Arsenic Alters ATP-Dependent Ca\(^{2+}\) Signaling in Human Airway Epithelial Cell Wound Response

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Arsenic is a natural metalloid toxicant that is associated with occupational inhalation injury and contaminates drinking water worldwide. Both inhalation of arsenic and consumption of arsenic-tainted water are correlated with malignant and nonmalignant lung diseases. Despite strong links between arsenic and respiratory illness, underlying cell responses to arsenic remain unclear. We hypothesized that arsenic may elicit some of its detrimental effects on the airway through limitation of innate immune function and, specifically, through alteration of paracrine ATP (purinergic) Ca\(^{2+}\) signaling in the airway epithelium. We examined the effects of acute (24 h) exposure with environmentally relevant levels of arsenic (i.e., < 4μM as Na-arsenite) on wound-induced Ca\(^{2+}\) signaling pathways in human bronchial epithelial cell line (16HBE14o-). We found that arsenic reduces purinergic Ca\(^{2+}\) signaling in a dose-dependent manner and results in a reshaping of the Ca\(^{2+}\) signaling response to localized wounds. We next examined arsenic effects on two purinergic receptor types: the metabolic P2Y and ionotropic P2X receptors. Arsenic inhibited both P2Y- and P2X-mediated Ca\(^{2+}\) signaling responses to ATP. Both inhaled and ingested arsenic can rapidly reach the airway epithelium where purinergic signaling is essential in innate immune functions (e.g., ciliary beat, salt and water transport, bactericidal production, and wound repair). Arsenic-induced compromise of such airway defense mechanisms may be an underlying contributor to chronic lung disease.

Key Words: purinergic signaling; calcium; 16HBE14o-; P2 receptor.

Arsenic is a natural metalloid toxicant that contaminates drinking water supplies in the United States and throughout the world. Human consumption of arsenic-tainted drinking water is associated with both malignant and nonmalignant lung diseases (Kapaj et al., 2006; Mazumder, 2007; Schuhmacher-Wolz et al., 2009). Exposure to higher levels of arsenic in drinking water (e.g., 300–1000 parts per billion [ppb or μg/l]) has been correlated with both restrictive and obstructive respiratory symptoms as well as the obstructive lung disease bronchiectasis (Mazumder, 2007). Bronchiectasis is thought to develop from a cycle of repeated infections and inflammation in part due to a weakened airway innate immunity. Direct inhalation of arsenic through industrial practices has long been known to lead to respiratory compromise, including lung cancer (IARC, 1980). Despite the strong correlations between respiratory illness and arsenic exposure, little is known about how arsenic contributes to these diseases.

Largely because of the adverse effects of high-level arsenic exposure, the United States Environmental Protection Agency and the World Health Organization have reduced acceptable maximum contaminant levels of arsenic in drinking water from 50 μg/l down to 10 μg/l. With these reduced limits, there has been an increase in studies of arsenic effects near these limits. Evidence is accumulating that submicromolar levels (e.g., < 50 μg/l) of arsenic exposure can have direct physiologic impact on human disease including diabetes (Navas-Acien et al., 2008) and lung cancer (Heck et al., 2009). Furthermore, high-throughput analyses of human and mouse lung consistently suggest a reduction in innate immune response and, specifically, cell migration and wound response (Andrew et al., 2007, 2008; Kozul et al., 2009b; Lantz and Hays, 2006). Mechanisms of action that underlie arsenic-induced toxicity remain ill defined, especially with respect to nonmalignant disease.

As a part of normal defense, airway epithelial cells coordinate cellular responses to prevent or repair damage due to toxicity. In studies using an in vitro wound repair model, we have shown that coordinated migration in human airway epithelial cells is reduced by arsenic exposure in part due to an upregulation of matrix metalloprotease-9 (MMP-9; Olsen et al., 2008). Others have shown that MMP-9 regulation and migration in airway epithelial cells can be linked to purinergic signaling (Wesley et al., 2007). Autocrine and paracrine airway epithelial cell purinergic signaling through ATP is important in a variety of innate immune functions, including ciliary beat, salt and water transport, wound closure, mucin secretion, and...
bactericide production (Lazarowski and Boucher, 2009). Extracellular nucleotides act as agonists of P2 receptors that are categorized into the metabotropic P2Y receptors and the ionotropic P2X receptors (Burnstock, 2007; Erb et al., 2006; Schwiebert and Zsembry, 2003). P2Y receptors are G protein–coupled receptors that are activated by a variety of nucleotides and include at least eight family members (Abbracchio et al., 2006). P2X receptors are cation-selective ion channels activated by ATP with seven family members (North, 2002). Activation of both receptor types can result in increased intracellular Ca	extsuperscript{2+} concentration ([Ca	extsuperscript{2+}]	extsubscript{i}).

