pHi responses to osmotic cell shrinkage in the presence of open-system buffers

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Heming, Thomas A., Gregory Boyarsky, Divina M. Tuazon, and Akhil Bidani. pHi responses to osmotic cell shrinkage in the presence of open-system buffers. J Appl Physiol 89: 1543–1552, 2000.—Changes in plasma volume in vivo cause rapid changes in extracellular pH by altering the plasma bicarbonate concentration at a constant PCO2 (Garella S, Chang BS, and Kahn SL Kidney Int 8: 279, 1975). Few studies have examined the possibility that changes in cell volume produce comparable changes in intracellular pH (pHi). In the present study, alveolar macrophages were exposed to hyperosmotic medium in the absence or presence of the open-system buffers CO2-HCO3–, propionic acid-propionate, or NH3-NH4+. In the absence of open-system buffers, exposure to twice-normal osmolarity (2T) produced a slow cellular alkalinization [change in pHi (ΔpHi) ∼ 0.38; exponential time constant (τ) ∼ 120 s]. In the presence of 5% CO2, 2T caused a biphasic pHi response: a rapid increase (ΔpHi ∼ 0.10, τ ∼ 15 s) followed by a slower pHi increase. Identical pHi increases were produced by 2T in the presence of propionic acid (20 mM). Conversely, 2T caused a rapid pHi decrease (ΔpHi ∼ −0.21, τ ∼ 10 s) in the presence of NH3 (20 mM). Thus osmotic cell shrinkage caused rapid pHi changes of opposite direction in the presence of a weak acid buffer (contraction alkalosis with CO2 or propionic acid) vs. a weak base buffer (contraction acidosis with NH3). Graded ΔpHi were produced by varying extracellular osmolality in the presence of open-system buffers; osmolality increases of as little as 5–10% produced significant ΔpHi. The rapid pHi responses to 2T were insensitive to inhibitors of membrane H+/HCO3– exchange and, under CO2-free conditions, to inhibitors of membrane H+/H2O transport (ethylisopropylamiloride and bafilomycin A1). The results are consistent with shrinkage-induced disequilibrium in the total cellular buffer system (i.e., intrinsic buffers plus added weak acid-base buffer).

alveolar macrophage; cell volume

REGULATION OF CELL VOLUME and regulation of intracellular pH (pHi) are important for normal cell functioning (5, 19, 21). In many cells, the two regulatory processes involve common membrane ion transporters, specifically the Na+/H+ exchanger and Cl-/HCO3– exchanger (7, 11). As a result, regulation of cell volume can entail changes in pHi, whereas regulation of pHi can entail changes in cell volume (7, 11). This interdependence raises interesting questions about the hierarchy of cell volume regulation vs. pHi regulation, especially in cells (e.g., macrophages) that exhibit coordinated changes in pHi and cell volume during cell activation.

The relationship between cell volume and pHi is complicated further by the potential effects of osmotically induced changes in cell volume on the equilibrium state of intracellular buffers. Osmotically induced dilution or concentration of a closed-system cytosolic buffer (i.e., a buffer such as intracellular protein, which has a cytosolic content that is essentially fixed) should exert similar effects on the concentrations of the buffer, its conjugate species, and H+. This would produce little, if any, change in pHi. However, the situation is different for open-system cytosolic buffers (i.e., buffers that readily permeate the plasma membrane and with a total cytosolic content that can vary rapidly). For example, consider cells that are shrunken by exposure to a hyperosmotic medium in the presence of physiological concentrations of CO2-HCO3–, an open-system buffer. One expects that osmotic cell shrinkage (reflecting a net loss of cytosolic water) would produce comparable increases in the cytosolic concentrations of HCO3– and H+ with little or no change in the cytosolic PCO2 (and hence CO2 concentration). In this instance, cell shrinkage would produce an intracellular CO2-HCO3–-H+ disequilibrium, leading to the net formation of CO2 from HCO3– and H+ and an increment in pHi (i.e., an intracellular contraction alkalosis). This change in pHi is comparable to the effect on plasma pH of reducing extracellular fluid volume in vivo (10). Weak acids (e.g., CO2) and bases are, by definition, buffers. Thus one might also expect osmotic changes in cell volume to disrupt pHi in the presence of other membrane-permeant weak acids or bases (e.g., lactic acid or NH3).

We have shown previously that osmotic shrinkage of resident alveolar macrophages activates the plasma-membrane Na+/H+ exchanger and, under CO2-free conditions, causes an increase in the steady-state pHi (15). In the present study, we determined the effects of osmotic cell shrinkage on the pHi of single alveolar macrophages in the presence of CO2. Macrophage ex-

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Exposure to hyperosmotic medium in the presence of CO₂ caused a biphasic rise in pHᵢ (i.e., an initial rapid increase, followed by a slower increment in pHᵢ), whereas osmotic shrinkage in the absence of CO₂ caused a slow monotonic rise in pHᵢ. We then conducted studies with other open-system buffers, specifically a membrane-permeant weak acid (propionic acid) or weak base (NH₃), to test the hypothesis that initially rapid change in pHᵢ on osmotic shrinkage reflected a disequilibrium in the total cellular buffer system (intrinsinc buffers plus added weak acid-base buffer).

METHODS

Cell preparation. Resident alveolar macrophages were isolated from rabbit lungs, as described previously (1, 2). The cells were suspended in RPMI 1640 supplemented with 25 mM HCO₃⁻, 25,000 U/ml penicillin, and 50 µg/ml gentamicin. The cells then were seeded on 18-mm round glass coverslips at densities of ~10⁶ macrophages per coverslip and maintained overnight in an incubator (37°C, 5% CO₂).

pHᵢ measurement. The methods for measurement of pHᵢ have been described previously in detail (6). Briefly, pHᵢ was measured using a Nikon inverted microscope coupled to a Spex cation measurement system. Cells were exposed to 10 mM 2’,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM (in standard HEPES-buffered solution) at room temperature for 2–10 min. The cells then were continuously superfused with a solution pre-equilibrated at 37°C. An ~10-µm-diameter spot of light was focused on a single cell on the stage of the microscope. The fluorescence of intracellular BCECF was measured and corrected for background fluorescence unrelated to BCECF. Intracellular BCECF was calibrated to pHᵢ using the standard high K⁺-nigericin technique (6, 22).

