Intracellular pH in Human Arterial Smooth Muscle

Regulation by Na\(^+\)/H\(^+\) Exchange and a Novel 5- (N-Ethyl-N-isopropyl)amiloride–Sensitive Na\(^+\)- and HCO\(_3\)^–-Dependent Mechanism

Craig B. Neylon, Peter J. Little, Edward J. Cragoe Jr., and Alex Bobik

We investigated in a physiological salt solution (PSS) containing HCO\(_3\)^– the intracellular pH (pH\(_i\)) regulating mechanisms in smooth muscle cells cultured from human internal mammary arteries, using the pH-sensitive dye 2',7'--bis(2-carboxyethyl)-5(6)--carboxyfluorescein (BCECF) and \(^{22}\)Na\(^+\) influx rates. The recovery of pH\(_i\) from an equivalent intracellular acidosis was more rapid when the cells were incubated in CO\(_2\)/HCO\(_3\)^–-buffered PSS than in HEPES-buffered PSS. Recovery of pH\(_i\) was dependent on extracellular Na\(^+\) (K\(_m\), 13.1 mM); however, it was not attenuated by 4-acetamido-4'--isothiocyanatostilbene-2,2'--disulfonic acid (SITS), indicating the absence of SITS-sensitive HCO\(_3\)^--dependent mechanisms. Recovery instead appeared mostly dependent on processes sensitive to 5- (N-ethyl-N-isopropyl)amiloride (EIPA), indicating the involvement of Na\(^+\)/H\(^+\) exchange and a previously undescribed EIPA-sensitive Na\(^+\)- and HCO\(_3\)^--dependent mechanism. Differentiation between this HCO\(_3\)^--dependent mechanism and Na\(^+\)/H\(^+\) exchange was achieved after depletion of cellular ATP. Under these conditions, the NH\(_4\)Cl-induced \(^{22}\)Na\(^+\) influx rate stimulated by intracellular acidosis was markedly attenuated in HEPES-buffered PSS but not in CO\(_2\)/HCO\(_3\)^--buffered PSS. EIPA also appeared to inhibit the two mechanisms differentially. In HEPES-buffered PSS containing 20 mM Na\(^+\), the EIPA inhibition curve for the intracellular acidosis--induced \(^{22}\)Na\(^+\) influx was monophasic (IC\(_{50}\), 39 nM), whereas in an identical CO\(_2)/HCO\(_3\)^--buffered PSS, the inhibition curve exhibited biphasic characteristics (IC\(_{50}\), 37.3 nM and 312 \(\mu\)M). Taken together, the results indicate that Na\(^+\)/H\(^+\) exchange and a previously undescribed EIPA-sensitive Na\(^+\)- and HCO\(_3\)^--dependent mechanism play an important role in regulating the pH\(_i\) of human vascular smooth muscle. The involvement of the latter mechanism depends on the severity of the intracellular acidosis, varying from approximately 25% in severe intracellular acidosis up to 50% at lesser, more physiological, levels of induced acidosis. (Circulation Research 1990;67:814–825)

Changes in intracellular pH (pH\(_i\)) have important effects on both the contractile and proliferative properties of vascular smooth muscle.\(^1\)\(^2\) However, little is known about the precise mechanisms that regulate smooth muscle pH\(_i\). Recent studies on pH\(_i\) control have focused solely on Na\(^+\)/H\(^+\) exchange and its regulation. In cultured rat aortic smooth muscle, this exchange has been shown to be an important mechanism by which the cells extrude protons from their cytoplasm.\(^3\)\(^4\) The properties of this exchange may be modulated through a number of membrane receptor systems, including those for growth factors,\(^5\) angiotensin II,\(^6\) and catecholamines,\(^7\) to either maintain or increase the basal steady state pH\(_i\). But although Na\(^+\)/H\(^+\) exchange is clearly important in pH\(_i\) control, other mechanisms, particularly those dependent on HCO\(_3\)^– ions, also could contribute.

Recently, Aalkjaer and Cragoe\(^8\) reported the presence in rat mesenteric microvessels of Na\(^+\)/H\(^+\) exchange and, in addition, a Na\(^+\)-dependent HCO\(_3\)^-- sensitive mechanism to 4-acetamido-4'--isothiocyanatostilbene-2,2'--disulfonic acid (SITS). Although the location of the HCO\(_3\)^-- transport process on smooth muscle was not conclusively demonstrated, the possibility exists that pH\(_i\) in vascular smooth muscle cells

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could be regulated by Na⁺/H⁺ exchange and a Na⁺-dependent Cl⁻/HCO₃⁻ exchange. Physiologically, HCO₃⁻-dependent processes may be particularly important in maintaining pHᵢ in smooth muscle during severe metabolic or respiratory acidosis. Under these conditions, the success or failure of the transport systems to maintain pHᵢ will greatly influence the responsiveness of smooth muscle to constrictor...
agents. In human vascular smooth muscle, the importance of $Na^+$/H$^+$ exchange and HCO$_3^-$-dependent pH$_i$ regulation has not been investigated. In the studies reported here, cultured smooth muscle cells from the internal mammary arteries of humans were used to characterize the processes involved in pH$_i$ regulation. We demonstrate that in human vascular smooth muscle, proton extrusion/neutralization from the cytoplasm is effected by two major transport systems: the amiloride-sensitive $Na^+/H^+$ exchange and a previously unreported $5'$-(N-ethyl-N-isopropyl)amiloride (EIPA)-sensitive $Na^+$- and HCO$_3^-$-dependent process.