In this report, we hypothesized that arsenic affects wound repair in airway epithelial cells through alteration of coordinated Ca	extsuperscript{2+} signaling. We examined the effects of submicromolar (0.8µM: −60 µl/g) and micromolar (3.9µM: −290 µg/l) acute (24 h) arsenic exposure on wound-induced Ca	extsuperscript{2+} signaling pathways in an immortalized human bronchial epithelial cell line, 16HBE14o-. This cell line expresses a variety of purinergic receptors, including P2Y1, P2Y2, P2Y4, P2Y6, P2X3, P2X5, P2X6, and P2X7 (Communi et al., 1999; Liang et al., 2005; Ma et al., 2006; Wong et al., 2009; Zsembry et al., 2003, 2004). We show that acute exposure to these low concentrations of Na-arsenite reduced the ability for lung epithelial cells to respond to wound-induced purinergic signaling in a dose-dependent manner. This alteration reduced responses to ATP via both P2Y and P2X receptors. In conclusion, submicromolar and micromolar concentrations of arsenic have direct effects on signaling pathways that contribute to airway epithelial innate immune function and in this manner may lead to or exacerbate lung disease.

MATERIALS AND METHODS

Materials. Minimum essential medium w/Earle’s salts (MEM), Lechner and LaVeck basal media (LHC), Hanks’ Balanced Saline Solution, glutamax, penicillin, streptomycin, TriZOL, Quant-iT OliGreen cDNA quantification kit, Platinum SYBR Green and qPCR SuperMix-UDG, GAPDH, and P2Y2 antibodies were from InVitrogen (Carlsbad, CA). Fibronectin and type I collagen were from Becton-Dickinson (Franklin Lakes, NJ). Fura-2-acetoxymethyl ester (fura-2-AM) and fura-2 were purchased from Calbiochem (La Jolla, CA). ATP, apyrase, and fetal bovine serum (FBS) were from Sigma-Aldrich (St. Louis, MO). iScript cDNA synthesis kit was from BioRad (Hercules, CA). P2X4 antibody was from Alomone labs (Jerusalem, Israel).

Real-time reverse transcriptase (RT)-PCR primers were purchased from IDT-DNA (Coralville, IA). All other chemicals were from Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA).

16HBE14o- cell culture. Growth conditions for 16HBE14o- cells (Gruenert et al., 1995) have been described (Olsen et al., 2008). Briefly, 16HBE14o- cells were grown on a collagen/fibronectin bovine serum albumin (CFB) matrix. Cells were expanded in flasks prior to passage onto 15-mm glass coverslips for microscopy studies. In both cases, cells were grown in a controlled growth medium (CGM) that consisted of MEM supplemented with 10% FBS, 2mM glutamax, penicillin, streptomycin, and amphotericin at 37°C in a 5% CO₂ atmosphere. CGM was replaced every other day until the cells reached confluence. At confluence (~5 days), CGM was replaced with CGM alone (arsenic-free) or with CGM supplemented with 0.8 or 3.9µM arsenic (added as Na-arsenite; 1µM = 74.92 µg/l) for 24 h prior to experimentation.

Intracellular Ca	extsuperscript{2+} concentration ([Ca	extsuperscript{2+}]	extsubscript{i}) measurements in 16HBE14o- cells. 16HBE14o- monolayer cultures were washed with a modified Hanks’ Balanced Saline Solution (HBSS: 1.3mM CaCl₂, 5.0mM KCl, 0.3mM KH₂PO₄, 0.5mM MgCl₂, 0.4mM MgSO₄, 137.9mM NaCl, 0.3mM Na₂PO₄, and 1% glucose additionally buffered with 25mM HEPES, pH 7.4) and loaded for 45 min in 5µM fura 2-AM in HBSS or HBSS supplemented with arsenic. Cells were removed from fura 2-AM loading solution and placed back in the matching HBSS for at least 20 min before Ca	extsuperscript{2+} imaging. For studies in Ca	extsuperscript{2+}-free (5.33mM KCl, 0.44mM KH₂PO₄, 137.9mM NaCl, 0.34mM Na₂PO₄, and 1% glucose additionally buffered with 25mM HEPES, pH 7.4) or Na-free (140mM N-methyl-D-glucamine; KCl, 5.0mM; CaCl₂, 3.0mM and additionally buffered with 10mM Hapes, pH 7.9) solutions, cells were transferred for 3-5 min in the respective solution, then washed again with this solution prior to imaging. Fura-2 fluorescence was observed on an Olympus IX70 microscope with a 40× oil immersion objective after alternating excitation between 340 and 380 nm by a 75 W Xenon lamp linked to a Delta Ram V illuminator (PTI, London, Ontario) and a gel optic line. Images of emitted fluorescence above 505 nm were recorded by an ICCD camera (PTI) and simultaneously displayed on a 21'' Vivitron color monitor. The imaging system was under software control (ImageMaster, PTI) and collected a ratio approximately every 0.6 s. Intracellular Ca	extsuperscript{2+} concentration ([Ca	extsuperscript{2+}]	extsubscript{i}) was calculated by ratiometric analysis of fura-2 fluorescence using equations originally published in Grynkiewicz et al. (1985). A typical field of view contained ~80–110 cells at a resting [Ca	extsuperscript{2+}] (1.24±0.032 nM), estimated to be ≤75nM. A change in [Ca	extsuperscript{2+}], was considered positive if the cell increased [Ca	extsuperscript{2+}], to 200nM or more.