Solutions. The standard HEPES-buffered solution contained (in mM) 135 NaCl, 5 KCl, 1 CaCl₂, 1 MgSO₄, 2 KH₂PO₄, 6 HEPES, and 5 glucose. HEPES-free CO₂-HCO₃⁻ solution was prepared by adding 25 mM NaHCO₃ (replacing NaCl and HEPES) and bubbling with 5% CO₂-balance air. Solutions with propionic acid-propionate were made by replacing 20 mM NaCl with 20 mM sodium propionate. Solutions with NH₄⁺-NH₃ were made by replacing 20 mM NaCl with 20 mM NH₄Cl. Extracellular osmolarity was routinely altered by adding mannitol (Sigma); a few experiments used sucrose (Mallinkrodt) or NaCl (Sigma). For calibration with the high K⁺-nigericin technique, the standard solution was used with Na⁺ replaced by K⁺ and 10 µM nigericin added. All solutions had a pH of 7.4 at 37°C, except for the calibrating solutions, which were titrated to different pH values using N-methyl-D-glucamine or HCl.

Statistics. The data points are presented as means ± SE. Mean differences between populations were compared with the use of the Student’s t-test. Nonlinear curve fitting was performed with the use of the Marquardt-Levenberg algorithm (NFIT, Island Products, Galveston, TX). Curve-fitted parameters are presented as means ± SD.

RESULTS

Effects of increased extracellular osmolarity in the absence of open-system buffers. Figure 1 shows the typical response of macrophage pHᵢ to osmotic cell shrinkage in the absence of open-system buffers (including CO₂-HCO₃⁻). Exposure to hyperosmotic solution [twice-normal osmolarity (2T) = ~600 mosM] caused the pHᵢ to increase slowly. The consequent change in pHᵢ (∆pHᵢ) averaged 0.38 ± 0.04 in 18 cells (Table 1). The effect was reversible. When the solution was returned to the original osmolarity, pHᵢ slowly recovered toward the initial starting value (Fig. 1). In 18 cells, the mean ∆pHᵢ after 2T removal was ~0.34 ± 0.04 (Table 1).

The time courses of the pHᵢ responses to 2T were well described by single exponentinals. For 18 cells, the mean exponential time constant (τ) was 121 s for the

Table 1. Changes in pHᵢ with doubling of extracellular osmolarity

<table>
<thead>
<tr>
<th>Experiment</th>
<th>n</th>
<th>pHᵢ before</th>
<th>pHᵢ after</th>
<th>∆pHᵢ</th>
<th>pHᵢ before</th>
<th>pHᵢ after</th>
<th>∆pHᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pHi before</td>
<td>pHi after</td>
<td></td>
<td>pHi before</td>
<td>pHi after</td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>18</td>
<td>6.92 ± 0.03</td>
<td>7.30 ± 0.04</td>
<td>0.38 ± 0.04*</td>
<td>7.29 ± 0.05</td>
<td>6.95 ± 0.05</td>
<td>-0.34 ± 0.04*</td>
</tr>
<tr>
<td>CO₂-HCO₃⁻</td>
<td>20</td>
<td>7.00 ± 0.04</td>
<td>7.10 ± 0.04</td>
<td>0.10 ± 0.01*</td>
<td>7.24 ± 0.05</td>
<td>7.10 ± 0.04</td>
<td>-0.14 ± 0.02*</td>
</tr>
<tr>
<td>Propionic acid-propionate</td>
<td>17</td>
<td>6.91 ± 0.04</td>
<td>7.03 ± 0.04</td>
<td>0.12 ± 0.01*</td>
<td>7.07 ± 0.04</td>
<td>6.96 ± 0.03</td>
<td>-0.10 ± 0.01*</td>
</tr>
<tr>
<td>+15 µM EIPA</td>
<td>6</td>
<td>6.93 ± 0.09</td>
<td>7.05 ± 0.09</td>
<td>0.13 ± 0.02*</td>
<td>7.08 ± 0.08</td>
<td>6.98 ± 0.07</td>
<td>-0.10 ± 0.04*</td>
</tr>
<tr>
<td>+5 µM bafilomycin A₁</td>
<td>7</td>
<td>6.72 ± 0.05</td>
<td>6.81 ± 0.05</td>
<td>0.09 ± 0.03*</td>
<td>6.82 ± 0.05</td>
<td>6.74 ± 0.05</td>
<td>-0.08 ± 0.03*</td>
</tr>
<tr>
<td>NH₄⁺-NH₃ + 300 mM mannitol</td>
<td>28</td>
<td>7.02 ± 0.02</td>
<td>6.81 ± 0.03</td>
<td>-0.21 ± 0.01*</td>
<td>6.80 ± 0.03</td>
<td>7.01 ± 0.02</td>
<td>0.21 ± 0.01*</td>
</tr>
<tr>
<td>+300 mM sucrose</td>
<td>6</td>
<td>7.00 ± 0.06</td>
<td>6.79 ± 0.06</td>
<td>-0.21 ± 0.01*</td>
<td>6.78 ± 0.06</td>
<td>6.99 ± 0.06</td>
<td>0.20 ± 0.01*</td>
</tr>
<tr>
<td>+150 mM NaCl</td>
<td>3</td>
<td>7.02 ± 0.08</td>
<td>6.84 ± 0.09</td>
<td>-0.19 ± 0.01*</td>
<td>6.84 ± 0.10</td>
<td>7.04 ± 0.09</td>
<td>0.20 ± 0.01*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of cells. Data for CO₂-HCO₃⁻ are for initial, rapid intracellular pH (pHᵢ) responses. EIPA, ethylisopropylamiloride. *Significantly different from zero, P < 0.0001.
On phase and 163 s for the Off phase (Table 2). The magnitudes of the pH$_i$ responses to 2T ($\Delta$pH$_i$ during On and Off phases) were not significantly dependent on the starting pH$_i$ (i.e., pH$_i$ at which 2T was applied or removed). Figure 2 shows the $\Delta$pH$_i$ vs. starting pH$_i$. The slopes of lines fitted to the data were not significantly different from zero ($P = 0.27$ for On data and 0.08 for Off data).

