Inhibition of hypoxia-induced calcium responses in pulmonary arterial smooth muscle by acetazolamide is independent of carbonic anhydrase inhibition

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poxic pulmonary vasoconstriction (HPV) occurs with ascent to high altitude and can contribute to development of high altitude pulmonary edema (HAPE). Vascular smooth muscle contains carbonic anhydrase (CA), and acetazolamide (AZ), a CA inhibitor, blunts HPV and might be useful in the prevention of HAPE. The mechanism by which AZ impairs HPV is uncertain. Originally developed as a diuretic, AZ also has direct effects on systemic vascular smooth muscle, including modulation of pH and membrane potential; however, the effect of AZ on pulmonary arterial smooth muscle cells (PASMCs) is unknown. Since HPV requires Ca2+ influx into PASMCs and can be modulated by pH, we hypothesized that AZ alters hypoxia-induced changes in PASMC intracellular pH (pHi) or Ca2+ concentration ([Ca2+]). Using fluorescent microscopy, we tested the effect of AZ as well as any other potent CA inhibitors, benzolamide and ethoxolamide, which exhibit low and high membrane permeability, respectively, on hypoxia-induced responses in PASMCs. Hypoxia caused a significant increase in [Ca2+], but no change in pHi. All three CA inhibitors slightly decreased basal pHi, but only AZ caused a concentration-dependent decrease in the [Ca2+]i response to hypoxia. AZ had no effect on the KCl-induced increase in [Ca2+], or membrane potential.

N-methyl-AZ (N-Meth-AZ), in which one of the amine hydrogens of the sulfonamide moiety of SO2NH2 is replaced by a methyl group, is an intracellular Ca2+ sensor that is insensitive to the pH of the extracellular space (21). When taken prior to ascent, acetazolamide (AZ), a carbonic anhydrase (CA) inhibitor, can prevent the development of acute mountain sickness (44) through its effects on renal bicarbonate reabsorption and chemoreceptor activity to increase ventilation and arterial oxygenation. Work in intact animals and isolated perfused lung preparations has demonstrated that AZ also attenuates the magnitude of HPV and slows the onset of the response (7, 8, 15). The mechanisms by which this occurs remain uncertain. As a respiratory stimulant, AZ raises alveolar PO2 and by this mechanism might blunt HPV; however, a direct effect independent of ventilation-induced changes in alveolar PO2 is also evident since AZ inhibits HPV when ventilation, alveolar PO2, and PCO2 are carefully controlled or held constant (7, 8, 15). Vascular smooth muscle contains CA (3), and AZ causes vasodilation in systemic blood vessels (5, 28, 29). Mechanisms involved may include modulation of K+ channels, membrane potential, and Ca2+ signaling, or pH (28, 29). Furthermore, in various cell types, CA inhibitors block Ca2+ channels (13, 17, 26, 55), inhibit a Cl−-dependent ATPase (5), and activate Ca2+-activated K+ channels (29, 46, 47). The effect of AZ and/or CA inhibition on PASMC function is unknown.

Based on these considerations, we hypothesized that AZ blunts HPV by CA inhibition via modulation of pHi, E\text{m}, or intracellular Ca2+ concentration ([Ca2+]i) in PASMCs. To test this hypothesis, we used fluorescent microscopy and the Ca2+ indicator fluorescein diacetate (Ca-Fluo-3, 10 μM) to monitor Ca2+ responses. These results suggest that AZ attenuates HPV by selectively inhibiting hypoxia-induced Ca2+ responses via a mechanism independent of CA inhibition, changes in pHi, or membrane potential.