Scrape and localized mechanical wounding of 16HBE14o- cells. Glass coverslips of culture fura-2 loaded 16HBE14o- monolayers were placed on the microscope described above and viewed in differential interference contrast mode. For scrape wounds, a glass micropipette (tip diameter approximately 1 µm) was positioned immediately above a single 16HBE14o- cell with a micromanipulator (Siskiyou, Inc., Grants Pass, OR) under motorized control. Scissors and double-cell wounds were characterized by a rapid loss of fura 2 dye. At the appropriate time, the probe was lowered to puncture an individual cell then dragged across the field of view for approximately 2 s to dislodge cells at which point the probe was raised above the confluent culture. For localized wounds (i.e., 1–2 cells), the glass probe was positioned and optics switched to Ca	extsuperscript{2+} imaging mode as above. At the appropriate time, the probe was lowered to puncture an individual cell (~0.25 s) and immediately retracted to a position well above the monolayer.

ATP dose response curves using the xCELLigence real-time cell analyzer. 16HBE14o- cells were plated in full medium onto 96 well E-plates (Roche Applied Science, Indianapolis, IN) coated with CFB solution and allowed to grow at 37°C and 5% CO₂ while relative impedance of each well was continuously monitored using the real-time cell analyzer (RTCA) device (Roche Applied Science). This device measures relative impedance changes over time at the 96 well surface to determine physiological changes in adherent cells that can be related to proliferation, cytotoxicity, or cellular signaling (e.g., Abassi et al., 2009; Xi et al., 2008). As per manufacturer’s instructions, relative impedance is expressed as a Cell Index in each of the graphs where: Cell Index = (Z	extsubscript{t} − Z	extsubscript{0})/Z	extsubscript{0}, and Z	extsubscript{0} is impedance at a given time point during the experiment (i.e., post-ATP addition), and Z	extsubscript{t} is impedance before the addition of agonist. For reference, a loss of adhesion, such as associated with cytotoxicity, would generate a lower Cell Index whereas an increase in cell adhesion, such as occurs following ATP activation of purinergic receptors, would result in an increase in Cell Index. After Cell Index reached a range indicative of ~80% confluence for the full complement of wells, full-growth medium was replaced with supplemented medium that contained 0, 0.8, or
3.9 \mu M arsenic for 24 h and monitored for cytotoxicity over this period. Cells were then washed with HBSS (supplemented with appropriate arsenic concentrations) and placed at room temperature for 30 min. Cells were then exposed to ATP (in HBSS and appropriate arsenic concentrations) at the listed concentrations. Cell Index responses to ATP were recorded every 30 s for 4 h. Cell responses were collected in triplicate and adjusted to a baseline by ratioing with recordings from cells washed with HBSS alone. Total integrated cellular responses were calculated from the area under the curve for each ATP dose.

**Real-time RT-PCR.** 16HBE14o- cells were grown to confluence in T75 flasks as described above, then exposed to 0, 0.8, or 3.9 \mu M arsenic-supplemented media. After 24 h exposure, RNA was isolated using TRIzol reagent according to the manufacturer’s protocol and quantified with a NanoDrop ND-1000 (ThermoFisher Scientific, Waltham, MA). cDNA was synthesized using iScript cDNA synthesis kit according to the manufacturer’s protocol on an iCycler thermocycler (Bio-Rad, Hercules, CA). cDNA was quantified using Quant-IT OliGreen quantification kit according to the manufacturer’s instructions on a TBS-380 mini-fluorometer (Turner BioSystems, Sunnyvale, CA). Total cDNA, 100 ng per reaction, was amplified with Platinum SYBR Green qPCR SuperMix-UDG kit according to the manufacturer’s instructions in a Rotor-Gene 3000 real-time thermal cycler (Corbett Robotics, San Francisco, CA) under the following conditions: initial hold for 2 min at 95°C and hold for 2 min at 95°C followed by 45 cycles consisting of denaturation 15 s at 94°C; anneal 30 s at 60°C for GAPDH, P2Y2, and P2Y4; 54°C for GAPDH, P2X4, P2X5, P2X6 and P2X7. Human gene-specific primer pairs were designed using IDT-DNA Primer Quest, Primer Bank (Wang and Seed, 2003) and/or MacVector Software. All primers were purchased from IDT-DNA and are listed in Supplementary Table 1. Individual analyses were performed in triplicate on cDNA samples obtained from at least three separate isolations for each experiment.

