 22 to 14 mM at 5% CO2 for 7 min. After MAc1, we return cells to the control solution for 10 min to impose MAc2. Using our definition of MAc resistance ([ΔpHi/ΔpHo] ≤ 40%), during MAc1, ~70% of CT26 and ~50% of C2C12 are MAc-resistant, whereas the other cell types are predominantly MAc-sensitive. During MAc2, some cells adapt (ΔpHi/ΔpHo) < (ΔpHi/ΔpHo)], particularly HCA, C2C12, and BMDM. Most maintain consistent responses [(ΔpHi/ΔpHo) ≥ (ΔpHi/ΔpHo)], and a few decouple [(ΔpHi/ΔpHo) ≥ (ΔpHi/ΔpHo)], particularly HCA, C2C12, and XB-2. Thus, responses to twin MAc challenges depend both on the individual cell and cell type.

Many diseases, including local ischemia, shock, diabetic ketoacidosis, inborn errors of metabolism, kidney failure, respiratory failure, and sleep apnea, produce acid-base disturbances in the extracellular fluid (21, 39, 43, 45, 50, 54–56, 74, 80, 89). Such disturbances, be they acute or chronic, almost invariably lead to modifications of intracellular pH (9, 16, 60, 69). Because virtually every biological process is sensitive to pH changes (13, 23, 27, 35, 41, 58), acid-base disturbances lead to cellular dysfunction (15, 37, 40, 88). For instance, in neurons, changes in extracellular pH (pHo) or intracellular pH (pHi) can disturb synaptic transmission and neuronal excitability by modulating the properties of ion channels (15, 25–27). In cardiac cells (12, 53, 75, 79, 88), osteoclasts (19), and other cell types (4, 57) acidosis is proapoptotic. On the other hand, many cancer cells acidify their extracellular environment (14, 30, 32, 61).

Given the importance of pHi for cell function, it is not surprising that nearly all cell types maintain the pHi within a narrow range. The steady-state pHi depends on the balance between the rates of acid-extruding processes (JH+) and acid-loading processes (JL). Acid-extruding processes include the uptake of HCO3− or CO2− by Na+–coupled members of the SLC4 family (17, 24, 68), Na-H exchange (24, 65), Na-K-Cl cotransporter (65), and H+-ATPase (66). Acid-loading processes include HCO3− influx mediated by CI-HCO3 exchange in the SLC4 family (also known as anion exchangers, or AE) and Na-H exchange in the SLC8 family (5, 68), or SLC36 family (6, 52), or mediated by anion exchangers (6).

Cells can experience two major types of challenges to their pHi homeostasis. In the first, an acute intracellular acid or alkali load—under stable environmental conditions and, thus, stable kinetics of the aforementioned transport mechanisms—causes a sudden shift in pHi. The cell responds by altering the rates of its various acid extruders and acid loaders, and (in theory) returns pHi to the initial value. This process, generally termed “pHi regulation” has been studied extensively. In the second type of challenge, a change in the environment of the cell presumably alters the kinetics of one or more processes affecting JH+ or JL and, thus, steady-state pHi. This process has been far less well studied. An example of the second kind of challenge is the fall in pHi caused by decrease in [HCO3−]o at a fixed [CO2]o, a disturbance called metabolic acidosis (MAc).

When challenged by MAc, cells generally exhibit a fall in pHi (2, 18, 22, 62, 66), consistent with the hypothesis that MAc shifts the balance between JE and JL in favor of JL (9, 16, 69). The magnitude of the MAc-induced decrease in pHi (ΔpHi), expressed as a fraction of the imposed change in pHo (ΔpHo), is diverse. Using the 14C-DMO technique to compute the steady-state pHi of rat diaphragm muscle, Adler et al. (2) found that MAc produces only a small fall in pHi between pHi 7.4 and ~6.9 (we calculate a ΔpHi/ΔpHo of ~0.04 from their Fig. 2, but a much larger decrease between pHi 7.4 and ~6.7 (ΔpHi/ΔpHo = 0.27). At least three groups have used fluorescent pH-sensitive dyes to monitor pHi during the imposition of MAc. Working with glomus cells from the rat carotid body, Buckler et al. (22) observed a steep dependence of pHi on pHi (ΔpHi/ΔpHo = 0.68 between pHi 7.4 and 7.0). Ritucci et al. (66) found that the AMPH/ΔpHo during MAc was greater in neurons from nucleus tractus solitarius (ΔpHi/ΔpHo = 0.84 ± 0.014) and ventrolateral medulla (0.72 ± 0.008) than from the hypothalamic nucleus (0.26 ± 0.014) and inferior olive (0.35 ± 0.014). Work on cultured rat neurons identified two types of ΔpHi responses to MAc in both rat hippocampal (HC) and medullary raphe (MR) neurons (18). Some neurons were MAc-sensitive (ΔpHi/ΔpHo of 0.71 between pHi 7.4 and 7.2) and...
others were MAK-resistant (ΔpH/ΔpH₀ of 0.09). Presumably, these MAK-resistant neurons were able to mount a strong pHₐ defense during MAK by increasing J₀ or decreasing Jᵢ (relative to their MAK-sensitive counterparts).

The main purpose of the present study was to establish the prevalence of MAK resistance and MAK sensitivity among cells in a broad range of cell types. We also explored whether cells improve their defense against MAK during a second exposure to MAK (e.g., does ΔpH/ΔpH₀ decrease?). To answer these questions, we use fluorescence imaging to monitor pHₐ as we impose our MAK-MAK protocol on 10 types of cultured cells or cell lines: primary-cultured mouse hippocampal neurons (HCₐ) and astrocytes (HCₐ), primary-cultured medullary raphé neurons (MRₐ) and astrocytes (MRₐ), CT26 colon carcinoma cells, C2C12 skeletal muscle cells, primary-cultured bone marrow-derived macrophages (BMDM), and dendritic cells (BMDC), Ink4a/ARF-null melanocytes (Ink4a), and XB-2 keratinocytes. Our results sometimes reveal a broad range of pHₐ responses to MAK-MAK among cells of a particular type. On the other hand, each cell type appears to have a characteristic set of pHₐ responses.

MATERIALS AND METHODS

Cell Culture

We performed experiments on 10 types of cells (presented sequentially in the RESULTS in Figs. 1–10) of murine origin, either produced in our own laboratory or obtained from others. In each case, the last preparative step before the physiological experiment was to culture the cells to subconfluence on 12-mm gridded coverslips (1916–91012; Belco Biotechnology, Vineland, NJ). Protocols for housing and handling of mice were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

Hippocampal and medullary raphé neurons and astrocytes. We obtained and used primary cocultures (never passaged) of neurons and astrocytes from HC and MR (presented in Figs. 1–4), as described previously (18, 33). Briefly, HC and MR regions were isolated from P0–P2 pups and digested in a HEPES-buffered saline solution that previously (18, 33). Briefly, HC and MR regions were isolated from obtained and used primary cocultures (never passaged) of neurons and cell lines: primary-cultured mouse hippocampal neurons (HCₐ) and astrocytes (HCₐ), primary-cultured medullary raphé neurons (MRₐ) and astrocytes (MRₐ), CT26 colon carcinoma cells, C2C12 skeletal muscle cells, primary-cultured bone marrow-derived macrophages (BMDM), and dendritic cells (BMDC), Ink4a/ARF-null melanocytes (Ink4a), and XB-2 keratinocytes. Our results sometimes reveal a broad range of pHₐ responses to MAK-MAK among cells of a particular type. On the other hand, each cell type appears to have a characteristic set of pHₐ responses.