Effects of increased extracellular osmolarity in the presence of CO$_2$. In the presence of CO$_2$, exposure to 2T caused a biphasic rise in pH$_i$. There was a rapid initial alkalization from a baseline value of 7.00 ± 0.04 to a shoulder value of 7.10 ± 0.04, followed by a slower rise over the course of >5 min to a new steady-state value of 7.24 ± 0.05 ($n = 20$ cells) (Fig. 3, Table 1). In 20 cells, the mean $\Delta$pH$_i$ was 0.10 ± 0.01 for the initial rapid portion of the On phase and 0.14 ± 0.04 for the subsequent slower portion. The Off response was monitored for only 1–2 min (Fig. 3). Macrophage pH$_i$ declined rapidly during that period of time to an apparently stable value ($\Delta$pH$_i$ ≈ 0.14; Table 1, Fig. 3).

The pH$_i$ responses to 2T in the presence of CO$_2$ occurred more rapidly than those in the absence of CO$_2$. The mean $\tau$ was 15 s for the initial On response in CO$_2$ and 24 s for the Off response (Table 2), considerably shorter than those in CO$_2$-free solution. Furthermore, in contrast to the case in CO$_2$-free solution, the rapid $\Delta$pH$_i$ produced by 2T (On and Off phases) in CO$_2$ was dependent on the starting pH$_i$ value, increasing with increments in the starting pH$_i$ (Fig. 4).

Effects of increased extracellular osmolarity in the presence of propionic acid. If the rapid pH$_i$ response to 2T in CO$_2$ reflected a disequilibrium in the total cellular buffer system, then other membrane-permeable weak acids would be expected to mimic the effects observed in CO$_2$. Furthermore, alveolar macrophages have HCO$_3^-$-dependent plasmalemma acid-base transporters (3) that could be operating during the osmotic challenge in CO$_2$. Propionic acid was selected as a membrane-permeant weak acid (4) that is unlikely to support HCO$_3^-$-dependent transport.

Figure 5 illustrates an experiment in which a cell was exposed to 20 mM propionic acid-propionate in the nominal absence of CO$_2$. The initial exposure to propionic acid caused a rapid cell acidification consistent with the entry of propionic acid, a fraction of which dissociated to form intracellular propionate and H$^+$ (4).

### Table 2. Exponential rate constant for pH$_i$ responses to doubling of extracellular osmolarity

<table>
<thead>
<tr>
<th>Experiment</th>
<th>n</th>
<th>On</th>
<th>Off</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>18</td>
<td>121 ± 16</td>
<td>163 ± 21</td>
</tr>
<tr>
<td>CO$_2$-HCO$_3^-$</td>
<td>20</td>
<td>15.4 ± 1.5</td>
<td>24.3 ± 2.3</td>
</tr>
<tr>
<td>Propionic acid-propionate</td>
<td>17</td>
<td>12.0 ± 0.7</td>
<td>16.2 ± 1.5</td>
</tr>
<tr>
<td>+15 μM EIPA</td>
<td>6</td>
<td>10.7 ± 1.6</td>
<td>16.7 ± 1.6</td>
</tr>
<tr>
<td>+5 μM bafilomycin A$_1$</td>
<td>7</td>
<td>11.0 ± 1.5</td>
<td>16.0 ± 2.3</td>
</tr>
<tr>
<td>NH$_4^+$/NH$_3$</td>
<td>28</td>
<td>10.4 ± 0.7</td>
<td>15.0 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of cells. $\tau$, Rate constant.
Fig. 4. pH_i dependence of responses to 2T in CO2-HCO3\(^{-}\) solution for 20 cells. •, \(\Delta\text{pH}_i\) produced by addition of 300 mM mannitol (On phase), as a function of the starting pH_i. The best-fit line to the data (solid line) had slope of 0.21 ± 0.05 and y-intercept of \(-1.34 \pm 0.21\) (±SD). Slope was significantly different from zero \((P = 0.0014)\) with \(R^2 = 0.44\). △, \(\Delta\text{pH}_i\) produced by return to normal osmolarity (Off phase), plotted with a positive sign. Best-fit line (dashed line) had slope of 0.25 ± 0.05 and y-intercept of \(-1.69 \pm 0.35\) (±SD). Slope was significantly different from zero \((P < 0.0001)\) with \(R^2 = 0.62\).

After the rapid acidification, pH_i recovered to a value near the original baseline, presumably through the actions of the plasmalemma H\(^+\) extruders. The cell was then briefly exposed to solutions of altered extracellular osmolarity, ranging from 110 to 300% of normal (1.1T to 3T). Changes in extracellular osmolarity under these conditions produced rapid, reversible changes in pH_i.