pulmonary vascular smooth muscle; intracellular Ca2+
METHODS

Isolation of PASMCs. All procedures and protocols in this study were approved by the Johns Hopkins University Animal Care and Use Committee. The method for obtaining single PASMCs has been described previously (39). Intrapulmonary arteries (200–600 μm outer diameter) were isolated from male Wistar rats (250–350 g) and cleaned of connective tissue. After removal of the endothelial cells, the arteries were allowed to recover for 30 min in cold (4°C) HBSS containing (in mM) 130 NaCl, 5 KCl, 1.2 MgCl₂, 1.5 CaCl₂, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), and 10 glucose, with pH adjusted to 7.2 with 5 M NaOH, followed by 20 min in reduced-Ca²⁺ (20 μM CaCl₂) HBSS at room temperature. The tissue was enzymatically digested in reduced-Ca²⁺ HBSS containing collagenase (type I; 1,750 U/ml), papain (9.5 U/ml), bovine serum albumin (2 mg/ml), and dithiothreitol (1 mM) at 37°C for 20 min. Following digestion, single smooth muscle cells were dispersed by gentle trituration with a wide-bore transfer pipette in Ca²⁺-free HBSS, and the cell suspension was placed on 25-mm glass cover slips. PASMCs were transiently cultured in Ham’s F-12 media supplemented with 0.5% fetal bovine serum and 1% penicillin/streptomycin for 24–48 h before study.

Measurement of [Ca²⁺]i. The methods for measurement of [Ca²⁺]i have been described previously (51). PASMCs were placed in reduced-Ca²⁺ (20 μM CaCl₂) HBSS at room temperature. The tissue was enzymatically digested in reduced-Ca²⁺ HBSS containing collagenase (type I; 1,750 U/ml), papain (9.5 U/ml), bovine serum albumin (2 mg/ml), and dithiothreitol (1 mM) at 37°C for 20 min. Following digestion, single smooth muscle cells were dispersed by gentle trituration with a wide-bore transfer pipette in Ca²⁺-free HBSS, and the cell suspension was placed on 25-mm glass cover slips. PASMCs were transiently cultured in Ham’s F-12 media supplemented with 0.5% fetal bovine serum and 1% penicillin/streptomycin for 24–48 h before study.

Measurement of [Ca²⁺]i. The methods for measurement of [Ca²⁺]i have been described previously (51). PASMCs were placed in a

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![Fig. 1](image1.png) Structure and properties of the carbonic anhydrase (CA)-inhibiting and -noninhibiting sulfonamides. MW, molecular weight; Kᵢ, dissociation constant for inhibitor binding; CA II, carbonic anhydrase II.

![Fig. 2](image2.png) Composite traces demonstrating the effect of hypoxia on intracellular pH (pHi) in the absence and presence of acetazolamide (AZ; 100 μM). Whereas AZ caused a small decrease in pHi, hypoxia had no effect on pHi in either control cells (n = 85 cells in 5 experiments) or cells treated with AZ (n = 55 cells in 4 experiments).
laminar flow cell chamber and perfused with modified Krebs bicarbonate solution containing (in mM) 118.3 NaCl, 4.7 KCl, 1.2 MgSO4, 25 NaHCO3, 1.1 glucose, and 1.2 KH2PO4. [Ca2+]i was measured in cells incubated with 5 μM fura-2 AM for 60 min at 37°C, then washed with physiological salt solution for 15 min at 37°C to remove extracellular dye and allow complete deesterification of cytosolic dye. Ratiometric measurement of fluorescence from the dye was performed on a workstation (Intracellular Imaging, Cincinnati, OH) consisting of a Nikon TSE 100 Ellipse inverted microscope with epifluorescence attachments. The light beam from a xenon arc lamp was filtered at 340 and 380 nm and focused onto the PASMCS under examination via a ×20 fluorescence objective (Super Fluor 20, Nikon). Light emitted from the cell at 510 nm was returned through the objective and detected by a cooled charge-coupled device (CCD) imaging camera. An electronic shutter (Sutter Instruments) was used to minimize photobleaching of dye. Protocols were executed and data collected online with InCyte software (Intracellular Imaging). [Ca2+]i was estimated from in vitro calibration solutions.