Materials

Cell cultures obtained from other laboratories. Each cell type was cultured according to the protocols used in the laboratories that provided us with the cells. The CT26 colon carcinoma cell line (Fig. 5) was kindly provided by the laboratory of G. Dubyak at Case Western Reserve University. These were originally obtained from American Type Culture Collection (ATCC; Manassas, VA) and cultured according to ATCC’s instructions (thawed and then passaged 10–20 times); we used them 24–48 h after plating onto gridded coverslips.

The C2C12 skeletal muscle cell line (Fig. 6; Ref. 3), originally obtained from ATCC, was kindly provided to us, frozen in liquid nitrogen, by the laboratory of J. Kirwan at the Cleveland Clinic Foundation. After thawing these cells, we passaged them 4 times, the last representing the plating onto the coverslips; we then waited 10–15 days for the cells to differentiate.

The Dubyak laboratory also supplied primary cultures (never passaged) of BMDM (Fig. 7; Ref. 63) and BMDC (Fig. 8; Refs. 7 and 81). Briefly, the cells were isolated from bone marrow, plated onto 100-mm culture dish for 5 days, lifted and plated onto coverslips, and then allowed to attach to coverslips for 24–72 h.

The Ink4a/ARF-null melanocyte (Fig. 9; Ref. 83) and XB-2 keratinocyte (Fig. 10; Ref. 8) cell lines were kindly provided by the laboratory of B. Bedogni at Case Western Reserve University. These cells were thawed and passaged 4 times, the last representing the plating onto the coverslips. We then allowed 24–48 h for the melanocytes to attach, and 24–72 h for the keratinocytes. For the above experiments on cells from other laboratories, the coverslips were coated with 0.1% poly-L-ornithine.

Solutions

We used three physiological solutions: (1) HEPES-buffered saline (HBS) had the following composition (in mM): 124 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 1.3 NaH₂PO₄, 32.5 HEPES, and 10.5 glucose. The solution was titrated to pH 7.4 with NaOH at 37°C. (2) Control CO₂/HCO₃⁻-buffered saline (Ctrl) had the following composition (in mM): 102 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 22 NaHCO₃, 1.3 NaH₂PO₄, 32.5 HEPES, and 10.5 glucose. The solution was then incubated with 5% CO₂, (3) The pH-7.2 solution (MAc) had the following composition (in mM): 110 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 14 NaHCO₃, 1.3 NaH₂PO₄, 32.5 HEPES, and 10.5 glucose. Solutions osmolalities were measured using a vapor pressure osmometer (5520; Wescor, Logan, UT) and adjusted to 300 ± 5 mosmol/kg H₂O. The HBS solution was gassed with 0% CO₂, 21% O₂, and balance N₂. The Ctrl and MAc solutions were gassed with 21% O₂, 5% CO₂, and balance N₂. For gas delivery, we used a computerized gas mixing system (Series 4000, Environics, Tolland, CT) to bubble solutions for 30–40 min before they were used in experiments.

Solutions were delivered to the experimental chamber on the stage of an inverted microscope at 4 ml/min using syringe pumps (model 33; Harvard Apparatus, Holliston, MA). The solutions were maintained at 37°C by water jacket system before delivered to the chamber.

Fluorescent Imaging of the Intracellular pH-Sensitive Dye

We followed the protocol used by Bouyer et al. (18). Briefly, gridded coverslips with cells attached were fixed to the perfusion chamber and served as the chamber floor. The cells were incubated for ~10 min at room temperature with HBS containing 10 µM of the acetoxymethyl ester of the pH-sensitive dye 2,7’-bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein (BCECF). The chamber was then mounted on Olympus IX-81 inverted microscope equipped for epifluorescence (oil immersion 40× objective, NA 1.35, with a ×1.5 magnification selector). Two excitation wavelengths at 440 nm and 490 nm were obtained using a 510-nm long-pass dichroic mirror, and two excitation filters (440 ± 5 nm and 495 ± 5 nm, U-N71001; B&B Microscope, Pittsburgh, PA) were mounted on a filter wheel placed in the excitation light path of a 75-W xenon arc lamp. The emitted light
was directed to a band-pass filter (530 ± 35 nm) and detected with an intensified EM-CCD camera (C9100–13; Hamamatsu, Bridgewater, NJ) with 512 × 512 pixels. We used Slidebook 5.0 data-acquisition software (Intelligent Imaging Innovation, Denver, CO). Each cell was defined as an area of interest (AOI) by circling the cell body using the outline tool in the software. Within each AOI, the average pixel intensity with a 490-nm excitation (I_{490}) was divided by the average pixel intensity with a 440-nm excitation (I_{440}). These fluorescence excitation ratios (I_{490}/I_{440}) were converted to pH_{i} value by using the high-K⁺/nigericin technique (84) at the end of each experiment to obtain three calibration points, first at pH 7.00, and then, if the cells remained viable (see following paragraph), also at pH 6.40 and 7.60. We averaged the data obtained at the three pH values to generate a unique three-point pH calibration curve for each cell type. For each cell, we used the nigericin data at pH 7.00 to obtain a single-point calibration for that cell (20).

MAC-MAC Protocol and Data Analysis

To study the effect of MAC-MAC protocol on pH_{i}, we started our experiments by flowing HBS for 5 min, followed by 10 min in the Ctrl solution. We then applied two 7-min MAC pulses separated by 10-min in the Ctrl solution. We finished our experiments by flowing the Ctrl solution for an additional 7 min, which causes another small pH_{i} decrease: (ΔpH)_{1} = −0.05. Thus, (ΔpH/ΔpH)_{1} = (−0.05)/(−0.20) = 0.25. This HCN is relatively MAC-resistant. We then return the neuron to the Ctrl solution for 10 min before switching to the MAC solution for an additional 7 min, which causes another small pH_{i} decrease: (ΔpH)_{2} = −0.06 and (ΔpH/ΔpH)_{2} = 0.30. Because (ΔpH/ΔpH)_{2} = (ΔpH/ΔpH)_{1}, this neuron has a relatively consistent response to MAC.

The red trace shows the pH_{i} response of a second HCN to the MAC-MAC protocol. However, in this case, the MAC-induced pH_{i} decreases are larger. In the first MAC pulse, (ΔpH)_{1} = −0.17 and (ΔpH/ΔpH)_{1} = 0.85. This neuron is relatively MAC-sensitive. Any numerical criterion for distinguishing MAC resistance from MAC sensitivity is somewhat arbitrary. Nevertheless, after examining data for the 10 cell types in the present study, as a working definition, we will call cells with a (ΔpH/ΔpH)_{1} ≤ 40% MAC-resistant, and those with a higher (ΔpH/ΔpH)_{1}, MAC-sensitive. Returning to the red trace, we see that in the second MAC pulse, the pH_{i} changes are similar to those in the first pulse: (ΔpH)_{2} = −0.18 and (ΔpH/ΔpH)_{2} = 0.90. Thus, this HCN, like the first, has a relatively consistent response to MAC.

The gray trace shows a third HCN. The first MAC pulse causes a very large decrease in pH_{i}, followed by a recovery. At the end of the MAC exposure, we compute (ΔpH)_{1} = −0.28 and (ΔpH/ΔpH)_{1} = 1.4. Thus, at this point, the neuron fulfills our criterion for being MAC-sensitive. However, when we challenge this HCN with a second MAC pulse, its pH_{i} decrease is substantially smaller than during the first pulse, with (ΔpH)_{2} = −0.04 and (ΔpH/ΔpH)_{2} = 0.20, and in fact, this neuron is now MAC-resistant after having undergone a substantial adaptation between MAC_{1} and MAC_{2}.

For the three neurons in Fig. 1A, Fig. 1B shows the −Δk_{440} records, which are indicators of the cell membrane integrity (11). We reject any cell with −Δk_{440} > 4% min⁻¹, and the neurons in Fig. 1A are well within range.