The pH_i responses to osmotic cell shrinkage in propionic acid were quantitatively identical to those in CO\(_2\). The magnitudes of the responses to 2T in propionic acid \((\Delta\text{pH}_i \approx 0.12\) during the On phase and \(-0.10\) during the Off phase) were not significantly different from those in CO\(_2\) (Table 1). Furthermore, the \(\Delta\text{pH}_i\) produced by 2T in propionic acid was sensitive to the starting pH_i value (data not shown). The regression slopes (±SD) of \(\Delta\text{pH}_i\) vs. starting pH_i were 0.27 ± 0.07 \((R^2 = 0.67)\) for the On phase and 0.19 ± 0.05 \((R^2 = 0.67)\) for the Off phase (each slope was significantly different from zero). The regression slopes in propionic acid were not significantly different from those in CO\(_2\) (see legend to Fig. 4). Finally, the kinetics of the pH_i responses to 2T in propionic acid \((\gamma = 12\) s during the On phase and 16 s during the Off phase) were not significantly different from those in CO\(_2\) (Table 2). These data indicate that the rapid pH_i responses to 2T were not unique to CO\(_2\)-HCO\(_3^-\) solutions.

Table 3 lists the mean \(\Delta\text{pH}_i\) observed for different sizes of osmolarity pulses in propionic acid. Statistically significant changes in pH_i were observed with as little as a 10% increase in external osmolarity. The \(\Delta\text{pH}_i\) varied directly with the magnitude of the osmotic challenge. Figure 6 plots the mean \(\Delta\text{pH}_i\) of 10 cells (On and Off phases; Off data plotted with a positive sign) as a function of the relative extracellular osmolarity. The curve in Fig. 6 is a fit of the data to the equation, \(A + B\log_{10}s\), where \(s\) represents an ideal shrinkage factor (i.e., osmolarity relative to 300 mosM). The best-fit relationship indicated that \(\Delta\text{pH}_i\) varied as 0.35 log_{10}s (see legend to Fig. 6).

It is unlikely that the rapid pH_i responses to hyperosmotic medium containing CO\(_2\) or propionic acid reflected shrinkage-induced activation of plasmalemma H\(^+\) extruders. Published studies indicate that there are two major contributors to plasmalemma H\(^+\) extrusion in alveolar macrophages: the V-type H\(^+\) pump and the Na\(^+\)/H\(^+\) exchanger (1). The pH_i responses to 2T in propionic acid were insensitive to 15 mM ethylisopropylamiloride (EIPA; a selective inhibitor of Na\(^+\)/H\(^+\) exchange) or 5 mM bafilomycin A\(_1\) (a selective inhibitor of V-type H\(^+\) pumps). The magnitudes of the pH_i responses to 2T \((\Delta\text{pH}_i\) during the On and Off phases) were unaffected by the transport inhibitors (Table 1).

Similarly, the mean \(\tau\) of the 2T responses (On and Off phases) were unaffected by EIPA or bafilomycin A\(_1\) (Table 2).

Effects of increased extracellular osmolarity in the presence of NH\(_3\). If the rapid pH_i response in the presence of a weak acid was due to changes in the equilibrium characteristics of the total cellular buffer system, then a different response should be observed in the presence of a membrane-permeant weak base. Now the total cellular buffer system consists of a weak base-conjugate acid buffer pair (e.g., NH\(_3\)-NH\(_4\)\(^+\)) plus the intrinsic buffers. Figure 7A illustrates an experiment in which a cell was exposed to 20 mM NH\(_3\)-NH\(_4\)\(^+\) in the nominal absence of CO\(_2\). This solution caused a rapid alkalinization, consistent with the entry of NH\(_3\), a fraction of which combined with intracellular H\(^+\) to form NH\(_4\)\(^+\). The pH_i subsequently recovered to near the original baseline value, reflecting a compensatory net...
H⁺ loading of the cell. The cell was then briefly exposed to NH₃ solutions of altered osmolarity. In marked contrast to the results with a weak acid buffer (CO₂ or propionic acid), increases in extracellular osmolarity caused cellular acidifications in the presence of NH₃. In the case with 2T, ΔpHi was 0.21 for the On response in NH₃ and 0.21 for the Off response (Table 1).

We visually confirmed that the hyperosmotic challenges produced the intended cell shrinkage. Using a reticle in the microscope eyepiece, we observed comparable decreases in cell diameter during exposures to hyperosmotic medium with or without CO₂, propionic acid, or NH₃. We also checked that similar pHᵢ responses were observed when the osmolarity of the NH₃ solution was altered with 300 mM sucrose or 150 mM NaCl instead of 300 mM mannitol (Fig. 7B, Table 1).

The pHᵢ responses to 2T in NH₃ were well fit by single exponentials (data not shown). The mean τ was 10 s for the On phase and 15 s for the Off phase (Table 2), similar to the results with CO₂ and propionic acid. Figure 8 illustrates the pHᵢ dependence of ΔpHi produced by 2T in NH₃. Neither On nor Off data showed a demonstrable dependence on starting pHᵢ, unlike the case for weak acids. The slopes of lines fitted to the data were not significantly different from zero (P > 0.0001). Figure 9 plots the mean ΔpHi during the On and Off phases in 24 cells (On data plotted with positive sign) as a function of the relative extracellular osmolarity. The absolute ΔpHi increased with increments in relative osmolarity between 1.05T and 3T. The curve in Fig. 9 is a fit of the data to the equation A + B log₁₀s. The observed ΔpHi values are means ± SE; n = 20 cells. *Significantly different from zero, P < 0.0001. †Significantly different from zero, P < 0.01.