In medium containing HCO$_3^-$, HCO$_3^-$ contributes between 18% and 50% to the overall recovery process, depending on the level of acidoesis induced. In contrast to $Na^+/H^+$ exchange, the HCO$_3^-$ process is relatively insensitive to cellular ATP depletion.

**Materials and Methods**

**Culture of Smooth Muscle Cells**

Discarded distal segments of internal mammary arteries were obtained from subjects (aged 50–70 years) undergoing coronary bypass surgery. The segments, usually about 1.5 cm in length, were cleaned of fat and connective tissue and cut longitudinally; their luminal surfaces were scraped to dislodge endothelium. Strips of media then were peeled away from the adventitial layer under a microscope. Segments of medial tissue (~2×2 mm) were placed into 90-mm-diameter tissue culture dishes (Sterilin, Ltd., Feltham, UK), covered with a washed, sterile glass coverslip (1×2 cm), and bathed with 10 ml of tissue culture medium containing 10 mM HEPES, 4 mM glutamine, 20 mM NaHCO$_3$, 60 μg/ml penicillin G, and 10% vol/vol fetal calf serum. This medium was replenished every 3–4 days. In the third week after explanting, smooth muscle cells began to appear, and by the fifth week, the extent of cell growth was sufficient to permit subculturing. The cells then were passaged once a week by harvesting with trypsin/versene and seeding at a 1:3 ratio.

For the ion flux experiments, cells between passage levels 5 and 15 were seeded into 30-mm-diameter dishes. For experiments on coverslips, the cells were passaged as above but plated into 30-mm-diameter culture dishes that contained two sterile coverslips. Experiments were conducted 5–7 days later on confluent layers of smooth muscle that were growth-arrested by serum deprivation for 24 hours. The composition of the depriving medium was identical to the growth medium except that the serum had been replaced with 0.46% wt/vol bovine serum albumin. The smooth muscle cultured in this manner grew as "hills and valleys" (see Figure 1), a characteristic typical of vascular smooth muscle.

**Measurement of pH$_i$**

The changes in pH$_i$ were monitored fluorometrically using the fluorescent pH-sensitive indicator 2',7'-bis(2-carboxyethyl)-5-(6)-carboxyfluorescein (BCECF). Briefly, the procedure involves washing (three times) the coverslips, to which the smooth muscle cells are attached, with physiological salt solution (PSS) and then incubating the cells at 37°C in PSS containing 6.2 μM BCEF-AM. After 30 minutes, the coverslips were again washed (three times) with PSS to remove any extracellularly located pH indicator. Examination of these BCECF-labeled cells under a fluorescence microscope indicated BCECF fluorescence to be evenly distributed through the cell cytoplasm.

For the determination of fluorescence, the coverslips with the BCECF-labeled cells were loaded into a vertical coverslip holding device that can be inserted into a standard fluorescence cuvette and permits the rapid exchange of extracellular medium or the addition of drugs to the medium without disturbing the cells or their orientation to the excitation beam. Fluorescence measurements were carried out at 37°C using a Perkin-Elmer LS-5 luminescence spectrometer (The Perkin-Elmer Corp., Norwalk, Conn.) with excitation wavelengths set at 495 and 440 nm (bandpass 10 nm) and emission wavelength at 530 nm (bandpass 10 nm). Under these conditions, BCECF fluorescence in the smooth muscle is maximal at 495 nm and dependent on pH$_i$, whereas the fluorescence at 440 nm (the isosbestic point) is not affected by changes in pH$_i$. The ratio of the 495/440 nm fluorescence values, corrected for cellular autofluorescence at these wavelengths, was used to estimate pH$_i$. Autofluorescence at 495 nm of the unlabeled cells represented less than 2% of the total fluorescence exhibited by BCECF-loaded cells. Calibration of the fluorescence signal from the labeled cells was achieved, as previously described, using high-concentration K$^+$ buffers of various pH values containing 7 μM nigericin.

**Intracellular Buffer Capacity**

The dependency of cytoplasmic buffer capacity on the pH$_i$ when smooth muscle cells are incubated in the nominal absence and the presence of HCO$_3^-$ was examined according to the procedure of Grinstein et al. Briefly, this procedure is dependent on initially determining the ability of intrinsic cellular components excluding CO$_2$/HCO$_3^-$ to buffer changes in the pH$_i$. This intrinsic buffer capacity was measured in HEPES-buffered PSS, pH 7.4, in which sodium ions had been replaced with N-methyl-d-glucamine. The smooth muscle cells, grown on coverslips and loaded with BCECF, were acidified at 37°C by the injection of nigericin (1–2 μg/ml) into the cuvette, and the intracellular BCECF fluorescence was monitored continuously. Once the desired pH$_i$ had been attained, the nigericin was removed rapidly by superfusing the cells with HEPES-buffered PSS containing N-methyl-d-glucamine to which bovine serum albumin (2 mg/ml) had been added. Ammonium chloride (5 mM) then was rapidly injected into the cuvette, and the peak increases in BCECF fluorescence ratio were recorded.
Measurement of capacity

Capacity at each pH was calculated from the equilibrium between $\text{NH}_4^+$, $\text{NH}_3$, and pH in the extracellular medium as determined by the Henderson-Hasselbach relation using a $pK_a$ for $\text{NH}_3$ of 9.21, and 2) the relation $\Delta[\text{base}]/\Delta\text{pH}$, as described in detail by Weintraub and Machen. The pH values at which the buffer capacity are quoted correspond to the midpoints of the changes in pH that occur on introducing the ammonium chloride. The buffer capacity of cells incubated in CO$_2$/HCO$_3^-$-buffered PSS containing N-methyl-D-glucamine, pH 7.4, were carried out in an identical manner.