pHi measurements. pH within PASMCs was monitored using the cell-permeant pH-sensitive dye BCECF-AM. After incubation with BCECF-AM for 60 min at 37°C under an atmosphere of 21% O2-5% CO2, cells were washed with Krebs solution for 15 min at 37°C to remove extracellular dye and allow complete deesterification of cytosolic dye. Cells were excited with light at 490 and 440 nm, and light emitted from the cells was detected at 530 nm. The ratio of 490-to-440-nm emission was calculated and converted into pH values by performing a calibration curve after each experiment. PASMCs were subjected to a high K+ buffer containing 10 μM nigericin, which allowed the cell to adopt the pH of the high K+ buffer. Two high K+/nigericin buffers were used to set pH to 6.5 or 7.5. pHi was estimated from in situ calibration after each experiment. Cells were perfused with a solution containing (in mM) 105 KCl, 1 MgCl2, 1.5 CaCl2, 10 glucose, 20 HEPES-Tris, and 0.01 nigericin to allow pHi to equilibrate to external pH (32). A two-point calibration was created from fluorescence measured as pHi was adjusted with KOH from 6.5 to 7.5. Intracellular H+ ion concentration ([H+]i) was determined from pHi using the formula pHi = -log([H+]i).

Table 1. Effect of carbonic anhydrase inhibitors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline [Ca2+]i (nM)</th>
<th>Treatment [Ca2+]i (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ (n = 73)</td>
<td>159.6±10.8</td>
<td>155.0±9.6</td>
</tr>
<tr>
<td>BZ (n = 50)</td>
<td>113.4±7.4</td>
<td>120.7±7.7</td>
</tr>
<tr>
<td>EZ (n = 88)</td>
<td>125.5±7.1</td>
<td>122.5±6.3</td>
</tr>
<tr>
<td>pH, AZ</td>
<td>7.26±0.02</td>
<td>7.22±0.03*</td>
</tr>
<tr>
<td>pH, BZ</td>
<td>7.31±0.02</td>
<td>7.25±0.03*</td>
</tr>
<tr>
<td>pH, EZ</td>
<td>7.28±0.04</td>
<td>7.19±0.04*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Effect of 3 different carbonic anhydrase (CA) inhibitors (AZ, acetazolamide; BZ, benzolamide; and EZ, ethoxzolamide) at a concentration of 100 μM on basal intracellular Ca2+ concentration and pH in pulmonary arterial smooth muscle cells. *Significant difference from baseline (P < 0.05).

![Fig. 3. A](http://ajplung.physiology.org/) Composite traces illustrating the effect of hypoxia on [Ca2+]i, in the absence (n = 115 cells in 5 experiments) and presence of AZ (10–100 μM). In the absence of AZ, hypoxia caused a rapid, reversible increase in [Ca2+]i. AZ caused a dose-dependent inhibition of the hypoxia-induced Ca2+ response with almost complete blockade at 100 μM (n = 73 cells in 5 experiments). B: bar graphs representing effect of AZ on the peak change in [Ca2+]i induced by hypoxia (n = 38 cells in 3 experiments for 10 μM; n = 60 cells in 4 experiments for 30 μM). C: concentration response plot demonstrating IC50 of AZ. *Significant difference from control value (P < 0.05).
Acetazolamide and Hypoxic Responses in PASMCs

Effect of hypoxia and AZ on pH. To test whether the mechanism by which AZ inhibited HPV in isolated lungs involved modulation of hypoxia-induced changes in PASMC pH, pH was measured during control conditions and after changing to hypoxic solution in the absence and presence of 100 μM AZ (Fig. 2). AZ alone caused a small but statistically significant decrease in resting pH (Table 1). Exposure to 4% O2 had no significant effect on PASMC pH, with pH measured at 7.23 ± 0.02 during normoxia and 7.24 ± 0.03 after 15 min of hypoxia (n = 85 cells in 5 experiments). Similarly, hypoxia had no effect on pH in PASMCs pretreated with AZ (7.22 ± 0.03 to 7.24 ± 0.03; n = 55 cells in 4 experiments).