For the 25 HC neurons that we studied using the MAC-MAC protocol shown in Fig. 1A, Fig. 1C summarizes the relationship between (ΔpH)_{1} and (ΔpH)_{2} for individual neurons. The black, red, and gray arrows point to symbols that represent the similarly colored pH_{i} records (i.e., neurons) in Fig. 1A. Note that both the black and red neurons lie close to the line of identity—the dashed gray line, for which (ΔpH)_{1} = (ΔpH)_{2}. That is, for a neuron near the line of identity, the pH_{i} response to the first MAC pulse is similar to the pH_{i} response to the second MAC pulse. We refer to this situation as consistency: that is, the cell neither adapts (i.e., exhibits a smaller ΔpH) nor decompensates (i.e., exhibits a larger ΔpH). Even though the neurons represented by the black and red traces each lie near the line of identity in Fig. 1C (i.e., they exhibit consistency), the neuron represented by the red trace exhibits much larger ΔpH values than the neuron represented by the black trace. The neuron represented by the black trace is MAC-resistant because (ΔpH/ΔpH)_{i} ≤ 40%, indicated by the dashed vertical gray line. On the other hand, the neuron represented by the red trace is MAC-sensitive because (ΔpH/ΔpH)_{i} > 40%. Hence, two cells can both exhibit consistency [i.e., (ΔpH)_{1} = (ΔpH)_{2}], even though one is resistant and the other, sensitive. By our criterion, 9 of the 25 neurons (36%) are MAC-resistant during MAC_{1}.
Fig. 1. Hippocampal neurons: responses to MAc-MAc protocol (25 neurons, 7 cover slips, 3 surgical preparations). A: examples of pHi responses in three HC neurons. The black record shows a MAc-resistant neuron; the red, a neuron that is MAc sensitive during the first MAc challenge (MAc1) and that does not adapt during the second MAc challenge (MAc2); and the gray, an neuron that is MAc sensitive during MAc1 but that markedly adapts during MAc2 (MAc2). The three records correspond to the neurons in A. C: cell-by-cell relationship between the ΔpHi during MAc1, (ΔpHi), and ΔpHi during MAc2, (ΔpHi). The three arrows identify points corresponding to the three records (black, red, gray) in A. In MATERIALS AND METHODS, we describe our procedure for computing (ΔpHi) and (ΔpHi). In Fig. 2A, we provide graphical examples of such calculations. D: cell-by-cell dependence of (ΔpHi) (purple squares, R² = 0.07) and (ΔpHi) (green squares, R² = 0.01) on the initial pHi before MAc1. The broken arrow identifies the gray neuron in panel A. E: frequency distribution of (ΔpHi) (purple bars) or (ΔpHi) (green bars), with a pH bin-width of 0.05. The values in parentheses are means ± standard deviations. The arrow identifies the (ΔpHi) (ΔpHi) shift of the gray neuron in A.

As a working definition, we will say that a cell adapts to MAc when (ΔpHi) and (ΔpHi) meet two criteria. First, with the assumption that we can make pHi measurements with perfect accuracy, log10(|ΔpHi|/ΔpHi)| < -0.05. That is, a cell adapts when (ΔpHi) is ~12.2% less than (ΔpHi). Second, we assume that the experimental error in measuring pHi using our technology is ±0.02 pH units. Combining these two criteria, we arrive at the confidence interval shown by the upper bending curve in Fig. 1C. By the aforementioned criteria, 6 of the 25 cells (25%) in Fig. 1C adapt, the most striking example of which is the gray, whose neuron lies near the left end of the x-axis and well above the line of identity. As a working definition, we will say that a cell decompensates to MAc when its (ΔpHi), (ΔpHi) point in Fig. 1C falls below the lower bending curve, which is the geometric reflection of the upper bending curve of the line of identity. By this definition, 8 of the 25 cells (32%) in Fig. 1C decompensate, the most extreme example of which is the neuron identified by the asterisk, which lies well below the line of identity.

Previous work on neurons showed that switching from a non-CO₂/HCO₃⁻ buffer to CO₂/HCO₃⁻ causes changes in steady-state pHi that depend on the initial steady-state pHi in the nominal absence of CO₂/HCO₃⁻ (10). The HCO₃⁻ dependent change in steady-state pHi becomes progressively more positive (indicating a net pHi increase) at lower initial pHi values, and progressively more negative (indicating a net pHi decrease) at higher initial pHi values (76). To determine whether the MAc-induced ΔpHi also depends on the pHi preceding the MAc, in Fig. 1D we plot, for all 25 neurons, (ΔpHi) [purple squares] and (ΔpHi) [green squares] as a function of the pHi prevailing just before MAc1. Thus, the purple and green squares for a particular neuron always have the same value on the x-axis. To illustrate, the two squares connected by the broken arrow in Fig. 1D represent the gray neuron in Fig. 1A. We can see from the position of the two points along the x-axis (see vertical line) that, before MAc1, this neuron had a pHi of 6.92. The first MAc produces a (ΔpHi) of −0.28, but the second MAc produces a (ΔpHi) of only −0.04, indicating strong adaption to MAc. An examination of all 25 MAc1 points and of all 25 MAc2 points by ordinary linear least-squares regression analysis reveals not even a weak linear relationship (MAc1, R² = 0.07, MAc2 R² = 0.01) between the pHi before MAc1 and either (ΔpHi) or (ΔpHi).

Finally, Fig. 1E shows the frequency distribution of ΔpHi during the first (purple bars) and second (green bars) MAc pulses. As indicated by the arrow, the gray neuron in Fig. 1A shifts from the far left to the far right of the frequency distribution. Nevertheless, at the population level, the distribution for the first MAc pulse (mean ΔpHi = −0.12, SD = 0.07) is indistinguishable from that for the second (mean ΔpHi = −0.12, SD = 0.05). Table 1 summarizes the pHi parameters in MAc-MAc protocols for HC neurons and the other nine cell types.

Hippocampal astrocytes. We also investigated in the present study whether astrocytes accompanying the neurons in the HC culture exhibit the same cellular responses to our MAc-MAc protocol. Fig. 2A, shows pH recordings for two HC astrocytes. The HC1, indicated by the black trace in Fig. 2A has a large (ΔpHi) of −0.18 [i.e., (ΔpHi)/(ΔpHi) = 0.90]. However, (ΔpHi) is much smaller, −0.08 [i.e., (ΔpHi)/(ΔpHi) = 0.4], reflecting a partial pH recovery during MAc2. The arrows in Fig. 2A represent the computed (ΔpHi) and (ΔpHi). Thus, this astrocyte adapts to MAc (Fig. 2C, black arrow). In the example represented by the red trace, (ΔpHi) is rather large, nearly −0.19 [i.e., (ΔpHi)/(ΔpHi) = 0.95], and (ΔpHi) is somewhat larger still, −0.22 [i.e., (ΔpHi)/(ΔpHi) = 1.1]. Although this astrocyte does not quite meet our definition of decompensating (Fig. 2C, red arrow), we note that the pHi continues to drift in the acid direction throughout MAc2.