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### Table 3. Changes in pHᵢ with variations in external extracellular osmolarity in the presence of propionic acid

<table>
<thead>
<tr>
<th>Relative Osmolarity</th>
<th>pHi before</th>
<th>pHi after</th>
<th>ΔpHi</th>
<th>pHi before</th>
<th>pHi after</th>
<th>ΔpHi</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>6.94 ± 0.02</td>
<td>6.96 ± 0.03</td>
<td>0.02 ± 0.004†</td>
<td>6.97 ± 0.03</td>
<td>6.95 ± 0.02</td>
<td>−0.02 ± 0.005†</td>
</tr>
<tr>
<td>1.25</td>
<td>6.96 ± 0.02</td>
<td>7.01 ± 0.03</td>
<td>0.05 ± 0.006‡</td>
<td>7.02 ± 0.03</td>
<td>6.97 ± 0.03</td>
<td>−0.05 ± 0.004*</td>
</tr>
<tr>
<td>1.5</td>
<td>6.97 ± 0.03</td>
<td>7.04 ± 0.03</td>
<td>0.08 ± 0.007‡</td>
<td>7.06 ± 0.04</td>
<td>6.99 ± 0.03</td>
<td>−0.07 ± 0.007‡</td>
</tr>
<tr>
<td>2.0</td>
<td>6.98 ± 0.04</td>
<td>7.10 ± 0.05</td>
<td>0.12 ± 0.01‡</td>
<td>7.12 ± 0.05</td>
<td>7.01 ± 0.04</td>
<td>−0.11 ± 0.01‡</td>
</tr>
<tr>
<td>3.0</td>
<td>7.01 ± 0.04</td>
<td>7.19 ± 0.06</td>
<td>0.18 ± 0.02‡</td>
<td>7.21 ± 0.06</td>
<td>7.03 ± 0.04</td>
<td>−0.17 ± 0.02‡</td>
</tr>
</tbody>
</table>

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Fig. 6. Effect of magnitude of osmotic challenge on ΔpHi in the presence of propionic acid-propionate. The mean ΔpHi values produced during both the On and Off phases (Off plotted with positive sign) for 20 cells are shown as a function of the relative osmolarity. The absolute ΔpHi increased with increments in relative osmolarity between 1.05T and 3T. The curve in Fig. 7 is a fit of the data to the equation A + B log₁₀s. The observed ΔpHi values are means ± SE; n = 20 cells. *Significantly different from zero, P < 0.0001. †Significantly different from zero, P < 0.01.

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Fig. 7. Representative effects on pHᵢ of osmotic challenges in the presence of NH₃-NH₄⁺. A: initial exposure to 20 mM total NH₃-NH₄⁺ produced a rapid alkalinization, as NH₃ entered the cell and combined with H⁺ to form NH₄⁺. In the continued presence of ammonium, the pHᵢ recovered back toward the starting value over a period of 10–15 min. The cell was then pulsed with differing amounts of added mannitol to alter extracellular osmolarity. B: similar responses to 2T in NH₃ were produced when extracellular osmolarity was altered using 300 mM mannitol, 300 mM sucrose, or 150 mM NaCl.
base buffer (NH$_3$) caused the pH$_i$ to decrease rapidly in the presence of a weak acid; it rapidly permeates the macrophage plasma membrane (4) and is unlikely to support plasmalemma acid-base transport (4) and is unlikely to support plasmalemma acid-base transport.

In the presence of weak acid buffers (CO$_2$ or propionic acid), exposure to 2T caused a rapid, reversible alkalization, the size of which was dependent on the starting pH$_i$ (see Figs. 3 and 4). Varying the magnitude of the osmotic challenge produced corresponding changes in the ΔpH$_i$ (see Fig. 6). This “intracellular contraction alkalosis” is comparable to the plasma acid-base disturbance described in vivo, wherein contraction alkalosis associated with reducing HCO$_3^-$ at a constant CO$_2$, with intrinsic (i.e., nonbicarbonate) buffers playing an important role in determining the size of the extracellular pH change (10). Conversely, increasing extracellular osmolarity in the presence of a weak base buffer (NH$_3$) caused the pH$_i$ to decrease rapidly (see Fig. 7), that is, an “intracellular contraction acidosis”. Changes in pH$_i$ in the presence of NH$_3$ were graded with the size of the osmotic challenge (see Fig. 9) but did not correlate with the starting pH$_i$ (see Fig. 8). In absolute terms, the ΔpH$_i$ produced by osmotic cell shrinkage in NH$_3$ was larger than that observed in CO$_2$ or propionic acid.

The appendix presents a simple qualitative analysis of the expected ΔpH$_i$ elicited by cell shrinkage in the presence or absence of membrane-permeant weak acid-base buffers (i.e., open-system buffers). A number of assumptions and simplifications are included in the analysis: ideality of the change in cell volume; no cell volume recovery during the pH$_i$ transients; high relative membrane permeabilities to H$_2$O, CO$_2$, propionic acid, and NH$_3$; and low relative membrane permeabilities to H$^+$, HCO$_3^-$, propionate, and NH$_4^+$. The analysis also ignores possible nonideality in changes in intracellular constituents during cell volume changes (e.g., changes in intracellular ionic strength), the relative rates of buffering reactions including catalyzed reactions (e.g., CO$_2$ hydration/dehydration), the dependence of intrinsic (nonbicarbonate) buffering power (β$_{int}$) on pH$_i$, the changes in β$_{int}$ produced by changes in cell volume, and the rates of plasmalemma acid-base transport that could be altered during the pH$_i$ transients or by changes in cell volume directly.