**Immunoblot.** For whole cell lysate fractions, cells were washed twice with ice cold phosphate-buffered saline and lysed in 10mM Tris–HCL (pH 7.4) containing 1% Triton X-100, 0.5% Nonidet P-40, 150mM NaCl, 1mM EDTA, 1mM NaF, and 1mM Na3VO4 with a 1:100 dilution of protease inhibitor (#P8340; Sigma). Cell lysate was then sonicated and centrifuged for 10 min at 4°C and 12,000 rpm and the supernatant collected. Hydrophobic phase protein isolates were obtained using the CellLytic MEM Protein Extraction Kit (Sigma) per the manufacturer’s instructions. Protein isolations were quantified using a Pierce bichinchoninic acid protein assay (ThermoFisher Scientific) per manufacturer’s instructions. Equal amounts of protein from experiments with 0, 0.8, or 3.9 \mu M arsenic were run out on 4–15% SDS-PAGE gels (Bio-Rad) under denaturing conditions. Proteins were transferred to nitrocellulose and blotted with primary antibodies specific for P2 receptors of interest and GAPDH as a loading control, followed by washes and appropriate HRP-linked secondary antibodies. Blots were developed with the SuperSignal West Femto kit (Pierce, Rockford, IL) per manufacturer’s instructions.

**Statistics.** Data were compared using a one-way ANOVA with Tukey’s multiple comparison test, unless otherwise noted. A value of \( p < 0.05 \) was used to establish significant difference between samples. Figures are graphed ± SEM.

### RESULTS

**Arsenic Reduces \( \mathrm{Ca}^{2+} \) Response following Wounding of Human Airway Epithelial Monolayers**

To evaluate if arsenic altered \( \mathrm{Ca}^{2+} \) response to scrape wounds in airway epithelial cultures, we initially monitored intracellular \( \mathrm{Ca}^{2+} \) concentration ([\( \mathrm{Ca}^{2+} \)]) of 16HBE14o- cells during and immediately following a single scrape wound of cell monolayers (Fig. 1). Monolayer cultures that were not supplemented with arsenic, scrape wounding elicit an immediate increase in [\( \mathrm{Ca}^{2+} \)], cells adjacent to the wound that was propagated throughout the field of view (Fig. 1, top panels). Cultures treated with 0.8 or 3.9 \mu M arsenic at the time of confluence and for 24 h prior to scrape wounding exhibited a reduced propagation of the \( \mathrm{Ca}^{2+} \) signal to adjacent cells; this was most prominent at the highest concentrations tested (Fig. 1, bottom panels). In summary, the scrape wound initiated a coordinated \( \mathrm{Ca}^{2+} \) wave to neighboring cells surrounding the wound, and this signaling was qualitatively reduced by a 24 h exposure to 0.8 or 3.9 \mu M arsenic.

To quantify mechanical wound-induced intercellular \( \mathrm{Ca}^{2+} \) signaling, we used a glass pipette to locally injure one or two airway epithelial cells and monitored intracellular and intercellular \( \mathrm{Ca}^{2+} \) signaling in the presence or absence of arsenic (Fig. 2; Supplementary movie). In untreated cells, localized mechanical wounds caused an immediate increase of [\( \mathrm{Ca}^{2+} \)], in cells surrounding the wounded area that resulted in an intercellular \( \mathrm{Ca}^{2+} \) wave of 27.4 ± 2.3 cells (\( n = 19 \); Fig. 2B). A 24-h incubation in arsenic-treated media resulted in a significant and dose-dependent reduction in the mechanical wound-induced \( \mathrm{Ca}^{2+} \) wave. Monolayers incubated with 0.8 \mu M arsenic exhibited \( \mathrm{Ca}^{2+} \) waves of 18.3 ± 2.0 cells (\( n = 11 \)) and monolayers incubated with 3.9 \mu M arsenic exhibited \( \mathrm{Ca}^{2+} \) waves of 13.2 ± 1.5 cells (\( n = 36 \)).

In addition to changes in the number of cells participating in the propagated \( \mathrm{Ca}^{2+} \) signal, we recorded arsenic-induced alterations in the intracellular \( \mathrm{Ca}^{2+} \) dynamics in cells that responded to the wounding of a neighboring cell. Clear differences in individual traces of wound-adjacent cells from single experiments were observed (Fig. 3A). In arsenic-free cultures, wound-adjacent cells responded with a rapid and coordinated increase in [\( \mathrm{Ca}^{2+} \)], that slowly recovered to baseline [\( \mathrm{Ca}^{2+} \)], over 60–120 s. Although the typical wound-adjacent cells treated with 0.8 \mu M arsenic similarly displayed a quick increase in [\( \mathrm{Ca}^{2+} \)], in response to the local wound, they typically showed a more rapid recovery to baseline [\( \mathrm{Ca}^{2+} \)]. In contrast, the wound-adjacent cells from cultures treated with 3.9 \mu M arsenic displayed a slower and reduced response in their [\( \mathrm{Ca}^{2+} \)] signaling.