Measurement of $^{22}\text{Na}^+$ Influx

$^{22}\text{Na}^+$ influx into smooth muscle cells was measured as previously described. Briefly, the procedure involved rinsing (three times) the cells in PSS containing 20 mM Na$^+$. The cells then were equilibrated at 37°C for 15 minutes in the 20 mM Na$^+-$PSS containing either 5.5 mM glucose or 2-deoxy-d-glucose in the absence and presence of ammonium chloride (see "Results"). After this period, the solutions were quickly aspirated and the cells rapidly rinsed once in 20 mM Na$^+-$PSS containing the appropriate sugar. $^{22}\text{Na}^+$ ($10^6$ cpm) then was added to the cells in appropriate 20 mM Na$^+-$PSS to which 2 mM ouabain also had been added. After a 2.5-minute incubation at 37°C, $^{22}\text{Na}^+$ uptake was terminated by rapidly washing (five times) the cells with ice-cold 0.1 M magnesium chloride. The cells were lysed by the addition of 0.1 M nitric acid, and $^{22}\text{Na}^+$ liberated from the cells was determined by liquid scintillation spectrometry. Cellular protein was determined by using the method of Lowry et al.

Measurement of ATP

Cellular ATP content was measured fluorometrically as described previously. The cells were thoroughly washed with ice-cold normal saline before extraction of the ATP with ice-cold 0.4 M perchloric acid. After neutralization of the cellular extracts with potassium carbonate, 100-μl aliquots were assayed for ATP in 2 ml of a buffered medium containing 100 mM Tris, 5 mM MgCl$_2$, 5 mM glucose, 10 μM NADP, and 3.5 units of glucose-6-phosphate dehydrogenase. Hexokinase (3.0 units) initiated the reaction, which was monitored fluorometrically with the spectrophotometer excitation and emission wavelengths set at 340 and 450 nm, respectively. Standardization of each sample was achieved by monitoring the change in fluorescence after addition of 2 nmol ATP to the reaction mixture.

Solutions

Unless otherwise stated, PSS used throughout the study had the following ionic composition (mM): Na$^+$ 135, K$^+$ 5, Ca$^{2+}$ 1.8, Mg$^{2+}$ 0.8, Cl$^-$ 144, SO$_4^{2-}$ 0.8, HCO$_3^-$ 20, and glucose 5.5. This solution was equilibrated at 37°C to pH 7.4 by gassing with 5% CO$_2$ in air. Bicarbonate-free PSS contained 10 mM HEPES adjusted to pH 7.4 with Tris base and was gassed with air. When the sodium concentrations were varied in the solutions, NaCl was replaced with an equimolar amount of N-methyl-D-glucamine, choline chloride, or lithium chloride. Solutions used in the calibration of fluorescence signals from the smooth muscle were nominally Na$^+$ and HCO$_3^-$-free PSS containing 140 mM KCl and 7 μM nigericin. All experiments were carried out at 37°C.
FIGURE 3. Dependence of the recovery of human smooth muscle cells from 15 mM NH4Cl-induced intracellular acidosis in CO2/HCO3- buffered physiological salt solution (PSS) (pH 7.4) on extracellular Na+ concentration. Inset: Double-reciprocal plot of the data that is consistent with the process responsible for the recovery from acidosis, having a mean $K_m$ of 13.1 mM for extracellular Na+ ions.

Source of Reagents

Cell culture medium M199 and fetal calf serum were from Flow Laboratories (Aust.) Pty. Ltd., South Yarra, Australia, and all other cell culture products were from the Commonwealth Serum Laboratories, Parkville, Australia. HEPES was from Calbiochem-Boehringer, La Jolla, Calif. BCECF-AM was purchased from Molecular Probes, Eugene, Ore. Ouabain, N-methyl-d-glucamine, nigericin, SITS, and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) were from Sigma Chemical Co., St. Louis. 22Na was purchased from Amersham International, Buckinghamshire, UK. EIPA was synthesized by Dr. E.J. Cragoe Jr. All other chemicals were of analytical or tissue culture grade and were purchased from local chemical suppliers.

Statistics

Results are expressed as mean±SEM. Statistical significance was evaluated by two-tailed Student’s $t$ test or analysis of covariance. The EIPA dose-response curves were modeled using a logistic func-

FIGURE 4. Mean recovery rates of cells from 15 mM NH4Cl-induced acidosis when equimolar amounts of lithium, choline, or N-methyl-d-glucamine (NMDG) ions were substituted for sodium in the CO2/HCO3- buffered physiological salt solution (pH 7.4). Results are expressed as mean±SEM of six determinations.