Effect of hypoxia and AZ on [Ca2+]. Perfusing cells with solution equilibrated with 4% O2, 5% CO2 produced a rapid, reversible increase in [Ca2+], (Fig. 3), from 176.2 ± 13.9 nM (Δ[Ca2+]i, 81.9 ± 9.5 nM; n = 115 cells in 5 experiments). The maximum change in [Ca2+], in response to hypoxia occurred within 7 min of beginning perfusion with hypoxic solution. On return to normoxia, [Ca2+], rapidly returned to normoxic levels. Application of AZ (10–100 μM) had no effect on basal [Ca2+], in PASMCs (Table 1). AZ caused a concentration-dependent decrease in the hypoxia-induced rise in [Ca2+], with a significant reduction in the peak change in [Ca2+], induced by hypoxia at both 30 and 100 μM AZ (Fig. 3). From the concentration-inhibition plot, the IC50 was estimated to be 50 μM.

Effect of AZ on KCl-induced Ca2+ responses. To examine whether the effect of AZ on hypoxia-induced Ca2+ responses was due to a nonspecific inhibition of Ca2+ signaling, we determined the effect of AZ on the response to KCl. Exposure to KCl (60 mM) caused a significant increase in [Ca2+], (153.2 ± 14.5 to 276.6 ± 19.8 nM; n = 40 cells in 4 experiments), reaching a mean peak change in [Ca2+], of 123.4 ± 20.6 nM (Fig. 4). Following pretreatment with 100

*Em* measurements. Changes in *Em* were measured using the fluorescence dye DiBAC4(3), which was excited at 490 nm and detected at 510 nm. Cells were loaded by continuous perfusion with Krebs solution containing 500 nM DiBAC4(3) for at least 15 min before beginning measurements to insure stable uptake of dye. Baseline DiBAC4(3) fluorescence was monitored for 5 min with control Krebs, followed by 10 min with Krebs in the presence or absence of AZ (100 μM). At the end of this period, cells were challenged with KCl (60 mM) or endothelin-1 (ET-1; 10−8 M). Since DiBAC4(3) fluorescence is detected at a single wavelength, any change in the concentration of the dye in the perfusion medium (i.e., differing concentrations between perfusion reservoirs) could result in a change in fluorescence that is not due to a change in *Em*. To minimize the possibility of this type of error, all experiments were performed by perfusing from a single reservoir into which AZ, KCl, or ET-1 were dissolved directly.

**CA inhibitors.** AZ and EZ were obtained from Sigma Scientific. BZ was a gift from Dr. Thomas H. Maren (University of Florida, Department of Pharmacology). The synthesis and purification of N-Meth-AZ followed that described by Maren (23). In N-Meth-AZ, one of the amine hydrogens of the sulfonamide moiety (SO2NH2) responsible for CA inhibition by AZ is replaced with a methyl group (SO2NCH3) to prevent binding to CA, but the rest of the molecule remains unaltered in terms of its general size, aromatic ring structure, and charge characteristics. Figure 1 shows the chemical structures of the four sulfonamides, molecular weight, inhibition constant against CA inhibitors, and charge characteristics. Figure 1 shows the chemical structures of the four sulfonamides, molecular weight, inhibition constant against CA inhibitors, and charge characteristics.

Statistical analysis. All data are presented as means ± SE. Statistical comparisons were performed using Student’s *t*-test (paired or unpaired) as appropriate. Differences were considered to be significant when *P* < 0.05.
μM AZ, the mean change in [Ca\textsuperscript{2+}]\textsubscript{i} in response to KCl was not altered, reaching a mean of 134.7 ± 18.2 nM (from 157.8 ± 11.9 to 292.6 ± 21.2 nM; n = 43 cells in 4 experiments).