To quantify localized wound-induced \( \mathrm{Ca}^{2+} \) signaling changes in response to arsenic, peak \( \mathrm{Ca}^{2+} \) changes in cells immediately adjacent to the wounded cell were aligned and averaged for arsenic-free (\( n = 76 \)) and arsenic-exposed (0.8 \mu M, \( n = 68 \); or 3.9 \mu M, \( n = 183 \)) cultures (Fig. 2B). Total \( \mathrm{Ca}^{2+} \) signal was estimated by calculating the area under the curve with an [\( \mathrm{Ca}^{2+} \)] of 200nM taken as a baseline for recovery from the active \( \mathrm{Ca}^{2+} \) signaling (shaded area in each graph). The area under the curve in the 0.8 \mu M arsenic-treated cells was only 82.1% of the arsenic-free cultures, and the 3.9 \mu M arsenic-treated cells was 64.3% of the untreated cells.
To evaluate how the relative $\text{Ca}^{2+}$ signal was altered, we first analyzed the peak $[\text{Ca}^{2+}]_i$ changes in all three treatments (Figs. 3B and 3D). In the arsenic-free cells, peak $[\text{Ca}^{2+}]_i$ rose to $569 \pm 18$ nM. This value was not significantly different than that observed in the 0.8 $\mu$M arsenic-treated cells ($565 \pm 21$ nM). An exponential fit to estimate recovery time from peak $[\text{Ca}^{2+}]_i$ to 200 nM for the arsenic-free and 0.8 $\mu$M wound-adjacent cells, however, revealed a faster recovery in the 0.8 $\mu$M arsenic-treated cells ($t_{1/2} = 7.7$ s) compared with the arsenic-free cells ($t_{1/2} = 13.8$ s). In contrast, the relative reduction in total $\text{Ca}^{2+}$ signal observed in the 3.9 $\mu$M-treated cells was largely due to a significant drop in peak $[\text{Ca}^{2+}]_i$ ($448 \pm 14$ nM; Figs. 3B–D). From these data, we concluded that localized wounds produce coordinated changes in $[\text{Ca}^{2+}]_i$ that were altered by 24-h exposure to submicromolar and micromolar concentrations of arsenic in a dose-dependent manner and that alteration in $\text{Ca}^{2+}$ signaling likely involved multiple mechanisms.

**Loss of ATP Signaling Limits $\text{Ca}^{2+}$ Wound Response in Airway Epithelial Cells**

Wound and mechanical stimulation-induced $\text{Ca}^{2+}$ signaling in airway epithelial cells have been associated with nucleotide agonists (e.g., Wesley et al., 2007). The scrape wound-induced $[\text{Ca}^{2+}]_i$ response in 16HBE14o- cells was largely eliminated in the presence of 50 U/ml apyrase, an enzyme that cleaves phosphates from nucleoside triphosphates and diphosphates and thus restricts purinergic signaling activities (Fig. 4A). Similar to the scrape wounds, apyrse significantly reduced the number of cells participating in the localized wound-induced $\text{Ca}^{2+}$ wave (Figs. 4B and 4C; Supplementary movie). In the presence of 50 U/ml apyrase, cultures grown with or without...
Arsenic displayed a severe reduction in the intercellular Ca^{2+} wave: arsenic-free cultures displayed Ca^{2+} waves of 2.8 ± 0.4 cells (n = 11); cultures treated with 0.8μM displayed Ca^{2+} waves of 4.2 ± 0.6 cells (n = 6); and cultures treated with 3.9μM arsenic displayed Ca^{2+} waves of 3.6 ± 0.5 cells (n = 18; Fig. 4C). We conclude that the wound-induced Ca^{2+} signaling observed in these studies is largely mediated by the recognition of extracellular nucleotides.

**FIG. 2.** Arsenic exposure reduces localized wound-induced intercellular Ca^{2+} waves in human airway epithelial cells. (A) Monolayers of 16HBE14o- cells were treated with arsenic-free or arsenic-supplemented media (0.8 or 3.9μM) for 24 h. Local wounding (1–2 cells; gray area) resulted in a propagated change in [Ca^{2+}]_{i} among neighboring cells that was reduced in arsenic-treated cultures. (B) Quantification of Ca^{2+} waves displayed a dose-dependent reduction in the number of cells participating in the Ca^{2+} wave as arsenic concentration was increased. Cells that reached a peak [Ca^{2+}]_{i} ≥ 200nM were included as participating in the Ca^{2+} wave. Data in (B) are graphed ± SEM. Symbols represent significant differences (p < 0.05) in comparison to arsenic-free (*) or to 0.8μM (^).
ATP-Dependent Ca\textsuperscript{2+} Signaling in Airway Epithelial Cells Is Disrupted by Arsenic