FIGURE 5. The effects of 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), and 5-(N-ethyl-N-isopropyl)amiloride (EIPA) each at 400 $\mu$M on $^{22}$Na+ uptake rates in CO2/HCO3- buffered 20 mM Na physiological salt solution (PSS) (pH 7.4) containing 2 mM ouabain after acidification of the cytoplasm by preexposing cells to 15 mM NH4Cl in CO2/ HCO3- buffered 20 mM Na PSS (pH 7.4) for 15 minutes. $^{22}$Na+ uptake rate in cells not acidified with NH4Cl is shown as “Basal.” Results are mean±SEM from six cultures.
Results

pH Regulation

The basal pH of the human smooth muscle isolated from the internal mammary arteries in CO₂/HCO₃⁻-buffered PSS, pH 7.4, averaged 7.18±0.02 (n=5). When 15 mM NH₄Cl was added to this medium, the pH rose rapidly to 7.56±0.03 (Figure 2). Despite the continual presence of NH₄Cl, the pH rapidly returned toward control values, attaining a pH of 7.36±0.04 4 min after the alkalosis was initiated. On removal of the NH₄Cl by perfusion with CO₂/HCO₃⁻-buffered PSS, pH rapidly fell to 6.78±0.01. Thereafter, it rapidly recovered, attaining control values 4–5 min after the alleviation of acidosis (Figure 2).

Because two sodium-dependent processes have been shown to be responsible for recovery from intracellular acidosis in rat blood vessels (i.e., a Na⁺/H⁺ exchange³,⁸ and a Na⁺-dependent HCO₃⁻ influx⁸), we examined the sodium dependency of this process in the human smooth muscle. Recovery from the 15 mM NH₄Cl-induced acidosis was also found to be predominantly dependent on extracellular sodium (Figure 2). However, in addition to the sodium-dependent recovery, a small recovery that represented 10–15% of the total initial recovery was apparent in nominally Na⁺-free CO₂/HCO₃⁻-buffered PSS. This latter sodium-independent system only contributed to pH recovery at low pH. Lineweaver-Burk analysis of the sodium-dependent component of the recovery of pH gave a Kₘ for extracellular sodium of 13.1 mM and a maximum recovery rate of 0.082±0.004 pH units/min (Figure 3). Other ions, in particular lithium, were poor substitutes for sodium. When 135 mM lithium was substituted for the sodium in the CO₂/HCO₃⁻-buffered PSS, the recovery rate from intracellular acidosis was only one quarter of that observed with sodium (Figure 4). Recovery rates from acidosis, measured in the presence of 135 mM choline, were greatly reduced and identical to those found in 135 mM CO₂/HCO₃⁻-buffered N-methyl-D-glucamine PSS.

The sensitivity of the sodium-dependent component of the recovery from acidosis to the HCO₃⁻/anion exchange inhibitors SITS and DIDS and to the Na⁺/H⁺ exchange inhibitor EIPA was examined by introducing these compounds during the induction of the intracellular acidosis after 15 mM NH₄Cl. Under these conditions, 100 µM DIDS (not shown) or 200 µM SITS had no measurable effect on the ability of the cells to recover from the acidosis (Figure 2). SITS (300 µM) did not alter the basal pH, and extending the exposure time to SITS to 4 min before the induction of acidosis had no additional effect on recovery (not shown). In contrast to this lack of effect of the HCO₃⁻/anion exchange inhibitors, 200 µM EIPA inhibited the recovery from intracellular acidosis (Figure 2).

²²Na⁺ Influx and Intracellular Acidosis

Because a SITS-sensitive Na⁺-dependent HCO₃⁻ influx mechanism has been shown to contribute to pH regulation and sodium influx in rat blood vessels,⁸ we also evaluated the effects of intracellular acidosis on ²²Na⁺ influx in the cultured human smooth muscle. In agreement with the above results on the recovery from pH, SITS at concentrations two times higher (400 µM) than those used to characterize the pH recovery processes had no significant effect on the ²²Na⁺ influx stimulated by the NH₄Cl-induced intracellular acidosis (Figure 5). DIDS also was without effect, whereas 400 µM EIPA inhibited approximately 92% of the ²²Na⁺ influx (see also Figure 9).

Bicarbonate-Independent pH Regulation

Because Na⁺/H⁺ exchange is an important regulator of pH in cultured rat aortic smooth muscle⁵,⁶ and
blood vessels and because the Na+/H+ exchange inhibitor EIPA impaired the ability of the human smooth muscle to recover from intracellular acidosis in the presence of bicarbonate, we examined the role of Na+/H+ exchange in regulating pH in the absence of any extracellular bicarbonate. As in the previous experiments, intracellular acidosis was induced by preexposing the cells to 15 mM NH₄Cl in HEPES-buffered PSS, pH 7.4. However, in contrast to the observations in PSS containing HCO₃⁻, a longer preincubation time with 15 mM NH₄Cl was required to achieve significant acidosis (Figures 2 and 6). This was the consequence of the very slow recovery of pH from the initial alkaline pH. For example, when the smooth muscle cells were initially exposed to 15 mM NH₄Cl in the HEPES-buffered PSS, pH rose rapidly from 7.33±0.02 to 7.77±0.01 and then, over the ensuing 4 minutes, recovered by only 14%. Perfusion of the cells with HEPES-buffered PSS at this time reduced pH to 7.13±0.02, approximately 0.30 pH units greater than that achieved in the CO₂/HCO₃⁻-buffered PSS. Because of this small reduction in pH, recovery toward control values was very slow (Figure 6). Prolonging the exposure time for NH₄Cl to 12 minutes led to a subsequent reduction in pH to values averaging 6.57. Recovery from this pH was rapid and could be completely attenuated by 200 μM EIPA (Figure 6). These effects are consistent with the presence in these cells of a Na+/H+ exchange that participated in proton removal.