Some of these issues have been addressed previously with alveolar macrophages (15). In suspensions of alveolar macrophages under CO$_2$-free conditions, addition of 320 mM sucrose to the external solution produced an approximate halving of cell volume, and macrophage volume remained at the smaller level for (at least) 10 min. The shrinkage was associated with an approximate doubling of β$_{int}$ at a given pH$_i$. Na$^+$/H$^+$ exchange (amiloride-sensitive and Na$^+$-dependent recovery from acid loads) was activated by osmotic cell shrinkage. This is consistent with the slow but large pH$_i$ increase observed in the present study in CO$_2$-free solution without added weak acid-base (see Fig. 1). In the presence of CO$_2$, additional acid-base transporters (e.g., Cl$^-$/HCO$_3^-$ exchangers) could contribute to changes in pH$_i$ after cell volume perturbations. For this reason, we chose propionic acid as a model weak acid; it rapidly permeates the macrophage plasma membrane (4) and is unlikely to support plasmalemma HCO$_3^-$-dependent transport. Osmotic cell shrinkage produced similar rapid pH$_i$ transients in the presence of NH$_3$. The mean ΔpH$_i$ values produced during both the On and Off phases (On plotted with positive sign) for 50 cells are shown as a function of the relative extracellular osmolarity. The curve is a fit of the data to A + B log$_{10}$x. The best-fit values (±SD) were $-0.011 ± 0.003$ for A and $0.74 ± 0.01$ for B.

![Fig. 8. pH$_i$ dependence of responses to 2T in the presence of NH$_3$-NH$_4^+$ for 28 cells. □ ΔpH$_i$ produced by exposure to 2T (On phase) was plotted with a positive sign, as a function of starting pH$_i$. △ ΔpH$_i$ produced by return to normal osmolarity (Off phase).](http://jap.physiology.org/)

![Fig. 9. Effect of magnitude of osmotic challenge on ΔpH$_i$ in the presence of NH$_3$-NH$_4^+$. The mean ΔpH$_i$ values produced during both the On and Off phases (On plotted with positive sign) for 50 cells are shown as a function of the relative extracellular osmolarity. The curve is a fit of the data to A + B log$_{10}$x. The best-fit values (±SD) were $-0.011 ± 0.003$ for A and $0.74 ± 0.01$ for B.](http://jap.physiology.org/)
of CO₂ or propionic acid. Thus it is unlikely that the responses were mediated via Cl⁻/HCO₃⁻ exchange. The rapid pH responses in propionic acid were insensitive to EIPA or baflomycin A₁. Hence, it also is unlikely that the responses were mediated via Na⁺/H⁺ exchange or the H⁺ pump. Furthermore, osmotic challenges caused pHᵢ to change in opposite directions in the presence of a weak acid buffer (i.e., contraction alkalosis) vs. a weak base buffer (i.e., contraction acidosis). It is difficult to imagine how shrinkage-activated acid-base transport could produce such divergent pHᵢ responses. These findings suggest that the rapid pHᵢ responses were not dependent on the effects of osmotic cell shrinkage on membrane ion transporters. The simplest explanation is that the rapid pHᵢ responses reflected disequilibria in the total cellular buffer system due to osmotic changes in cell volume.

This interpretation of the data is consistent with all salient features of the pHᵢ responses. As shown in the appendix, a shrinkage-induced disequilibrium in the total cellular buffer system is predicted to produce a cytosolic alkalization in the presence of a weak acid buffer (e.g., CO₂ or propionic acid) and a cytosolic acidification in the presence of a weak base buffer (e.g., NH₃). Such paradoxical changes in pHᵢ were observed experimentally. The analysis in the appendix also predicts that the magnitude of the pHᵢ response (ΔpHᵢ) should be dependent on the size of the osmotic challenge (s) and βᵢnt, reaching a maximum value of \(±\log_{10}^s\) when βᵢnt is zero (direction of change in pH determined by presence of weak acid vs. weak base). The observed ΔpHᵢ values varied directly with s (see Figs. 6 and 9) and were 35–74% of the predicted maximum, in keeping with the presence of intrinsic buffers in alveolar macrophages (15).

If cell shrinkage induced a disequilibrium in the total cellular buffer system, then reequilibration should be determined by competing reactions between intrinsic buffers and the added weak acid-base buffer (i.e., CO₂-HCO₃⁻, propionic acid-propionate, or NH₃-NH₄⁺). Although the kinetics of these competing reactions are not known, we can compare the relative contribution of each reaction to the final equilibrium state by examining the buffering powers of the individual buffers. Alveolar macrophages in isotonic solution have an βᵢnt of ~20 mM/pH at pH 7.1 (15). At the same pHᵢ in the presence of 5% CO₂, the intracellular HCO₃⁻ concentration is calculated to be ~12 mM, yielding an intracellular bicarbonate buffering power (βbicarb) of ~28 mM/pH (assuming the apparent CO₂-HCO₃⁻ equilibrium constant and CO₂ concentration are the same inside and outside the cell and that CO₂-HCO₃⁻ behaves as an open-system buffer such that βbicarb is 2.303 times the concentration of HCO₃⁻). In the presence of 20 mM propionic acid-propionate or NH₃-NH₄⁺, the intracellular propionate buffering power (βprop) is calculated to be ~23 mM/pH and the intracellular ammonium buffering power (βamm) to be ~92 mM/pH (using similar assumptions as above). The absolute ΔpHᵢ elicited by osmotic cell shrinkage was larger in the presence of NH₃ than weak acids, whereas similar ΔpHᵢ values were detected in experiments with CO₂ and propionic acid, in keeping with βamm > βbicarb > βprop.

The approach of comparing buffering powers can also be used to explain the different sensitivities of ΔpHᵢ to starting pHᵢ in experiments with weak acids vs. weak bases. βbicarb, βprop, βamm, and βᵢnt are all sensitive to pH. In experiments with CO₂ or propionic acid, the conjugate base concentration (and hence, βbicarb or βprop) increased with increments in pHᵢ, following the law of mass action. In contrast, the βᵢnt of alveolar macrophages decreased at higher pHᵢ values (15). Thus the relationship between the buffering powers of intrinsic buffers vs. the added weak acid buffer (hence, the magnitude of the pHᵢ responses to osmotic cell shrinkage) should be relatively sensitive to starting pHᵢ in the presence of CO₂ or propionic acid. On the other hand, in experiments with NH₃, the conjugate acid concentration (hence, βamm) decreased with increments in pHᵢ along with the pH-induced decrements in βᵢnt. Thus the relationship between the buffering powers of intrinsic buffers vs. the added weak base buffer (hence, ΔpHᵢ) should be relatively insensitive to starting pHᵢ in the presence of NH₃. In keeping with this interpretation of the data, ΔpHᵢ was dependent on the starting pHᵢ value in weak acid experiments but was not correlated with the starting pHᵢ in NH₃ experiments.