Figure 2C shows that all of the HC astrocytes in our study have rather large responses to the first MAc pulse [(ΔpHi) < −0.10, (ΔpHi)/(ΔpHi) > 0.4], meeting our criterion for being MAc-sensitive. For 6 of the 15 astrocytes (40%), the response to the second MAc pulse is sufficiently small that these cells meet our criteria for adaptation. Note that the cells with a low
Table 1. Comparison of pH\textsubscript{i} data among cell types for MAc-MAc protocol

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Steady-State pH\textsubscript{i} Before MAc\textsubscript{1}</th>
<th>Steady-State pH\textsubscript{i} Before MAc\textsubscript{2}</th>
<th>(\frac{\Delta\text{pH}}{\Delta\text{pH}_{\text{R1}}})</th>
<th>(\frac{\Delta\text{pH}}{\Delta\text{pH}_{\text{R2}}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCN (Fig. 1)</td>
<td>7.17 ± 0.03</td>
<td>7.13 ± 0.03</td>
<td>0.60 ± 0.07</td>
<td>0.59 ± 0.05</td>
</tr>
<tr>
<td>HCA (Fig. 2)</td>
<td>7.00 ± 0.03</td>
<td>7.02 ± 0.02</td>
<td>0.65 ± 0.04</td>
<td>0.62 ± 03</td>
</tr>
<tr>
<td>MR\textsubscript{A} (Fig. 3)</td>
<td>7.05 ± 0.02</td>
<td>7.04 ± 0.02</td>
<td>0.95 ± 0.01</td>
<td>0.93 ± 06</td>
</tr>
<tr>
<td>MR\textsubscript{N} (Fig. 4)</td>
<td>7.04 ± 0.02</td>
<td>6.99 ± 0.01</td>
<td>0.33 ± 0.03</td>
<td>0.34 ± 04</td>
</tr>
<tr>
<td>CT26 (Fig. 5)</td>
<td>7.23 ± 0.03</td>
<td>7.16 ± 0.04</td>
<td>0.40 ± 0.05</td>
<td>0.24 ± 04*</td>
</tr>
<tr>
<td>C2C12 (Fig. 6)</td>
<td>7.08 ± 0.03</td>
<td>6.98 ± 0.03</td>
<td>0.75 ± 0.06</td>
<td>0.69 ± 05</td>
</tr>
<tr>
<td>BMDC (Fig. 7)</td>
<td>7.01 ± 0.04</td>
<td>6.91 ± 0.04</td>
<td>1.00 ± 0.05</td>
<td>0.86 ± 06*</td>
</tr>
<tr>
<td>BMDC (Fig. 8)</td>
<td>7.18 ± 0.03</td>
<td>7.00 ± 0.03</td>
<td>0.68 ± 0.05</td>
<td>0.69 ± 04</td>
</tr>
<tr>
<td>Ink4-a (Fig. 9)</td>
<td>7.31 ± 0.02</td>
<td>7.20 ± 0.02</td>
<td>0.85 ± 0.07</td>
<td>0.86 ± 06</td>
</tr>
<tr>
<td>XE-2 (Fig. 10)</td>
<td>7.16 ± 0.02</td>
<td>7.04 ± 0.02</td>
<td>0.89 ± 0.04</td>
<td>0.80 ± 01</td>
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</table>

MAc, metabolic acidosis; pH\textsubscript{i}, intracellular pH; HCN, primary cultured hippocampal; HCA, primary cultured astrocytes; MR\textsubscript{N}, cultured medullary raphe neurons; MR\textsubscript{A}, cultured medullary astrocytes; BMDC, bone marrow-derived macrophages; BMDC, bone marrow ddrict cells. *Significant difference between this value and the corresponding value in the adjacent column (\(P < 0.01\)).

(\(\Delta\text{pH}_{\text{R1}}\)) magnitude tend to adapt in the second MAc pulse, whereas astrocytes with a high (\(\Delta\text{pH}_{\text{R1}}\)) magnitude tend not to adapt.

Figure 2D shows that the HC astrocytes have a much narrower range of initial pH\textsubscript{i} values than their neuronal counterparts (see Fig. 1D) and that the astrocytes that have an initial steady-state pH\textsubscript{i} lower than 7.0 are most likely to exhibit adaptation. We observe a moderately strong, negative linear relationship between initial pH\textsubscript{i} and (\(\Delta\text{pH}_{\text{R1}}\)) (\(R^2 = 0.57\)) and a stronger one for (\(\Delta\text{pH}_{\text{R2}}\)) (\(R^2 = 0.76\)). Thus, the magnitude of the MAC-induced \(\Delta\text{pH}\) increases as the initial pH\textsubscript{i} increases.

Figure 2E reveals a single population for (\(\Delta\text{pH}_{\text{R1}}\)), but suggests two populations for (\(\Delta\text{pH}_{\text{R2}}\))—one shifted to the right for astrocytes that adapt, and one at the extreme left for those that do not. Table 1 summarizes the pH\textsubscript{i} parameters in MAC-MAc protocols for HC astrocytes.

**Medullary raphé neurons.** The second area from the CNS that we investigated is the medullary raphé. Figure 3A shows the responses of two MR neurons to the MAC-MAc protocol. The black MR\textsubscript{N} has a smaller pH\textsubscript{i} decrease during MAc\textsubscript{1} [i.e., (\(\Delta\text{pH}_{\text{R1}}\)) = −0.09, (\(\Delta\text{pH}/\Delta\text{pH}_{\text{R1}}\)) = 0.45] and, thus, barely meets the criteria for a MAC-sensitive cell. The response to MAc\textsubscript{2} is slightly smaller [i.e., (\(\Delta\text{pH}_{\text{R2}}\)) = −0.08, (\(\Delta\text{pH}/\Delta\text{pH}_{\text{R2}}\)) = 0.40]. The red neuron, on the other hand, is clearly MAC-sensitive during both MAc\textsubscript{1} [i.e., (\(\Delta\text{pH}/\Delta\text{pH}_{\text{R1}}\)) = −0.27, (\(\Delta\text{pH}/\Delta\text{pH}_{\text{R2}}\)) = 1.35] and MAc\textsubscript{2} [i.e., (\(\Delta\text{pH}/\Delta\text{pH}_{\text{R2}}\)) = −0.19, (\(\Delta\text{pH}/\Delta\text{pH}_{\text{R2}}\)) = 0.95]. Figure 3C shows that the MR neurons have a wide range of responses to MAc\textsubscript{1}, with (\(\Delta\text{pH}\)) ranging between −0.06 and −0.27. Nevertheless, only 3 MR neurons (11%) are MAC-resistant during MAc\textsubscript{1} (about one-third the ratio for HC neurons), and only one MR\textsubscript{N} barely meets the criteria for MAC resistance for both the first and second pulses. Figure 3C also shows that the responses of MR neurons to MAc\textsubscript{2} are generally very similar to those of MR\textsubscript{A}, that is, MR neurons are much less likely to adapt or decompensate than HC neurons. Figure 3D shows no relationship between initial steady-state pH\textsubscript{i} of MR neurons and their (\(\Delta\text{pH}\)) responses to MAc\textsubscript{1} (\(R^2 = 0.04\)) and MAc\textsubscript{2} (\(R^2 = 0.01\)). The frequency distributions of MR neurons in Fig. 3E shows that (\(\Delta\text{pH}\)) responses of MR neurons are much more tightly grouped than those of HC neurons. Table 1 summarizes the pH\textsubscript{i} parameters in MAC-MAc protocols for MR neurons.