**Na+/H+ Exchange and pH Regulation**

To examine the importance of Na+/H+ exchange in regulating pH in bicarbonate-containing media, we compared the rates of pH recovery after increased levels of intracellular acidification induced by either a 6-minute or a 4-minute NH₄Cl pulse in the absence and presence of CO₂/HCO₃⁻-buffered medium, respectively. As expected, removal of equivalent concentrations of NH₄Cl from cells bathed in these solutions induced a greater level of intracellular acidification in cells incubated in CO₂/HCO₃⁻-containing PSS (not shown). The rates at which pH recovered from similar initial levels of intracellular pH were greater in the cells incubated in the CO₂/HCO₃⁻-buffered PSS (Figure 7, upper panel). Furthermore, differences in the cytoplasmic buffer capacity between cells incubated in CO₂/HCO₃⁻-buffered and HEPES-buffered PSS could not account for the more rapid recovery in the presence of bicarbonate. The intrinsic buffer capacity of the cells in the HEPES-buffered PSS, pH 7.4, calculated from the magnitude of the alkalinization that occurred on introduction of NH₄Cl (5 mM) into the nominally sodium-free HEPES-buffered PSS, did not vary significantly (p>0.10) over the pH range 6.53–7.12 (Table 1), averaging 41.3±1.5 mM per pH unit. The total buffering capacity in the zero-sodium CO₂/HCO₃⁻-buffered PSS containing N-methyl-d-glucamine, estimated by the same procedure, was approximately 40% higher, averaging 54.6±5.1 and 57.8±6.4 mM per pH unit when the cytoplasmic pH was 7.04 and 6.54, respectively. The somewhat higher buffering capacity of the smooth muscle cytosol at pH 6.54 than would be predicted from the passive move-

**FIGURE 7.** Upper panel: Apparent rates of intracellular pH recovery of the cultured human smooth muscle from different levels of intracellular acidification induced by a graded NH₄Cl prepulse in CO₂/HCO₃⁻-buffered (▲) and HEPES-buffered (●) physiological salt solution (PSS) (pH 7.4). Lower panel: Apparent rates of acid extrusion/neuralization of the cultured smooth muscle (expressed as mmol/l/min) after intracellular acidification in CO₂/HCO₃⁻-buffered (▲) and HEPES-buffered (●) PSS (pH 7.4) represented as a function of intracellular pH and total buffering capacity.

**TABLE 1. Intracellular pH and Buffer Capacity in Human Vascular Smooth Muscle**

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>pH</th>
<th>Buffer capacity (mM/pH unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES-buffered PSS</td>
<td>7.12±0.08</td>
<td>42.2±2.2</td>
</tr>
<tr>
<td></td>
<td>6.80±0.02</td>
<td>38.8±1.3</td>
</tr>
<tr>
<td></td>
<td>6.53±0.03</td>
<td>43.2±3.9</td>
</tr>
<tr>
<td>CO₂/HCO₃⁻-buffered PSS</td>
<td>7.04±0.07</td>
<td>54.6±5.1</td>
</tr>
<tr>
<td></td>
<td>6.54±0.02</td>
<td>57.8±6.4</td>
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</table>

Values are mean±SEM. PSS, physiological salt solution.
Acidosis-Stimulated $^{22}$Na$^+$ Uptake and Metabolic Energy

Previous studies in a number of cell lines including cultured rat aortic smooth muscle have provided evidence for an indirect dependency of Na$^+$/H$^+$ exchange activity on metabolic energy.\textsuperscript{4,13,21} To further examine the possibility that differences in the rate of proton extrusion/neutralization may represent two independent processes, we examined the effects of depleting cellular ATP content on $^{22}$Na$^+$ uptake after intracellular acidification with various NH$_4$Cl concentrations in HEPES-buffered 20 mM Na$^+$-PSS, pH 7.4, and CO$_2$/HCO$_3^-$-buffered 20 mM Na$^+$-PSS, pH 7.4. Preincubation of the cells for 30 minutes in CO$_2$/HCO$_3^-$-buffered PSS, in which an equimolar amount of 2-deoxy-d-glucose was substituted for the glucose, reduced cellular ATP content by 91%, from 15.9±0.9 to 1.5±0.6 mmol ATP/mg protein. As expected from earlier studies on the indirect dependency of Na$^+$/H$^+$ exchange on metabolic energy,\textsuperscript{15} ATP depletion was associated with a marked reduction in $^{22}$Na$^+$ influx when $^{22}$Na$^+$ influx was measured in NH$_4$Cl-acidified cells incubated in the HEPES-buffered 20 mM Na$^+$-PSS (Figure 8). This effect was not observed when the ATP-depleted cells were acidified in the CO$_2$/HCO$_3^-$-buffered 20 mM Na$^+$-PSS. Under these conditions, $^{22}$Na$^+$ influx rates in control and ATP-depleted cells, in response to various degrees of intracellular acidification, were similar ($p>0.10$).