In an effort to identify rank order of nucleotides and to preliminarily identify nucleotide receptors on the 16HBE14o-cells, we used a variety of nucleotide agonists to activate Ca\textsuperscript{2+} signaling pathways (Fig. 5; \( n \geq 4 \) for all treatments). The ensuing Ca\textsuperscript{2+} response sensitivity pattern: ATP > UTP >> UDP \( \approx \) ADP \( \approx \) AMP was consistent with activation of P2Y\textsubscript{2} as the primary P2Y nucleotide receptor in our cell model (Burnstock, 2007). Because ATP is released during wounding and was the best nucleotide agonist tested, we used a high throughput, cell population-based screen (RTCA, for description, see Methods) to directly evaluate the physiological response to ATP on arsenic-exposed airway epithelial cells (Abassi et al., 2009; Xi et al., 2008). We treated 16HBE14o-cells to varying concentrations of arsenic (0, 0.8, and 3.9\mu M) for 24 h and monitored relative cellular impedance as a result of exogenously applied ATP (Fig. 6). The 24-h exposure to arsenic-free or arsenic-supplemented medium resulted in similar impedance measurements, consistent with a lack of arsenic-induced cytotoxicity previously demonstrated by live/dead fluorescent assays of 16HBE14o-cells (Olsen et al., 2008). The response to ATP by arsenic-free and arsenic-exposed (0.8 or 3.9\mu M) 16HBE14o-cells, however, was significantly reduced by arsenic exposure (Fig. 6). Examination of physiological to supraphysiological signaling doses of ATP (250nM to 10\mu M; \( n = 6 \) for each arsenic/ATP dose; Figs. 6A–C) shows a reduction in both the peak signaling response and in the integrated signaling response measured over a 4-h experiment. Full dose-response curves of arsenic-free and arsenic-treated cells (300nM to 100\mu M ATP) constructed from the peak response (not shown) or the integrated physiological output (area under the curve) showed a significant loss of

![Graphs showing effects of arsenic on Ca\textsuperscript{2+} signaling](https://academic.oup.com/toxsci/article-abstract/121/1/191/1640492)
16HBE14o- response to ATP following 24-h arsenic exposure (Fig. 6D).

Using these studies as a baseline, we tested lung epithelial Ca\(^{2+}\) signaling in response to varying concentrations of ATP following 24-h exposure to arsenic with digital imaging microscopy. As shown in Figures 1–4, Ca\(^{2+}\) signaling is an important early response to cellular wounding and provides both a sensitive and quantifiable early signaling response to nucleotides. In response to physiologically high amounts of ATP (10\(\mu\)M), control (arsenic-free; \(n = 4\)) and 0.8\(\mu\)M arsenic-treated (\(n = 4\)) 16HBE14o- cultures displayed increases of [Ca\(^{2+}\)] of at least 200nM or more within 60 s of application in > 95% of the cells in culture. Cells treated with 3.9\(\mu\)M arsenic (\(n = 3\)) displayed a slightly lower delayed and more variable response (79.2 ± 14.4%), however, the percentage of cells activated over the 3-min experiment was not significantly different from the other treatments (Fig. 7A). When ATP concentration was reduced to levels reported to be within signaling range of airway epithelial cells (Okada et al., 2006), a significant loss of Ca\(^{2+}\) response was detected in the arsenic-treated cultures compared with the untreated cultures. In confluent cultures without added arsenic, 60.4 ± 8.9% (\(n = 10\))
of 16HBE14o- cells responded to 1 μM ATP (Figs. 7A and 7B). In contrast, cultures treated for 24 h with 0.8 μM or 3.9 μM arsenic prior to exposure to 1 μM ATP displayed [Ca^{2+}]_{i} increases in 30.3 ± 7.3% (n = 13) and 29.8 ± 7.1% (n = 6), respectively (Figs. 7A and 7B). When ATP concentrations were lowered further to 500nM, the arsenic inhibition of Ca^{2+} response persisted: 27.3 ± 5.3% (n = 7) of control cells responded while only 4.4 ± 2.6% (n = 4) of the cells treated with 0.8μM arsenic and 1.7 ± 1.1% (n = 3) of the cells treated with 3.9μM arsenic responded (Fig. 7A). Similar to the wound experiments above, the duration of [Ca^{2+}]_{i} increase appeared shortened in cells treated with arsenic. These data demonstrate that a 24-h exposure to environmentally relevant levels of arsenic reduces ATP-dependent Ca^{2+} responses in airway epithelial cells.

Determination of P2Y Receptor Contribution to Arsenic-Inhibited, Purinergic Ca^{2+} Signaling