**Medullary raphé astrocytes.** Figure 4A shows pH\textsubscript{i} traces of one MAC-resistant [black: (\(\Delta\text{pH}_{\text{R1}}\)) = −0.04, (\(\Delta\text{pH}_{\text{R2}}\)) = −0.09] example of a raphé astrocyte. The black trace shows the responses of an astrocyte to the MAC-MAc protocol. The black trace shows that the astrocyte has a much smaller pH\textsubscript{i} decrease during MAc\textsubscript{1} [i.e., (\(\Delta\text{pH}_{\text{R1}}\)) = −0.09, (\(\Delta\text{pH}/\Delta\text{pH}_{\text{R1}}\)) = 0.45] and, thus, barely meets the criteria for a MAC-sensitive cell. The response to MAc\textsubscript{2} is slightly smaller [i.e., (\(\Delta\text{pH}_{\text{R2}}\)) = −0.08, (\(\Delta\text{pH}/\Delta\text{pH}_{\text{R2}}\)) = 0.40]. The red neuron, on the other hand, is clearly MAC-sensitive during both MAc\textsubscript{1} [i.e., (\(\Delta\text{pH}/\Delta\text{pH}_{\text{R1}}\)) = −0.27, (\(\Delta\text{pH}/\Delta\text{pH}_{\text{R2}}\)) = 1.35] and MAc\textsubscript{2} [i.e., (\(\Delta\text{pH}/\Delta\text{pH}_{\text{R2}}\)) = −0.19, (\(\Delta\text{pH}/\Delta\text{pH}_{\text{R2}}\)) = 0.95]. Figure 3C shows that the MR neurons have a wide range of responses to MAc\textsubscript{1}, with (\(\Delta\text{pH}\)) ranging between −0.06 and −0.27. Nevertheless, only 3 MR neurons (11%) are MAC-resistant during MAc\textsubscript{1} (about one-third the ratio for HC neurons), and only one MR\textsubscript{N} barely meets the criteria for MAC resistance for both the first and second pulses. Figure 3C also shows that the responses of MR neurons to MAc\textsubscript{2} are generally very similar to those of MR\textsubscript{A}, that is, MR neurons are much less likely to adapt or decompensate than HC neurons. Figure 3D shows no relationship between initial steady-state pH\textsubscript{i} of MR neurons and their (\(\Delta\text{pH}\)) responses to MAc\textsubscript{1} (\(R^2 = 0.04\)) and MAc\textsubscript{2} (\(R^2 = 0.01\)). The frequency distributions of MR neurons in Fig. 3E shows that (\(\Delta\text{pH}\)) responses of MR neurons are much more tightly grouped than those of HC neurons. Table 1 summarizes the pH\textsubscript{i} parameters in MAC-MAc protocols for MR neurons.
and one MAC-sensitive MR astrocyte [red: \((\Delta pHi)_1 = -0.25, (\Delta pHi)_2 = -0.20\)]. Figure 4C shows that only 3 of 17 MR astrocytes (18%) are MAC-resistant during MAC1, and reveals little tendency of these cells to adapt or decompensate from MAC1 to MAC2. Figure 4D shows a mild, negative relationship between the initial steady-state pHi and the MAC responses (MAC1 \(R^2 = 0.32\), MAC2 \(R^2 = 0.30\)). Figure 4E shows that MR astrocytes tend to have larger, more broadly distributed \(\Delta pHi\) responses than do either HC astrocytes (Fig. 2D) or MR neurons (Fig. 3D). Table 1 summarizes the MR astrocytes MAC-MAC parameters.

**Cells From Outside CNS**

To determine whether MAC resistance vs. sensitivity and adaptation vs. decompensation are characteristics that extend beyond cells cultured from the CNS, we applied our MAC-MAC protocol on selection of cell types cultured from outside CNS.

**CT26 colon cancer.** Figure 5A shows the responses of two CT26 cells in MAC-MAC experiments. The cell represented by the black record exhibits small \(\Delta pHi\) responses to both MAC1 and MAC2. For the red cell, pH drifts downward during MAC1, the inter-MAC period, and MAC2.

Figure 5C reveals that a high fraction of CT26 cells fits our criterion for being MAC-resistant during MAC1 (29 cells out of 40 = 73%), and 8 cells have \((\Delta pHi)_1/(\Delta pHi)_0\) values above 50%. The second MAC pulse reveals that seven cells (18%) adapt and nine cells (22%) decompensate. Figure 5D shows a moderate positive relationship between steady-state pHi and MAC1 \((R^2 = 0.47)\) that becomes weaker with MAC2.
Thus, the magnitude of the MAC-induced \( \Delta \text{pHi} \) tends to decrease as the initial \( \text{pHi} \) increases. The population distribution in Fig. 5E shows that the \( \Delta \text{pHi} \) responses tend to be clustered toward the right during MAC1 and MAC2, confirming that CT26 cancer cells are generally MAC-resistant. Table 1 summarizes the \( \text{pHi} \) parameters in MAC-MAC protocols for CT26 cells.

**Skeletal muscle.** The black \( \text{pHi} \) record for the C2C12 skeletal muscle cell line in Fig. 6A exhibits very small increase in its \( \text{pHi} \) in response to MAC1, and then a very small decrease with MAC2. The red record shows a very different set of responses. In MAC1, the cell has a \( \Delta \text{pHi} \) of \(-0.09\) [i.e., \( \text{pHi}_1 / \Delta \text{pHi}_1 = 0.45 \)], and thus fits our criterion for MAC sensitivity. However, in MAC2 the cell has larger \( \Delta \text{pHi} \) of \(-0.14\) [i.e., \( \text{pHi}_2 / \Delta \text{pHi}_2 = 0.70 \)]; thus, the cell decompensates. The gray C2C12 record is from a cell on the same coverslip as the red cell. During MAC1, \( \Delta \text{pHi} \) is \(-0.14\) [i.e., \( \text{pHi}_2 / \Delta \text{pHi}_2 = 0.70 \)], which fits our criterion for MAC sensitivity. During MAC2, however, \( \text{pHi} \) drifted slightly upward. Moreover, after the removal of MAC2, \( \text{pHi} \) now recovers rather robustly. We cannot rule out the possibility that a small mismatch in \([\text{CO}_2]_0\) values between the control and MAC solutions may have contributed to the small, abrupt changes in \( \text{pHi} \) at the transitions to/from MAC. Nevertheless, it is clear that this cell fully resists the tendency to acidify during MAC2, and may even alkalinize.

Figure 6C shows that, during MAC1, 13 of 28 C2C12 cells (46%) are MAC-resistant. A comparison of \( \Delta \text{pHi}_1 \) and \( \Delta \text{pHi}_2 \) values reveals that 7 of 28 (25%) cells lie near the line of identity; 13 adapt to varying degrees, often quite robustly; and 8 decompensate to a minor extent. Figure 6D reveals no
obvious correlation between the starting pH and C2C12 MAC sensitivity (MAC1 $R^2 = 0.01$, MAC2 $R^2 = 0.00$). The population distribution, Fig. 6E, shows that MAC responses of C2C12 cells are clustered toward small acidifications during MAC1, and toward even smaller acidifications during MAC2. Table 1 summarizes the pH$_i$ parameters in MAC-MAC protocols for C2C12 cells.

**Bone marrow-derived macrophages.** Figure 7A shows pH$_i$ traces of a MAC-resistant [black: ($\Delta$PH)$_1 = -0.04$, ($\Delta$PH)$_2 = -0.05$] and a MAC-sensitive BMDC cell [red: ($\Delta$PH)$_1 = -0.25$, ($\Delta$PH)$_2 = -0.19$]. Figure 7C shows that BMDC are almost exclusively MAC-sensitive. Only 2 of 24 macrophages (8%) are MAC-resistant. This panel also reveals that BMDC have little tendency to decompensate (1 cell). On the other hand, 8 cells (33%) adapt during MAC2. Figure 7D shows no relationship for MAC$_1$ ($R^2 = 0.00$) or a weak negative relationship [for MAC$_2$ ($R^2 = 0.10$)] between the steady-state pH$_i$ before MAC1 and the MAC responses. Figure 7E shows that BMDC have broadly distributed $\Delta$PH$_i$ responses to both MAC1 and MAC2. Table 1 summarizes the pH$_i$ parameters in MAC-MAC protocols for BMDC.