These results indicate the different ATP dependencies of the Na$^+$/H$^+$ exchange and EIPA-sensitive HCO$_3^-$-dependent $^{22}$Na$^+$ uptake pathways. This difference in energy dependency is consistent with the presence in the human vascular smooth muscle of a Na$^+$/H$^+$ exchange and an EIPA-sensitive Na$^+$- and HCO$_3^-$-dependent mechanism that is activated during conditions that initiate intracellular acidosis.

Inhibition of Acidosis-Stimulated $^{22}$Na$^+$ Influx by EIPA

The possibility that two pharmacologically distinguishable processes may account for the Na$^+$-dependent recovery from intracellular acidosis in the presence of HCO$_3^-$ was further evaluated by examining the ability of EIPA to inhibit acidosis-stimulated $^{22}$Na$^+$ influx from a CO$_2$/HCO$_3^-$-buffered PSS containing 20 mM Na$^+$, pH 7.4, compared with a HEPES-buffered PSS containing 20 mM Na$^+$, pH 7.4. In the latter medium, Na$^+$/H$^+$ exchange is most probably the only Na$^+$-dependent mechanism operating to restore pH. Maximal stimulation of $^{22}$Na$^+$ influx was achieved by rapidly removing 30 mM NH$_4$Cl to which the cells had been exposed for 15 minutes. After this time, the pH of cells incubated in HEPES- or CO$_2$/HCO$_3^-$-buffered PSS were similar, averaging 6.42±0.01 and 6.39±0.01, respectively. In HEPES-buffered PSS, EIPA at 1 mM inhibited 87.8±4.3% ($n=3$) of the total $^{22}$Na$^+$ influx. The EIPA inhibition curve for $^{22}$Na$^+$ influx was monophasic, with 50% attenuation of $^{22}$Na$^+$ uptake (IC$_{50}$) occurring at 39.0 mM (Figure 9, left panel). In CO$_2$/HCO$_3^-$-buffered PSS, EIPA at 1 mM inhibited 96.7±0.9% ($n=4$) of the total $^{22}$Na$^+$ influx; however, the dose-response curve was biphasic, as indicated by a highly

![Figure 8](http://circres.ahajournals.org/figure8.png)
significant ($p<0.016$) improvement in regression fit compared with the monophasic curve fitted to the same data (Figure 9, right panel). The IC$_{50}$ of EIPA for the two components of the inhibition curve averaged 37.3 nM and 312 μM. The former is consistent with the inhibition of Na$^+$/H$^+$ exchange (see above). Under the conditions of these experiments, the high- and low-affinity components accounted for 79.2% and 18.7%, respectively, of the total $^{22}$Na$^+$ influx, indicating a greater contribution to the $^{22}$Na$^+$ influx of Na$^+$/H$^+$ exchange compared with the HCO$_3^-$-dependent Na$^+$ influx mechanism. The inhibition of Na$^+$, K$^+$, and Cl$^-$ cotransport activity with bumetanide (100 μM) failed to affect either the basal or acidosis-stimulated $^{22}$Na$^+$ uptake into cells incubated in either the HEPES- or CO$_2$/HCO$_3^-$-buffered PSS (data not shown).

**Discussion**

We have demonstrated that the well-described Na$^+$/H$^+$ antiport and a previously unreported EIPA-sensitive Na$^+$- and HCO$_3^-$-dependent mechanism are major contributors to pH$_i$ regulation in human arterial smooth muscle. Three independent lines of evidence support the involvement of the novel EIPA-sensitive Na$^+$- and HCO$_3^-$-dependent mechanism in pH$_i$ regulation. First, from identical levels of intracellular acidosis, pH$_i$ recovery rate is faster in cells incubated in CO$_2$/HCO$_3^-$-buffered PSS compared with nominally HCO$_3^-$-free HEPES-buffered PSS. Second, in contrast to the Na$^+$/H$^+$ antiport, the HCO$_3^-$-dependent mechanism is not significantly impaired, at least initially, by severe cellular ATP depletion. Third, in cells incubated in CO$_2$/HCO$_3^-$-buffered PSS, the EIPA dose-response curve for the inhibition of $^{22}$Na$^+$ influx accompanying recovery from acidosis shows a high-affinity component, indicating inhibition of Na$^+$/H$^+$ exchange, and a previously unreported low-affinity component, indicating the involvement of a HCO$_3^-$-dependent mechanism. The contribution of this EIPA-sensitive Na$^+$ and HCO$_3^-$ system to $^{22}$Na$^+$ uptake and pH$_i$ recovery from intracellular acidosis varied between 25% and 50%, depending on the initial level of acidosis induced. The contribution of the HCO$_3^-$-dependent mechanism was greater at lesser, possibly more physiological, levels of induced acidosis.