Extracellular ATP can induce Ca^{2+} signaling in airway epithelial cells through G protein–coupled P2Y receptors that leads to release of Ca^{2+} from intracellular stores. To isolate the contribution of P2Y receptors to the reduction of overall wound-induced Ca^{2+} signal by arsenic, we repeated our ATP application and local wound experiments in the absence of extracellular Ca^{2+} (Fig. 8). Under these conditions, [Ca^{2+}]_{i} is limited to release from intracellular stores and thus eliminates the contribution from ionotropic P2X receptors. Removal of extracellular Ca^{2+} also potentiates 16HBE14o- Ca^{2+} release in a manner that is independent of arsenic exposure (compare Figs. 7A and 8A). In the Ca^{2+}-free conditions, 10μM ATP was sufficient to activate > 98% of the cells in untreated (n = 3) or 0.8μM arsenic-treated (n = 3) and > 92% of the cells from the 3.9μM arsenic-treated (n = 4) samples (Fig. 8A). Unlike the Ca^{2+}-containing solutions, the results after application of 1μM ATP in Ca^{2+}-free solutions were similar between the arsenic-
FIG. 7. Arsenic reduces ATP-induced Ca\textsuperscript{2+} response in human airway epithelial cells. 16HBE14o- cells were grown to monolayers and treated with arsenic-free or arsenic-supplemented media (0.8 or 3.9 µM) for 24 h. Cultures were monitored for [Ca\textsuperscript{2+}] changes in response to bath application of varying ATP concentrations. (A) After application of 10 µM ATP, there were no significant differences in the percentage of cells responding (increased [Ca\textsuperscript{2+}], to ≥ 200 nM) between 0, 0.8, or 3.9 µM treated cells. In comparison with the arsenic-treated cultures, a significantly higher percentage of cells in the arsenic-free cultures displayed [Ca\textsuperscript{2+}] responses when 1 µM ATP was applied. At 500 nM ATP, a significantly limited response was observed in the cells treated with 0.8 or 3.9 µM arsenic, whereas untreated samples displayed responses in > 25% of cells. (B) Single field of cells from cultures treated with or without arsenic and monitored over time for [Ca\textsuperscript{2+}], after the addition of 1 µM ATP. An increased number of arsenic-free cells show an elongated Ca\textsuperscript{2+} response when compared with the arsenic-treated cultures. Data in (A) are graphed ± SEM; ‘‘*’’ represents significant difference in Ca\textsuperscript{2+} response between arsenic-free and [ATP]-matched arsenic-treated cells (p < 0.05).
free and 0.8 μM treated samples (77.8 ± 10.4%, n = 3 and 78.4 ± 6.1%, n = 3, respectively), however, the 3.9 μM treated samples were significantly reduced in their response (24.6 ± 2.1%, n = 3). At the lowest ATP dose tested (500 nM), the arsenic exposure displayed a full dose-response effect. Arsenic-free monolayers showed a positive response to ATP (90.6 ± 2.2%, n = 3), 0.8 μM arsenic-treated monolayers displayed a significantly reduced response (64.1 ± 11.3%, n = 4), and another significant reduction in signaling was seen in the 3.9 μM arsenic-treated monolayers (29.4 ± 8.2%, n = 3).

To clarify the role for P2Y receptors in wound-induced Ca²⁺ signaling under arsenic exposure, we repeated the localized wound experiments in the Ca²⁺-free HBSS. In the absence of extracellular Ca²⁺, the overall Ca²⁺ wave size was reduced as arsenic exposure was increased. The absence of extracellular Ca²⁺ also potentiated this response to ATP, extending the wound-induced response to cells outside the field of view and precluding a full quantification between Ca²⁺ wave sizes similar to that shown in Ca²⁺-containing medium (e.g., Fig. 2B). The evaluation of the wound-adjacent cells, however, pointed to a functional loss of response of P2Y receptors only at the highest arsenic doses tested (Figs. 8B–D).

FIG. 8. Role for P2Y receptors in arsenic-altered ATP-dependent Ca²⁺ signaling. 16HBE14o- cells were grown as described in Figure 3. Prior to experimentation, cultures were washed with Ca²⁺-free media to assay Ca²⁺-release pathways independent of Ca²⁺ influx. (A) Direct application of ATP resulted in changes in [Ca²⁺]. Cultures exposed to 0.8 μM arsenic for 24 h displayed a significant reduction in Ca²⁺ response compared with arsenic-free cultures at the 500 nM ATP application. Cultures treated with 3.9 μM arsenic for 24 h displayed a significantly lower response to 1 μM and 500 nM ATP. (B–F) Localized mechanical wounding also resulted in changes in [Ca²⁺] in wound-adjacent cells and in Ca²⁺ waves that frequently propagated outside the field of view. (B) Ca²⁺ responses in wound-adjacent cells were near 100% in arsenic-free and 0.8 μM arsenic-treated cultures, whereas cultures with 3.9 μM showed a significant decrease. (C) Aligned mean Ca²⁺ response (±SEM) in wound-adjacent cells are plotted for arsenic-free, 0.8 μM, and 3.9 μM arsenic-treated cultures; gray area represents relative Ca²⁺ signal with 200 nM [Ca²⁺], taken as a signaling baseline. (D) Peak Ca²⁺ change and relative Ca²⁺ signal in wound-adjacent cells are significantly reduced in the 3.9 μM arsenic-treated cultures. (E) Real-time RT-PCR of 16HBE14o- cells shows that P2Y₂ mRNA is reduced in a dose-dependent manner following arsenic exposure and the less robustly expressed P2Y₄ receptor does not change expression following arsenic exposure. (F) Immunoblot of hydrophobic phase protein isolates stained positively for P2Y₂ expression in arsenic-free and arsenic-treated cultures. For all experiments, “*” indicates significant difference from arsenic-free cultures, “^” indicates significant difference from 0.8 μM arsenic-treated cultures (p < 0.05).
arsenic-treated cells (89.9 ± 6.7%, n = 15) but was significantly lower in the 3.9 μM arsenic-treated samples (79.5 ± 8.4, n = 9; p < 0.05 vs. control; Fig. 8B). When the average Ca^{2+} signals in wound-adjacent cells were plotted and compared (Fig. 8C), the difference in the time from peak to recovery between the arsenic-free and 0.8 μM arsenic-treated samples observed in conditions with Ca^{2+} in the medium was no longer evident (i.e., not from arsenic effects on P2Y receptors). Similarly, the peak [Ca^{2+}] changes and the relative Ca^{2+} signal (area under the curve) were only significantly different at the highest arsenic doses tested (Fig. 8D). These data support P2Y-mediated Ca^{2+} release as the primary response that underlies the observed [Ca^{2+}] change in wound-adjacent cells and 24-h exposure to 3.9 μM significantly reduces this P2Y-mediated Ca^{2+} release.