The present findings are probably not unique to alveolar macrophages. We have previously observed rapid changes in pHᵢ during changes in extracellular osmolarity in the presence of CO₂-HCO₃⁻ in renal mesangial cells (Boyarsky, unpublished results). Conditions exist in vivo in which cells are exposed to diverse osmotic microenvironments in the presence of multiple open-system buffers (e.g., CO₂ and NH₃), particularly in the kidney. The effects on pHᵢ of alterations in extracellular osmolarity in a multiple buffer system are unclear. Furthermore, although the present studies focussed on the responses to hyperosmotic cell shrinkage, the pHᵢ effects were reversed on return to the original tonicity (with consequent cell swelling to recover macrophage volume). This observation suggests that “expansion” acidoses and alkaloses might occur during hypotonic cell swelling in the presence of weak acids and weak bases, respectively.

The experimental cells were highly sensitive to increases in extracellular osmolarity. Statistically significant changes in pHᵢ occurred with only 5–10% increases in extracellular osmolarity (see Figs. 6 and 9). This raises the possibility that the responses have physiological/pathophysiological relevance. A large body of evidence indicates that recovery of cell volume following osmotic cell shrinkage involves the activation of plasma membrane acid-base transporters and often is accompanied by transporter-mediated changes in pHᵢ with a time course of several minutes (8, 9, 12–15, 18, 20). The present results show that cell shrinkage under physiological conditions (5% CO₂) can produce rapid increases in pHᵢ with a time course of several seconds, most likely due to disequilibria in the total cellular buffering system. The duration of these pHᵢ shifts was
not determined. Nonetheless, such rapid responses will define the initial changes in pH$_i$ that occur after osmotic cell shrinkage under physiological conditions. It is worthwhile to consider, therefore, that rapid buffer-associated pH$_i$ changes are involved in the allosterical activation of the acid-base transporters that play a role in the subsequent recovery of cell volume.

Regardless of the specific mechanisms involved (transporter-mediated or buffer-associated), any change in pH$_i$ that accompanies changes in cell volume will effectively reset the relationship between pH$_i$ and extracellular pH. As such, pH$_i$ responses to cell volume changes will influence the functioning of physiological systems that track extracellular acid-base status. For example, in animal studies, Kasserra and co-workers (16, 17) found that intravenous infusion of a hyperosmotic solution caused a prolonged blood acidosis (dilution acidosis) and hypercarbia without eliciting a compensatory increase in ventilation. The hyperosmotic challenge also produced alkaline shifts in the tissue pH of skeletal muscle (i.e., a tissue contraction alkalosis).

Assuming that a similar pH shift occurred in chemoreceptive cells (intracellular alkalosis), the authors suggested that aniosmotic-induced changes in pH$_i$ caused the chemoreceptors to mistrack blood acid-base status, and this impaired respiratory compensation of the acid-base disturbance. The present data suggest that uncoupling of intracellular and extracellular pH can have a rapid onset, before the involvement of volume-activated plasmalemma acid-base transporters.

**APPENDIX: ANALYSIS OF pH$_i$ CHANGES PRODUCED BY CHANGES IN CELL VOLUME**

The assumptions and simplifications of this analysis are outlined above. Volume changes are expected to produce little change in pH$_i$ in the presence of a closed-system buffer (i.e., a situation in which the total amount of cytosolic buffer remains constant). In this case, we assume that equilibrium of the buffer (Buf) is described as follows (subscript 1 denotes initial conditions)

$$Buf + H^+ \leftrightarrow HBuf^+$$

where $K_{eq}$ is the equilibrium constant. If the cell volume is shrunk by a factor s, then, initially, the concentrations of all constituents increase by the same factor (subscript 2). Now, the buffer is no longer in equilibrium (dropping concentration brackets and superscripts)

$$\frac{Buf_2 H_2}{HBuf_2} = \frac{(sBuf_1)(sH_1)}{(sHBuf_1)} = sK_{eq}$$

and the following reaction occurs until reestablishment of equilibrium (subscript 3)

$$Buf + H \rightarrow HBuf$$

The extent of an equilibrating reaction ($x$) is given by the following relationships

$$Buf_x = Buf_x - x \quad HBuf_x = HBuf_x + x \quad H_3 = H_2 - x$$

Equation 4 assumes that there is no other buffer present, which obligates a 1:1 consumption of $H^+$ along with Buf in producing HBuf. One approach to understanding why the pH returns to normal (i.e., $H_3 \approx H_1$) is to plug into Eq. 3 from Eqs. 1, 2, and 4

$$\frac{(sBuf_1 - x)(sH_1 - x)}{(sHBuf_1 + x)} = K_{eq}$$

Rearrangement yields

$$\frac{(sH_1 - x)}{K_{eq} HBuf_1} = \frac{(sBuf_1 - x)}{Buf_1}$$

Ignoring the $x$ terms on the right (because $x$ is only nM compared with the mM of Buf and HBuf) and canceling the $s$ terms leaves the following

$$\frac{(sH_1 - x)}{(sBuf_1 - x)} = \frac{K_{eq} HBuf_1}{Buf_1} = H_1$$

The last equation results from the original equilibrium definition (Eq. 1). Thus, by rearrangement