Previous studies on pH$_i$ control in the vascular system have concentrated on characterizing the Na$^+$-dependent pH$_i$ regulating systems in smooth muscle cultured from rat aorta. In these cells, grown either in primary culture or subcultured, the Na$^+$/H$^+$ exchange has been shown to be the major mechanism responsible for intracellular proton removal in the absence of extracellular HCO$_3^-$ ions.$^{3,4,6}$ Even in the presence of HCO$_3^-$, the apparent insensitivity of pH$_i$ recovery from acidosis to the anion exchange inhibitor SITS suggests that the Na$^+$/H$^+$ exchange is the predominant mechanism for controlling pH$_i$ in rat aortic smooth muscle cells.$^4$ More recently, a SITS-sensitive pH$_i$ control system, presumably the Na$^+$-dependent Cl$^-$/HCO$_3^-$ exchange, has been shown to contribute to pH$_i$ recovery from acidosis in microvessels of the rat mesenteric vasculature.$^8$ However, the location of this SITS-sensitive system to a specific cell type within the microvessel was not demonstrated.

Our experiments on smooth muscle cultured from the human internal mammary artery indicate that neither a SITS-sensitive Na$^+$-dependent Cl$^-$/HCO$_3^-$ exchange nor a SITS-sensitive Na$^+$/HCO$_3^-$ cotransport system contributes significantly to recovery from acidosis in these cells. Although the lack of a SITS-sensitive Na$^+$-dependent pH$_i$ regulating mechanism in these smooth muscle cells does not appear to be a...
Consequence of culturing the cells, because Na\(^{+}\)-dependent SITS-sensitive mechanisms frequently have been reported in other subcultured cell lines,\(^{22,23}\) this possibility cannot be excluded. A small recovery of pH\(_i\) was observed at low pH\(_i\) in nominally sodium-free medium, presumably due to Cl\(^-\)/HCO\(_3^-\) exchange. In human smooth muscle, the major Na\(^{+}\)- and HCO\(_3^-\)-dependent system that contributed to pH\(_i\) homeostasis was EIPA sensitive. The activity of this system, as well as that of the Na\(^{+}/H^+\) exchange, is dependent on the electrochemical gradient for sodium ions and is progressively activated by increasing levels of intracellular acidosis, presumably via allosteric mechanisms. Analysis of the dependency of pH\(_i\) recovery in the presence of HCO\(_3^-\) ions on extracellular sodium, when both the Na\(^{+}/H^+\) exchange and the EIPA-sensitive Na\(^{+}\)- and HCO\(_3^-\)-dependent mechanisms are operating, indicated that the two mechanisms have similar \(K_m\) values for extracellular sodium, averaging 13.1 mM. This value is similar to those previously reported for Na\(^{+}/H^+\) exchange in cultured rat aortic smooth muscle\(^3\) and for the SITS-sensitive Na\(^{+}\)-dependent Cl\(^-\)/HCO\(_3^-\) exchange in fibroblasts.\(^{22}\) However, despite our finding that the two EIPA-sensitive processes could not be distinguished by their \(K_m\) values for sodium, the biphasic nature of the concentration-dependent inhibition curves for Na\(^{+}\) uptake by EIPA, when the cells were acidified in the presence of bicarbonate ions, strongly supports our hypothesis for the involvement of an additional EIPA-sensitive Na\(^{+}\)- and HCO\(_3^-\)-dependent mechanism contributing to pH\(_i\) recovery in human arterial smooth muscle cells. Comparison of the EIPA inhibition curves for Na\(^{+}\) uptake cells acidified in HEPES- and CO\(_2\)/HCO\(_3^-\)-buffered PSS, respectively, strongly suggests that EIPA may be more potent at inhibiting Na\(^{+}/H^+\) exchange than this Na\(^{+}\)- and HCO\(_3^-\)-dependent mechanism.

ATP-dependent mechanisms, presumably involving phosphorylation, also have been shown to be important modulators of Na\(^{+}\)-dependent pH\(_i\) regulating systems.\(^{4,15,21}\) In rat aortic smooth muscle, ATP depletion markedly attenuates the ability of the Na\(^{+}/H^+\) exchange to respond to intracellular acidosis by reducing its sensitivity to changes in pH\(_i\) as well as affecting its maximal activity.\(^{15}\) Similar effects on Na\(^{+}/H^+\) exchange activity were observed in human smooth muscle cells. However, in contrast to the effects on Na\(^{+}/H^+\) exchange, the EIPA-sensitive Na\(^{+}\)- and HCO\(_3^-\)-dependent system showed no such dependency on cellular ATP.\(^{22}\) Na\(^{+}\) uptake in response to intracellular acidosis was not attenuated when the cells, depleted of most of their ATP, were incubated in CO\(_2\)/HCO\(_3^-\)-buffered 20 mM Na\(^+-\)PSS. This differential dependency of the two Na\(^+\) transport processes on cellular ATP supports the hypothesis that the amiloride-sensitive Na\(^{+}/H^+\) exchange and the Na\(^{+}\)- and HCO\(_3^-\)-dependent systems are two independent pH\(_i\) regulating mechanisms. The alternative explanation for this differential dependency on ATP is that HCO\(_3^-\) ions prevent inactivation of the Na\(^{+}/H^+\) exchange, for example, by preventing its dephosphorylation. This is unlikely in view of the greater rate at which proton elimination/neutralization occurs when the cell cytoplasm is acidified in the presence of extracellular HCO\(_3^-\) ions. Whether phosphorylation is involved in regulating the activity of this HCO\(_3^-\)-dependent process could not be excluded in the present study. The reduced dependency on cellular ATP compared with the Na\(^{+}/H^+\) exchange simply may reflect a more efficient phosphorylation system operating at lower ATP concentrations or using different ATP stores. Clearly, further work will be necessary to elucidate these questions. However, the important physiological implication of our finding is that severe ATP depletion in human arterial vessels will not necessarily impair pH\(_i\) regulation in the smooth muscle, despite the attenuation of Na\(^{+}/H^+\) exchange activity.