**Effect of EZ and BZ on Ca\textsuperscript{2+} responses.** As with AZ, neither EZ nor BZ altered basal [Ca\textsuperscript{2+}]\textsubscript{i} (Table 1). However, in contrast to the effects of AZ, EZ (Fig. 5) and BZ (Fig. 6) had no significant effect on the hypoxia-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i} at any of the concentrations tested (10–100 μM). As with AZ, both EZ and BZ caused a significant decrease in resting pH\textsubscript{i} (Table 1). These results suggest that CA inhibition in PASMCs results in intracellular acidosis but that this is not related to the inhibition of the hypoxia-mediated rise in [Ca\textsuperscript{2+}]\textsubscript{i} by AZ.

**Effect of N-Meth-AZ on hypoxia-induced Ca\textsuperscript{2+} responses.** To further test the hypothesis that AZ inhibited [Ca\textsuperscript{2+}]\textsubscript{i} response to hypoxia via a mechanism unrelated to CA inhibition, we determined the effects of an AZ-derivative compound in which the sulfonamide group responsible for CA inhibition was substituted with a methyl group (N-Meth-AZ). The purity of the compound was assessed by high-resolution fast atom bombardment mass spectrometry, which showed only one species to be present in the recrystallized sample. In contrast to AZ, BZ, and EZ, N-Meth-AZ (100 μM) had minimal effect on basal pH\textsubscript{i} (7.13 ± 0.02 to 7.12 ± 0.01; n = 81 cells in 5 experiments; Fig. 7). Pretreatment with N-Meth-AZ for 10 min caused a very small but statistically significant increase in basal [Ca\textsuperscript{2+}]\textsubscript{i} (193.4 ± 11.0 to 200.9 ± 11.3 nM; n = 63 cells in 4 experiments). The increase in [Ca\textsuperscript{2+}]\textsubscript{i} induced by hypoxia in control PASMCs was markedly reduced in PASMCs treated with N-Meth-AZ, from 141.1 ± 32.2 nM (n = 78 cells in 4 experiments) to 33.2 ± 17.4 nM (n = 63 cells in 3 experiments) (Fig. 8A). To test whether the inhibitory effect of N-Meth-AZ on hypoxia-induced Ca\textsuperscript{2+} responses was due to generalized cell
toxicity, we also tested the effect of N-Meth-AZ on KCl-induced Ca^{2+} responses. The mean increase in [Ca^{2+}]_i in response to KCl (60 mM) was similar in the presence (n = 73 cells in 4 experiments) and absence (n = 83 cells in 4 experiments) of 100 µM N-Meth-AZ (Fig. 8B).

**Effect of AZ on \( E_m \).** AZ has recently been proposed to alter the activation of Ca^{2+}-activated K^+ channels, causing hyperpolarization (29, 46, 47). Since depolarization is believed to contribute to the hypoxia-induced increase in [Ca^{2+}]_i, AZ-induced hyperpolarization could prevent activation of voltage-gated Ca^{2+} channels and an increase in [Ca^{2+}]_i in response to hypoxia. To test this possibility, we used the \( E_m \)-sensitive fluorescent dye DiBAC4(3). The ability of the dye to measure changes in \( E_m \) was first verified by measuring the response to 1) the K^+ channel opener, pinacidil, which causes hyperpolarization; 2) KCl, which causes depolarization by reducing the gradient for K^+ efflux; and 3) ET-1, which causes depolarization via inhibition of K^+ channels (Fig. 9). Baseline fluorescence was relatively stable over time, decreasing by 4.11 ± 0.4% over 10 min (n = 90 cells in 3 experiments). In response to pinacidil (100 µM), fluorescence decreased 19.76 ± 3.3% (n = 58 cells in 3 experiments), whereas fluorescence increased 61.58 ± 12.8% in response to 60 mM KCl (n = 109 cells in 4 experiments) and 75.6 ± 13.4% in response to ET-1 (n = 82 cells in 5 experiments). Application of AZ (100 µM) had no significant effect on basal \( E_m \) (-2.86 ± 4.5%; n = 178 cells in 8 experiments) and did not alter the increase in fluorescence induced by either KCl (53.13 ± 14.8%; n = 90 cells in 3 experiments) or ET-1 (66.0 ± 10.4%; n = 87 cells in 5 experiments).