**Bone marrow-derived dendritic cells.** Figure 8A shows two pH$_i$ traces for two BMDC cells. The black trace represents a cell with a ($\Delta$PH)$_1 = -0.13$, and a ($\Delta$PH)$_2 = -0.10$, and the red trace, ($\Delta$PH)$_1 = -0.22$ and ($\Delta$PH)$_2 = -0.10$. The cell represented by the red trace not only adapts substantially, but also exhibits a robust pH$_i$ recovery after the removal of MAC$_2$. Figure 8C shows that BMDC tend to be MAC-sensitive; only one cell out of 23 has a ($\Delta$PH$_1$/MAC$_1$) $\leq$ 40%. The figure also shows that 10 cells out of 23 (43%) exhibit adaptation during
MAc2, and only one cell decompensates. The steady-state pHi before MAc1 does not correlate with the BMDC response to MAc1 ($R^2 = 0.06$; Fig. 8D); however, with MAc2, we see a mild, negative correlation ($R^2 = 0.35$). The population distribution in Fig. 8E shows a broad distribution of relatively large ΔpHi responses to both MAc1 and MAc2. Table 1 summarizes the pHi parameters in MAc-MAc protocols for BMDC cells.

**Ink4a/ARF-null melanocyte cell line.** Figure 9A shows traces for two melanocytes. The cell represented by the black trace has a (ΔpHi)$_1$ = −0.10 and (ΔpHi)$_2$ = −0.07. The cell represented by the red trace has a (ΔpHi)$_1$ = −0.16 and slightly decompensates in MAc2 to (ΔpHi)$_2$ = −0.19. Figure 9C reveals that 5 cells out of 25 (20%) decompensate to some degree in MAc2, whereas 3 cells (16%) adapt to a modest extent. Figure 9D shows a tight grouping of initial pHi values and no correlation between steady-state pHi before MAc1 and either the MAc1 ($R^2 = 0.04$) or MAc2 ($R^2 = 0.03$) response. Figure 9E shows that melanocytes have ΔpHi distributions with moderate standard deviations and tend to be MAc-sensitive. Table 1 summarizes the pHi parameters in MAc-MAc protocols for Ink4a cells.

**XB-2 keratinocyte cell line.** Figure 10A shows the traces of two keratinocytes. Both the black ([ΔpHi]$_1$ = −0.15] and the red traces ([ΔpHi]$_2$ = −0.15] meet our criteria for being MAc-sensitive. The black trace adapts during MAc2 ([ΔpHi]$_2$ = −0.03), whereas the red trace decompensates ([ΔpHi]$_2$ = −0.22]. Figure 10C shows that only 1 XB-2 cell out of 26 has a (ΔpHi/ΔpHi)$_1$ = 40%; the rest meet our definition of being MAc-sensitive during MAc1. This panel also reveals that 10 out of 26 cells (38%) adapt and 8 cells (31%) decompensate in response to
MAC. Figure 10D shows that steady-state pH before MAC correlates mildly in the positive direction with the MAC response ($R^2 = 0.21$) and that the correlation weakens for MAC $R^2 = 0.08$. Finally, Fig. 10E reveals that the ΔpH distributions are broad and shifted toward high ΔpH values. Table 1 summarizes the pH parameters in MAC-MAC protocols for XB-2 cells.

**DISCUSSION**

**Importance of Metabolic Acidosis**

Metabolic acidosis is one of the most prevalent and important conditions encountered in emergency and intensive-care settings (48, 51, 85). Perhaps the most common type of MAC is lactic acidosis, generally the result of tissue hypoxia, which, in turn, is usually a consequence of hypoperfusion (e.g., shock; Ref. 67) or ischemia (50, 70). Other examples of MAC include diabetic ketoacidosis (54), a consequence of an excess release of ketoads; renal-tubule acidosis, the inability of the kidneys to excrete sufficient HCO$_3^-$ (36); and acidoses caused by inborn errors of metabolism (21).

In vivo, metabolic acidoses of various etiologies involve changes in multiple parameters other than pH, and [HCO$_3^-$], changes that are characteristic of each MAC etiology. In the present study, we lay the groundwork for understanding the mechanisms by which metabolic acidoses affect pH by studying in 10 selected cell types the simplest example of MAC. Namely, we decrease [HCO$_3^-$], and pHo at a fixed [CO$_2$], but in the absence of other metabolic intermediates (e.g., lactic acid, ketone bodies), and without changing other parameters (e.g., [K$^+$], [O$_2$], [CO$_2$]) that can accompany MAC in vivo.

To our knowledge, the present study is the first to investigate the effects of MAC on CT26, C2C12, BMDM, BMDC, Ink4a, or XB-2 cells. In fact, we are not aware of previous pH measurements in any of these six cell types, except for BMDC during antigen cross-presentation (72, 73), a process in which dendritic cells present proteolytic peptides to lymphocytes. These BMDC studies report initial pH$_i$ values of ~7.20, which is very close to our value of 7.18 (Table 1). To our knowledge, the present study is also the first to address the pH responses to sequential MAC episodes. Finally, to facilitate comparisons across cell types, we employ a uniform technology to measure pH$_i$ and use a uniform MAC protocol (e.g., duration and ΔpH$_o$).

**Limitations of our Protocol**

We designed our MAC-MAC protocol to monitor cells during two MAC pulses for as long as possible, and yet still keep the experiment short enough—a total of 44 min in a CO$_2$/HCO$_3^-$ buffer—to have a reasonable success rate. An unavoidable limitation is that the cells often did not attain a steady state during MAC pulses or the subsequent recoveries in the Ctrl solution. Therefore, our ΔpH$_i$ values are operationally defined on the basis of the pH$_i$ values achieved after 7 min in MAC. In cells for which pH$_i$ was still drifting after 7 min in MAC, it is impossible to know just how low/high pH$_i$ would have continued to drift, given sufficient time.

**Comparison of Our Data to the Work of Others**

Our results are generally consistent with those of previous reports (2, 18, 22, 62, 66). For instance, Adler et al. (2) reported that a decrease in pH$_i$ from 7.4 to 6.9, maintained for 4 to 6 h caused pH$_i$ to fall by ~0.04 in rat diaphragm muscle. We find that a 7-min decrease in pH$_i$ from 7.4 to 7.2 causes an average ΔpH$_i$ of 0.08 in the skeletal muscle cell line C2C12. Although our ΔpH$_i$ (especially if extrapolated to a ΔpH$_i$ of 0.5) is appreciably larger, the long incubation period of Adler et al. (2) may have permitted the muscle cells to synthesize new proteins and gradually reduce the ΔpH$_i$. Ritu et al. (66) reported that rat neurons from the chemosensitive NTS have a ΔpH$_i$/ΔpH$_o$ of 0.84, whereas neurons from the nonchemosensitive hypoglossal nucleus have a ΔpH$_i$/ΔpH$_o$ of 0.26. We find that the average ΔpH$_i$/ΔpH$_o$ is ~0.62 for neurons from the chemosensitive MR region, and ~0.60 for neurons from the nonchemosensitive HC region. Our results agree with those of Bouyer et al. (18), who reported that the majority of rat MR neurons are MAC-sensitive. However, unlike Bouyer’s study, our data on mice show that the majority of HC neurons are also MAC-sensitive.
Parameters That Govern the pH$_i$ Response to MAc