$$x = (s - 1)H_1$$

Plugging into the value for $H_3$ (Eq. 4) and $H_2$ (Eq. 2), one obtains the following

$$H_3 = H_4 - x = sH_1 - (s - 1)H_1 = H_1$$

**Rapid Loss of Cellular Water in the Presence of a Weak Acid**

The response is different in the presence of an open-system buffer (i.e., a situation in which the total amount of cytosolic buffer can vary). We will assume that CO$_2$-HCO$_3^-$ is present and that CO$_2$ rapidly equilibrates across the cell membrane. Then, the CO$_2$-HCO$_3^-$ buffer pair obey the following equilibrium

$$H^+ + HCO_3^- \leftrightarrow CO_2 + H_2O$$

$$K_{eq} = \frac{[H^+][HCO_3^-]}{[CO_2][H_2O]}$$

Assuming there is a pure loss of water producing cell shrinkage by $s$, we can consider two extreme cases. In the first case, there are no intrinsic buffers ($\beta_{int} = 0$). The concentrations of HCO$_3^-$ and $H^+$ then change by $s$. CO$_2$ freely permeates the plasma membrane and, consequently, remains constant. A disequilibrium is created

$$HCO_3^{-} = sHCO_3^{-}$$

$$CO_2_{s=2} = CO_2_{s=1}$$

$$H_2 = sH_1$$

The following reaction occurs until a new equilibrium is reached (subscript 3)

$$H^+ + HCO_3^- \rightarrow CO_2 (\uparrow)$$

$$\frac{(H_3)(HCO_3_{s=3})}{(CO_2_{s=3})} = K'_{eq} = \frac{(H_2)(HCO_3_{s=2})}{(CO_2_{s=2})} = sK_{eq}$$

Inasmuch as $\beta_{int} = 0$, the $H^+$ concentration decreases with little change in the HCO$_3^-$ concentration because stoichiometric coupling of the changes in $H^+$ (nM) and HCO$_3^-$ (mM) force reequilibration with nanomolar changes in each. Ignor-
ing the nanomolar change in HCO$_3^-$ concentration, Eq. 12 can be rearranged as follows

$$\text{(H}_3\text{)} = \frac{(HCO_3^-)}{(H)_{\text{equ}}}(H)_{\text{equ}}$$

$$\text{HCO}_3^- = HCO_3^- + sHCO_3^-$$

$$\text{H}_1 = (1/s)H_1$$

Thus, with $\beta_{\text{int}} = 0$, the H$^+$ concentration decreases by a factor 1/s or pH increases by log$_{10}$s. In the second extreme case, $\beta_{\text{int}}$ is assumed to be infinite. Here, we expect to see no change in the H$^+$ concentration but large changes in the HCO$_3^-$ concentration. Shrinkage produces changes in the HCO$_3^-$ concentration, which result in disequilibrium

$$\text{HCO}_3^2 = sHCO_3^-1$$

$$\text{CO}_{2} = \frac{H_2}{(H)}(\text{CO}_3)$$

Reestablishment of equilibrium is again accomplished by the reaction in Eq. 12

$$\text{H}_3 = \frac{(HCO_3^-)}{(H)}(H)_{\text{equ}}$$

$$\text{HCO}_3^- = HCO_3^- + H_1$$

Thus osmotic cell shrinkage in the presence of a weak acid causes a $\Delta$H$_i$, which ranges in magnitude from zero (infinite $\beta_{\text{int}}$) to a maximum of log$_{10}$s (infinite $\beta_{\text{int}}$). In experiments with CO$_2$ and propionic acid, hyperosmotic challenges produced alkalinizations equivalent to 0.35 log$_{10}$s (i.e., 35% of the expected maximum), consistent with the expected $\beta_{\text{int}}$ of alveolar macrophages (15).

**Rapid Loss of Cellular Water in the Presence of a Weak Base**

We will assume that NH$_3$-NH$_4^+$ is present and that NH$_3$ rapidly equilibrates across the cell membrane. Then, the NH$_3$-NH$_4^+$ buffer pair obeys the following equilibrium

$$\text{H}^+ + \text{NH}_3 \leftrightarrow \text{NH}_4^+ \quad K_{\text{eq}} = \frac{[\text{H}^+][\text{NH}_3]}{[\text{NH}_4^+]}$$

We again consider the cell loses water to undergo shrinkage by s and that this produces proportional increases in all constituents except NH$_3$ (which freely permeates the cell membrane)

$$\text{NH}_3 = \text{sNH}_3^-$

$$\text{H}_2 = \text{sH}_1$$

$$\text{NH}_3 = \text{sNH}_3$$

$$\text{H}_3 = \text{sH}_1$$

$$\text{HCO}_3^- = \text{sHCO}_3^-$$

During osmotic cell shrinkage, the ammonium buffer pair remains in equilibrium with the increase in H$^+$ proportionally balanced by the increase in NH$_4^-$. Thus, in the extreme case with $\beta_{\text{int}} = 0$, the H$^+$ concentration remains elevated at s[H$^+_i$] and pH changes by $-\log_{10}s$, that is, pH decreases. On the other hand, in the presence of intrinsic buffers (i.e., $\beta_{\text{int}} > 0$), although the ammonium buffer pair remains in equilibrium during osmotic cell shrinkage, the intrinsic buffers do not

$$\text{Buf}_2 = s\text{Buf}_1$$

Thus cell shrinkage in the presence of a weak base causes a decrease in pH that ranges in magnitude from $-\log_{10}s$ (infinite $\beta_{\text{int}}$) to zero (infinite $\beta_{\text{int}}$). In experiments with NH$_3$, hyperosmotic challenges produced acidifications equivalent to $-0.74\log_{10}s$ (i.e., 74% of the expected maximum), consistent with the modest $\beta_{\text{int}}$ of alveolar macrophages (15).

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