**DISCUSSION**

In this study, we found that inhibition of CA with AZ, BZ, or EZ caused a small but significant acid shift in PASMC \( pHi \), consistent with a mild acidosis arising from loss of CA-
mediated facilitated CO₂ diffusion (44). Challenge with moderate hypoxia caused a significant increase in PASMC [Ca²⁺], that was prevented by AZ but not BZ or EZ. The lack of effect of BZ and EZ on the hypoxic response was not due to ineffective concentrations of these two more powerful CA inhibitors, as both reduced basal pH_i to a similar or greater extent than did AZ. Moreover, N-Meth-AZ, which has no CA-inhibiting action (23) and had no effect on pH_i, also prevented the hypoxia-induced increase in [Ca²⁺]. The inhibitory action of AZ and N-Meth-AZ on Ca²⁺ signaling was specific for hypoxia, as no effect on KCl-induced Ca²⁺ responses was observed. Finally, AZ had no effect on either resting Eₘₙ or agonist-induced depolarization. These results suggest that AZ prevents a rise in [Ca²⁺], in response to hypoxia in PASMCs by a mechanism that does not involve intracellular acidification or a change in Eₘₙ and is independent of CA inhibition.

CA inhibitors were originally developed as diuretics with primary action on the kidney to interfere in acid-base related ion transport events. Although their use as diuretics has been supplanted by more potent alternatives, they are presently used to treat glaucoma, metabolic alkalosis, epilepsy, and acute mountain sickness, conditions in which alteration in systemic acid-base status or CA-dependent ion transport can be beneficial. CA is expressed in most cells, and there have been 14 different isozymes identified. This rich diversity of expression has led some to search for nonclassical (i.e., non-acid-base) functions of the enzyme. Swenson et al. (45) showed that CA inhibition impairs ventilation-perfusion matching in the lung, a process that is thought to result from both O₂- and CO₂-dependent changes in bronchial and vascular smooth muscle tone. To directly test whether CA inhibition alters HPV, a major mechanism that maintains ventilation perfusion (VA/Q) matching, Deem et al. (7) found that AZ blunts and slows HPV in the isolated perfused rabbit lung. Iturriaga et al. (16) found similar effects on another hypoxia-sensitive response: the neural output of the peripheral receptors of the carotid body, which contain CA, as does vascular smooth muscle (3).

The mechanism by which AZ prevents HPV in vivo may be multifactorial. The respiratory stimulation generated by an induction of metabolic acidosis leads to increased ventilation and thus enhanced alveolar PO₂ at any given inspired PO₂ (44) and thus a reduction of the primary stimulus to HPV. However, the prevention of HPV by AZ in isolated perfused lung preparations and awake animals, where ventilation and/or alveolar PO₂ are carefully controlled (7, 8, 15), indicate that AZ can reduce HPV via mechanisms other than elevation of alveolar PO₂.

In this study, we show that AZ acts directly on the vascular smooth muscle to inhibit [Ca²⁺], responses to hypoxia and that these effects do not depend on CA inhibition or intracellular acidification. In our cells, all three CA inhibitors (AZ, BZ, and EZ) resulted in a small but significant acid shift in basal pH_i. This is consistent with CA inhibition-induced acidosis described in corneal endothelium (4), nonpigmented ciliary epithelium (52), lactotrophs (11), neuronal cells (19, 20), and rat heart, brain, liver, and spleen tissue (36), and rules out the possibility that, at least in the case of highly diffusible EZ, there was a lack of intracellular penetration of the drug. Our findings suggest that CA actively participates in regulation of basal pH_i in PASMCs under normal conditions.