Sodium-independent HCO\(_3^-\) processes also appear to contribute to pH\(_i\) control in cultured human smooth muscle cells. At low pH\(_i\) partial restoration of pH\(_i\) could be achieved in nominally Na\(^{+}\)-free CO\(_2\)/HCO\(_3^-\)-buffered N-methyl-d-glucamine PSS. This recovery at low pH\(_i\) is consistent with a net influx of HCO\(_3^-\) ions when the internal HCO\(_3^-\) concentration is transiently decreased by severe acidosis. Under these circumstances, the outwardly directed chloride ion gradient may be great enough to drive external HCO\(_3^-\) ions inward through the Cl\(^-\)/HCO\(_3^-\) exchange.\(^{19}\) Such a process could be contributing to the maintenance of an apparently stable intracellular buffering capacity in the presence of cytoplasmic acidification when the smooth muscle cells are bathed in PSS containing CO\(_2\)/HCO\(_3^-\). Cl\(^-\)/HCO\(_3^-\) exchange previously has been shown to contribute to the recovery from intracellular acidosis in mouse soleus muscle fibers\(^24\) and squid giant axons.\(^25\) The presence of the Cl\(^-\)/HCO\(_3^-\) exchange in these cells would also account for the HCO\(_3^-\)-dependent acceleration of pH\(_i\) recovery from intracellular alkalosis. This exchange accounts for the major component of the ability of canine kidney cells,\(^26\) alveolar epithelial cells,\(^27\) and cultured rat aortic cells (A. Bobik, unpublished data, 1989) to recover from intracellular alkalosis. However, under normal circumstances, this system contributes little to the maintenance of basal pH\(_i\), as judged from the inability of high concentrations (300 \(\mu\)M) of the anion exchange inhibitor SITS to alter the basal pH\(_i\) of the cells in CO\(_2\)/HCO\(_3^-\)-buffered PSS, pH 7.4. It is also possible that other mechanisms, such as a more rapid endogenous production of acidic products via, for example, the stimulation of anaerobic metabolism in HCO\(_3^-\) containing medium or a more rapid influx of NH\(_4^+\) via K\(^+\) channels, also could be contributing to the more rapid recovery of pH\(_i\) from the NH\(_4\)Cl-induced intracellular alkalosis when the smooth muscle cells were incubated in medium containing HCO\(_3^-\) ions.

Our observation that pH\(_i\) is consistently 0.15 pH units lower in CO\(_2\)/HCO\(_3^-\)-buffered PSS than in
HEPES-buffered PSS is consistent with previous observations on pH in vascular smooth muscle. This effect can be attributed to a HCO₃⁻/CO₂ shuttle movement in which the continuous efflux of HCO₃⁻ ions down their electrochemical gradient imposes a constant acid load on the cell by the continuously dissociating carbonic acid. It is not understood why the Na⁺/H⁺ exchange and the EIPA-sensitive Na⁺- and HCO₃⁻-dependent mechanisms do not return pH to control levels, particularly when their combined ability to extrude/neutralize intracellular protons is increased when compared with cells bathed in nominally HCO₃⁻-free PSS. However, the physiological consequence of this effect of CO₂ is that pH will be lower in a respiratory acidosis than an equivalent metabolic acidosis. Metabolic acidosis is known to have a less profound effect in reducing the contractile ability of vessels than an equivalent respiratory acidosis. This could well be accounted for by the lower pH achieved during respiratory acidosis. Reductions in pH in arterial vessels are known to increase the intracellular calcium requirement for tension development and the activation of Mg²⁺-activated ATPase. These effects of CO₂ are known to be more pronounced in certain vascular beds. For example, arterial hypercapnia dilates the cerebral arteries, thereby increasing cerebral blood flow, whereas arterial hypocapnia reverses these effects. The main mechanism for the effect of CO₂ is a direct action on the cerebral vascular smooth muscle via effects on pHi. Coronary vascular resistance also has been shown to vary inversely with the perfusing CO₂ concentration, both in the direction of vasoconstriction and vasodilatation. In the future, it will be of interest to examine whether the differences in the sensitivities of vessels to CO₂ can be largely explained on the basis of the magnitude of the changes in pH. In conclusion, our results indicate that, in addition to Na⁺/H⁺ exchange, a previously undescribed EIPA-sensitive Na⁺- and HCO₃⁻-dependent mechanism plays an important role in regulating the pH of human vascular smooth muscle. The HCO₃⁻-dependent mechanism has a similar dependency on extracellular sodium ions as that of the Na⁺/H⁺ exchange but exhibits a lower dependency on cellular ATP. Under conditions of cellular ATP depletion, this EIPA-sensitive Na⁺- and HCO₃⁻-dependent mechanism becomes the major system preventing the development of intracellular acidosis in smooth muscle from the human skeletal muscle vascular beds. Future studies will further explore the regulation of this newly identified process and its contribution to pHi regulation in smooth muscle from other vascular beds.

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References

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