The model. The hypothetical model in Fig. 11A, shows several major HCO$_3^-$-dependent and HCO$_3^-$-independent acid-base transport pathways that affect pH$_i$. Steady-state pH$_i$ (point “a” in Fig. 11B) depends on the balance of $J_E$ vs. $J_L$ (9, 69), which, in turn, depends on the extent to which the cell expresses variants of particular acid-base transporters (e.g., see Ref. 17) and how it regulates them. The simplest mechanism by which MAc could cause pH$_i$ to fall would be by increasing the H$^+$ leak into or the OH$^-$ leak out of the cell. However, [H$^+$] and [OH$^-$] are so low that they may not make major contributions (69), except under special circumstances, as with the activation of Hv channels under depolarized conditions, in which the H$^+$ electrochemical gradient is outward (34, 64, 71, 90). We expect MAc, and the accompanying decrease in [HCO$_3^-$]$_o$ and [CO$_3^{2-}$]$_o$, to inhibit acid extruders (downward shift of $J_E$ curve in Fig. 11C) and at the same time to stimulate acid loaders (upward shift of $J_L$ curve in Fig. 11C). In addition, the fall in [HCO$_3^-$]$_o$ or pH$_o$ could trigger hypothetical sensors that modulate $J_E$ or $J_L$. According to the fundamental law of pH$_i$ regulation, that is, dpH$_i$/dt = $\rho$($J_E$ - $J_L$)/$\beta$ (9), where dpH$_i$/dt is the rate of pH$_i$ change, $\rho$ is the cell’s surface-to-volume ratio, and $\beta$ is the intrinsic buffering power—the MAc-induced imbalance between $J_E$ and $J_L$ ($J_E$ < $J_L$) will lead to a fall in pH$_i$ (i.e., dpH$_i$/dt < 0). As pH$_i$ falls, $J_E$ gradually rises along the black dashed curve in Fig. 11C and $J_L$ gradually falls along the red dashed curve, until $J_E$ and $J_L$ once again come into balance at a new steady-state pH$_i$ (point “b” in Fig. 11C).

Role of $\rho$ and $\beta$. Why do different cells exhibit different $\Delta$pH$_i$ values during MAC$_1$, for example? According to the above discussion, the magnitude of $\Delta$pH$_i$ depends solely on how MAc affects the pH$_i$ dependencies of $J_E$ vs. $J_L$. In turn, these pH$_i$ dependencies could be under the influence of 1) pH$_o$ or [HCO$_3^-$]$_o$ sensors, as the cell begins to experience the MAc, or 2) pH$_i$-dependent processes, as the pH$_i$ begins to fall. Note that, $\rho$ and $\beta$ have no bearing on the initial steady-state pH$_i$ or the MAc-induced $\Delta$pH$_i$ (assuming the cell reaches a steady state), only on dpH$_i$/dt (i.e., the rate at which pH$_i$ approaches

Fig. 12. pH responses of the ten cell types (A–J) during the MAC-MAC protocol. The values for the first Ctrl, MAC$_1$, the second Ctrl, MAC$_2$, and the last Ctrl correspond to the values calculated for the last minute of the period (see part A Figs. 1–10), as described in MATERIALS AND METHODS.
the new steady state during MAc). Thus, two otherwise-identical cells would exhibit the same MAc-induced ΔpHi; however, the cell with the higher β would reach the new steady-state pHi more slowly.

Response to the removal of MAc. Why, after the first MAc pulse, does pHi in some cells return to approximately the pre-MAc1 value (Fig. 1A, red trace), whereas in other cells pHi remains lower than the pre-MAc1 value (Fig. 4A, red trace), and in still others pHi rebounds to a value greater than the pre-MAc1 pHi (Fig. 1A, gray trace)? According to the above discussion, a shift in steady-state pHi from the pre-MAc1 state to the post-MAc1 state reflects a change in the pH dependencies of JE vs. JL. In turn, these pH dependencies could be under the influence of 1) the lingering effects of MAc1 and the transition to the Ctrl solution (via pHe or [HCO3]- sensors) on signal transduction processes, and 2) the history of pHi. Figure 12 summarizes the pHi histories (recorded at the end of each of the periods) for each of the cells in the present study. Four cell types have the tendency to return to the pre-MAc value: HC neurons (Fig. 12A) and astrocytes (Fig. 12B), and MR neurons (Fig. 12C) and astrocytes (Fig. 12D). On the other hand, five cell types tend to drift to values lower than pre-MAc pHi. The cancer cell line CT26 (Fig. 12E) is unusual because we can clearly see two populations of cellular responses. In the first population of the cells with higher initial pHi values, the pHi is lower than the pre-MAc pHi. In the second population, the cells with lower initial pHi, the post-MAc pHi tends to be lower than the pre-MAc pHi.

Role of complicating circumstances. Finally, one might ask how the pHi response to MAc might depend on other parameters, such as a rise in $[K^+]_o$, a common consequence of acidosis (44). Cells subjected to an increase in $[K^+]_o$ generally will depolarize (i.e., membrane voltage will shift in a positive direction). In astrocytes, which have a high activity of the electrogenic Na/HCO3 cotransporter NBCe1, depolarization increases the NBCe1-mediated net uptake of what appears to be one Na+ ion with two HCO3- ions. The resulting increase in $J_E$ causes pHi to rise, a so-called depolarization-induced alkalinization (DIA; Refs. 28, 29, 46, 47, 59, 82)—first described in renal proximal tubule cells (77, 78). If a rise in $[K^+]_o$ , with its attendant increase $J_E$, were to occur at the same time as a MAc, we would expect the increased $[K^+]_o$ to attenuate the MAc-induced decrease in pHi, and to do so most effectively in cells with a high NBCe1 activity. One could use the above approach, together with Fig. 11, to analyze the effects of other complicating circumstances on $J_E$ and $J_L$, and thus on MAc-induced pH changes.

The pHi Response to the First MAc Challenge

Table 2 summarizes the effect of MAc on naïve cells. Eight of the ten cell types are predominantly (i.e., 60% or more) MAc-sensitive: HC neurons and astrocytes, MR neurons and astrocytes, BMDM, BMDC, Ink4-a, and XB-2. One cell type is about evenly split between sensitive and resistant: CT26. Only one cell type is prominently MAc-resistant: the colon carcinoma line CT26.

In the context of Fig. 11, MAc-sensitive cells behave as illustrated in Fig. 11C, where the projection of the dotted arrow on the x-axis represents a relatively large (ΔpHi/ΔpHio)1. MAc-resistant cells behave as illustrated in Fig. 11D, where the dotted arrow represents a relatively small (ΔpHi/ΔpHio)1. Understanding why (ΔpHi/ΔpHio)1 is larger in some cells than in others, requires knowing $J_E$ vs. pHi and $J_L$ vs. pHi relationships (as in Fig. 11, C or D) both under control and MAc conditions. Such complete data are not available for any cell type. Another issue is why—especially among MAc-sensitive cells—the initial MAc-induced acidification rate is rapid in some cases (e.g., the red and gray HC traces in Fig. 1A) but slow in others (e.g., Fig. 5A). A rapid initial decrease in pHi could be the result of

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**Table 2. Comparison among cell types of MAc1 characteristics**