That AZ decreased pH_i in PASMCs presented a possible mechanism for preventing HPV. Intracellular pH can modulate a number of cell functions, including PASMC contraction (9, 18). While we observed no change in pH_i when oxygen concentration was reduced, consistent with reports in systemic vascular smooth muscle (10, 12, 37), previous studies in feline PASMCs demonstrated that moderate hypoxia caused an increase in pH_i that was associated with smooth muscle cell contraction (22). Moreover, HPV in isolated perfused rat lungs was enhanced when pH_i was increased by addition of weak bases, whereas decreasing pH_i by addition of weak acids or by inhibition of Na⁺/H⁺ exchange blunted HPV (33). If alkalization of PASMCs during hypoxia contributes to HPV, a decrease in pH_i, as seen with CA inhibitors, could have adverse effects on HPV and provide a mechanism for AZ-induced antagonism of HPV. This hypothesis is opposed, however, by results from experiments we performed to assess the effect of CA inhibition on PASMC Ca²⁺ signaling during hypoxia. In PASMCs, hypoxia caused a significant, rapid, and reversible increase in [Ca²⁺]. This increase in [Ca²⁺] in response to hypoxia had previously been shown to require Ca²⁺ influx (1, 6, 50) and was required for generation of HPV (25, 27, 34, 40–42, 48). We found that AZ inhibited the rise in [Ca²⁺], induced by hypoxia, suggesting an ability to mod-

Fig. 7. Composite traces demonstrating the effect of 100 μΜ AZ (top) and N-methyl-AZ (N-Meth-AZ; bottom) on pH_i in pulmonary arterial smooth muscle cells (PASMCs). AZ caused a small decrease in pH_i (n = 55 cells in 4 experiments), whereas N-Meth-AZ (n = 81 cells in 5 experiments) had no significant effect on pH_i.

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ulate Ca\(^{2+}\) signaling. The effect of AZ was concentration dependent with a calculated IC\(_{50}\) of 50 \(\mu\)M. This concentration corresponds to the concentrations of AZ that inhibited HPV in isolated lungs and intact animals (7, 8). In contrast, neither BZ nor EZ was able to alter the hypoxia-induced change in [Ca\(^{2+}\)]\(_i\), yet caused similar, if not greater, acidification of PASMCs.

We further investigated the possibility that the effects of AZ on HPV and hypoxia-induced Ca\(^{2+}\) responses were independent of CA inhibition using N-Meth-AZ. As expected from data demonstrating a loss of CA inhibitory action in vitro (23), substitution of one of the amines in the sulfonamide group with a methyl group resulted in the inability of the compound to induce acidification. However, N-Meth-AZ reduced the increase in [Ca\(^{2+}\)]\(_i\), in response to hypoxia to a similar extent as the same concentration of AZ. Based on our data, we can therefore rule out inhibition of CA and consequent acidification as the mechanism by which AZ prevents hypoxia-induced Ca\(^{2+}\) mobilization.

AZ has been proposed to cause membrane hyperpolarization (5, 28, 29, 47), which could act to decrease Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. We found that AZ had no effect on resting \(E_m\) in our cells, results consistent with findings in rat kidney (14). The lack of a measurable effect of AZ on \(E_m\) was not due to an inability to detect hyperpolarization in our system, as a decrease in cell fluorescence was readily observed in response to pinacidil, a K\(^+\) channel activator. One limitation of this study was that we were unable to consistently demonstrate a change in \(E_m\) in response to hypoxia with DiBAC\(_4\)(3) (data not shown) and thus could not determine directly the effect of AZ on hypoxia-induced depolarization. It is possible that the rate of change in PO\(_2\) in our system was sufficiently slow to prevent observation of an \(E_m\) response. Another possibility is that our cells do not exhibit hypoxia-induced depolarization, and/or the magnitude of change is below the threshold for detection. This would be consistent with data from several investigators who were able to elicit hypoxia-induced depolarization in isolated PASMCs only when cells were first depolarized with other agents (49) or severe PO\(_2\) or chemical hypoxia were used (30, 53, 54). Although significant depolarization was observed in canine PASMCs (31), the reproducibility of the response was unclear since only a single representative trace was presented. Other indirect evidence that would point away from AZ-induced hyperpolarization as the mechanism for repressed Ca\(^{2+}\) mobilization during hypoxia would be that EZ was as effective as AZ in opening KCa channels (46) but had no effect on the hypoxia-induced rise in [Ca\(^{2+}\)]\(_i\) in our cells. It is also unlikely that AZ acts to repress depolarization by preventing inhibition of K\(^+\) channels since AZ did not alter depolarization in response to ET-1 or KCl (Fig. 9), which increase \(E_m\) via inhibition of voltage-gated K\(^+\) channels.

![Composite traces (left) and bar graphs (right) demonstrating the effect of hypoxia (4% O\(_2\)) (A) and 60 mM KCl (B) on [Ca\(^{2+}\)]\(_i\), in the absence and presence of 100 \(\mu\)M N-Meth-AZ. Hypoxia caused a significant increase in [Ca\(^{2+}\)]\(_i\), in control cells (n = 78 cells in 4 experiments) but had no effect on [Ca\(^{2+}\)]\(_i\), in the presence of N-Meth-AZ (n = 63 cells in 3 experiments). In contrast, [Ca\(^{2+}\)]\(_i\), increased to a similar extent in response to KCl in the absence (n = 83 cells in 4 experiments) and presence (n = 73 cells in 4 experiments) of N-Meth-AZ. *Significant difference from control value (P < 0.05).](http://ajplung.physiology.org/content/292/4/L1009)
channels (39) and by reducing the gradient for $K^+$ efflux, respectively.

While the results from experiments examining the effect of AZ on $E_m$ appear to rule out hyperpolarization-driven reduction in $Ca^{2+}$ influx as a mechanism of inhibited $Ca^{2+}$ mobilization in response to hypoxia, inhibitors of CA have also been shown to directly block a range of voltage-dependent $Ca^{2+}$ channels (13, 17, 26, 55), some of which have been shown to participate in HPV (25, 40–42, 48). Thus we also tested the ability of AZ and N-Meth-AZ to inhibit $Ca^{2+}$ mobilization in response to KCl, which depends on $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels (38, 51). In PASMCs, the KCl-induced increase in $[Ca^{2+}]_i$ was similar in magnitude to that induced by hypoxia but was unaffected by pretreatment with AZ or N-Meth-AZ. These results indicate that AZ had no effect on the activation of voltage-gated $Ca^{2+}$ channels and suggest that the effect of AZ on PASMC $Ca^{2+}$-signaling during hypoxia was not due to toxicity or a nonspecific inability to mobilize intracellular $Ca^{2+}$. The differential inhibitory effect of AZ on the increases in $[Ca^{2+}]_i$ in response to hypoxia and KCl might also point to the intriguing implication that hypoxia causes $Ca^{2+}$ influx through pathways other than $L$-type $Ca^{2+}$ channels, as recently suggested (51).

In summary, we found that AZ blocks the rise in PASMC $[Ca^{2+}]_i$ that occurs in response to hypoxia by a mechanism independent of CA inhibition and its generation of a mild intracellular acidosis. AZ does not appear to exert its inhibitory effect on the hypoxia-mediated increase in $[Ca^{2+}]_i$ in PASMCs by either changes in $E_m$ or by blockade of voltage-sensitive calcium channels. The molecular target or pathway that is altered by AZ, but not other sulfonamide CA inhibitors, and is responsible for HPV inhibition remains to be discovered. The use of compounds such as N-Meth-AZ, which are similar in structure to AZ but lack its CA-inhibiting activity (and attendant side effects), may prove beneficial in elucidating the action of AZ in blunting HPV and provide new directions to explore regarding therapeutic strategies.

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ACETAZOLAMIDE AND HYPOXIC RESPONSES IN PASMCs

L1011


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