<table>
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<th>Cell Type</th>
<th>Sensitive</th>
<th>Resistant</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>HCN (Fig. 1)</td>
<td>16 (64%)</td>
<td>9 (36%)</td>
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<td>HCA (Fig. 2)</td>
<td>15 (100%)</td>
<td>0 (0%)</td>
<td>15</td>
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<tr>
<td>MRN (Fig. 3)</td>
<td>24 (89%)</td>
<td>3 (11%)</td>
<td>27</td>
</tr>
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<td>MRN (Fig. 4)</td>
<td>14 (82%)</td>
<td>3 (18%)</td>
<td>17</td>
</tr>
<tr>
<td>CT26 (Fig. 5)</td>
<td>11 (27%)</td>
<td>19 (73%)</td>
<td>30</td>
</tr>
<tr>
<td>C2C12 (Fig. 6)</td>
<td>15 (54%)</td>
<td>13 (46%)</td>
<td>28</td>
</tr>
<tr>
<td>BMDM (Fig. 7)</td>
<td>22 (96%)</td>
<td>2 (8%)</td>
<td>24</td>
</tr>
<tr>
<td>BMDC (Fig. 8)</td>
<td>22 (96%)</td>
<td>1 (4%)</td>
<td>23</td>
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<tr>
<td>Ink4-a (Fig. 9)</td>
<td>23 (92%)</td>
<td>4 (8%)</td>
<td>27</td>
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<td>XB-2 (Fig. 10)</td>
<td>25 (96%)</td>
<td>1 (4%)</td>
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<tr>
<td>Total</td>
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**Table 3. Comparison among cell types of MAc2 characteristics**

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<th>Cell Type</th>
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<th>Remain Consistent</th>
<th>Deparam</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCN (Fig. 1)</td>
<td>6 (24%)</td>
<td>11 (44%)</td>
<td>8 (32%)</td>
<td>25</td>
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<tr>
<td>HCA (Fig. 2)</td>
<td>6 (40%)</td>
<td>9 (60%)</td>
<td>0 (0%)</td>
<td>15</td>
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<tr>
<td>MRN (Fig. 3)</td>
<td>7 (26%)</td>
<td>17 (65%)</td>
<td>3 (11%)</td>
<td>27</td>
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<tr>
<td>MRN (Fig. 4)</td>
<td>3 (18%)</td>
<td>11 (64%)</td>
<td>1 (4%)</td>
<td>15</td>
</tr>
<tr>
<td>CT26 (Fig. 5)</td>
<td>7 (18%)</td>
<td>24 (60%)</td>
<td>9 (22%)</td>
<td>40</td>
</tr>
<tr>
<td>C2C12 (Fig. 6)</td>
<td>13 (46%)</td>
<td>7 (25%)</td>
<td>8 (29%)</td>
<td>28</td>
</tr>
<tr>
<td>BMDM (Fig. 7)</td>
<td>8 (33%)</td>
<td>15 (63%)</td>
<td>1 (4%)</td>
<td>24</td>
</tr>
<tr>
<td>BMDC (Fig. 8)</td>
<td>10 (43%)</td>
<td>12 (53%)</td>
<td>1 (4%)</td>
<td>23</td>
</tr>
<tr>
<td>Ink4-a (Fig. 9)</td>
<td>3 (16%)</td>
<td>16 (64%)</td>
<td>5 (20%)</td>
<td>25</td>
</tr>
<tr>
<td>XB-2 (Fig. 10)</td>
<td>10 (38%)</td>
<td>8 (31%)</td>
<td>8 (31%)</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>250</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Fig. 13. Summary of mean ΔpHi responses of the 10 cell types to the first and second MAc challenges. Purple bars represent the responses of the cells to the first MAc challenge, and the green bars represent the responses to the second MAc challenge. The differences between the means for (ΔpHi1) and (ΔpHi2) are not significant ($P = 0.05$) except in C2C12 and BMDC, as it is identified by *. Error bars represent means ± SEs.
any combination of 1) a large increase in \( J_l \) (e.g., mediated by AE3 in neurons), 2) a large decrease in \( J_h \) (e.g., NBCn1 in a neuron) on the background of a large \( J_L \), 3) a large \( p_r \), or 4) a small \( \beta \).

The \( pHi \) Response to a Second MAc Challenge

Because our work is the first to study sequential MAc challenges, the \( C \) panels of Figs. 1–10 offer a novel approach for analyzing the response to a MAc-MAc challenge. Table 3 summarizes the \( pHi \) responses of cells to a second MAc, relative to the first MAc. For five cell types, \( \Delta pHi/\Delta pHo \) is predominantly consistent between MAc1 and MAc2 (i.e., in the \( C \) panels of Figs. 1–10, 60% or more of the cells fall between the curvilinear confidence limits). These cell types are HC astrocytes, MR neurons, and astrocytes, CT26, and BMDM. For three cell types, 40% or more of the cells adapt: HC astrocytes, C2C12, and BMDC. Decompensation seems to be a less common event, representing \( \sim 30\% \) of cells in only three cell types: HC neurons, C2C12, and XB-2. Note that C2C12, which is about evenly split in distribution between sensitive and resistant cells, is the cell type that is least likely to remain consistent from MAc1 to MAc2. It is interesting that Fig. 13—a more traditional approach to analyzing \( \Delta pHi/\Delta pHo \) data—shows that only two cell types (i.e., C2C12 and BMDC) whose mean \( \Delta pHi \) values differ significantly from MAc1 to MAc2. Thus, Fig. 13 provides little insight into the diversity of responses among individual cells that we see in the \( C \) panels in Figs. 1–10.

In the context of Fig. 11, adaptation would cause a cell in Fig. 11C to become more like that in \( D \), whereas decomposition would cause a cell like that in \( D \) to become more like that in \( C \). A question that arises is why should the \( pHi \) response of a cell to MAc2 ever differ from that to MAc1? During the imposition of (or recovery from) MAc1, the cell must sense a change in \( pHi \) or \( pHo \), or a parameter related to \( pHi \), such as \([HCO_3^-]\) (Fig. 11). Bouyer et al. (18) hypothesized that the sensing of \([HCO_3^-]\) is important for determining whether a naïve cell is MAc-sensitive or MAc-resistant. As to the mechanism of implementing adaptation vs. decomposition (i.e., altering the kinetics of \( J_L \) or \( J_h \)), the effect is too rapid to involve protein synthesis, and must, therefore, involve a change in the activity or number of individual transport-related proteins in the cell membrane.

Perspectives and Significance

From the perspective of a single cell, it seems that it would be advantageous to respond to MAc1 with a small \( pHi \) decrease, or to respond to MAc2 by adapting or at least maintaining a consistent \( \Delta pHi \). From the perspective of the tissue, it might be advantageous for some “altruistic” cells to respond to MAc1 by taking up relatively more net acid, thereby exhibiting a relatively large \( \Delta pHi/\Delta pHo \), so that the local fall in \( pHo \) would be relatively small, thereby protecting other “selfish” cells. Similarly, it might be advantageous for “especially altruistic” cells to decompensate. An interesting observation from the \( E \) panels of Figs. 1–10, as well as from Fig. 13 and Table 2 is that two of the cell types most likely to undergo large MAc-induced decreases in \( pHi \) are astrocytes, whereas the neurons cultured from the same brain region are likely to undergo much smaller \( pHi \) decreases.

Two other cell types likely to undergo large MAc-induced \( pHi \) decreases are BMDM and BMDC. The advantage conferred to these immune cells by high MAc sensitivity is unclear. However, it is interesting that CT26, the lone cancer cell line that we studied, is by far the most likely to be MAc-resistant. If MAc sensitivity is a more general property of immune cells, and MAc-resistance is a general property of cancer cells, it would help explain how cancer cells survive in acidic environments (61).

Conclusions

At the population level, each cell type that we examined has a characteristic distribution of \( \Delta pHi \) responses to MAc1 and MAc2 (panels \( C, D, E \) in Figs. 1–10). Nevertheless, within a particular cell type, individual cells often exhibit a wide range of responses.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.I.S. and W.F.B. conception and design of research; A.I.S. and V.A.R. performed experiments; A.I.S. analyzed data; A.I.S. interpreted results of experiments; A.I.S. prepared figures; A.I.S. drafted manuscript; A.I.S., V.A.R., and W.F.B. edited and revised manuscript; A.I.S., V.A.R., and W.F.B. approved final version of manuscript.

REFERENCES

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