To further explore arsenic effects on P2Y receptor Ca^{2+} signaling, we used real-time RT-PCR to evaluate changes in mRNA expression following 24-h arsenic exposure. We focused our evaluations on P2Y2 and P2Y4 receptors because they are expressed in airway epithelial cells (Communi et al., 1999; Lazarowski and Boucher, 2009), displayed nucleotide responses consistent with our findings (Fig. 5), and were the prominent P2Y receptors expressed in 16HBE14o- cells under our growth conditions (not shown). In response to 24-h arsenic

**FIG. 9.** Role for P2X receptors in arsenic-altered ATP-dependent Ca^{2+} signaling. 16HBE14o- cells were grown as described in Figure 3. Prior to experimentation, cultures were washed with Na-free medium to assay Ca^{2+}-influx pathways via digital imaging microscopy. (A) Individual traces for arsenic-free and arsenic-treated cells (24 h in 0.8 or 3.9 μM arsenic-supplemented media) in response to increasing doses of zinc (Zn) used to directly activate P2X receptors (Liang et al., 2005). Arsenic-free cultures respond to 5 μM Zn as well as subsequent 10 μM and 20 μM additions with increases in [Ca^{2+}]; washout of Zn restores [Ca^{2+}] to baseline. As arsenic is increased, an increase in Zn is required for activation of Ca^{2+} influx. (B) Representative [Ca^{2+}] traces of individual cells following addition of 10 μM Zn. As arsenic exposure goes up, the response to Zn is reduced. (C) The percentage of cells responding to addition of 10 μM Zn with a change in [Ca^{2+}] ≥ 200 nM are graphed for 24 h treatment with 0, 0.8, or 3.9 μM arsenic-supplemented media. There is a dose-dependent loss of Ca^{2+} response as arsenic is increased. (D) Real-time RT-PCR for P2X4 and P2X5 receptor mRNA expression after 24 h treatment with 0, 0.8, or 3.9 μM arsenic-supplemented medium. P2X4 mRNA was significantly reduced only at the highest arsenic dose tested. (E) Immunoblot of protein from whole-cell lysates showed no visible changes in P2X4 protein expression between arsenic-free and arsenic-treated cell cultures. For all experiments, ‘‘*’’ indicates significant difference for arsenic-free cultures, ‘‘^’’ indicates significant difference from 0.8 μM cultures (p < 0.05).
exposure, the P2Y2 receptor displayed a dose-dependent reduction in mRNA expression, whereas the P2Y4 receptor remained unchanged (Fig. 8E). Using immunoblot, we detected P2Y2 protein expression in arsenic-free and arsenic-exposed cells (Fig. 8F). Similar to other reports (e.g., Klepeis et al., 2004), it is noted here that the available antibodies to detect native P2Y2 receptors display protein bands at the predicted molecular weight as well as those outside of the predicted molecular weight. This multiple banding precludes densitometric analysis and thus comparisons of protein expression across arsenic exposure. These data are qualitatively consistent with P2Y2 receptor as the primary sensor for ATP-induced Ca2+ signaling in 16HBE14o- cells and that P2Y2 is expressed in arsenic-free as well as 24-h arsenic-treated cultures.

**Determination of P2X Receptor Contribution to Arsenic-inhibited, Purinergic Ca2+ Signaling**

Extracellular ATP can act as an agonist of the ligand-gated, nonspecific cation channels in the P2X receptor family to alter Ca2+ signaling in airway epithelial cells (Erb et al., 2006; Schwiebert and Zsembery, 2003). We used Zinc (Zn) in Na+-free medium to directly activate airway epithelial P2X receptors (Liang et al., 2005) and evaluate arsenic-specific effects that contribute to the observed altered Ca2+ signaling (Figs. 9A–C). Under these conditions, airway epithelial cells increased [Ca2+]i in response to 5µM Zn and displayed an increasingly robust response as extracellular Zn was increased to 10 or 20µM (n = 4; Fig. 9A). In contrast, cells exposed to 0.8µM arsenic for 24 h were largely unresponsive to 5µM Zn and displayed a reduced Ca2+ response to 10µM Zn (n = 3). Analysis of Zn response in the 3.9-µM arsenic-treated monolayers after a 24-h incubation showed a reduced response to all three Zn concentrations tested (n = 4). In separate experiments to evaluate a dose-dependent reduction in P2X receptor activity, exogenous application of 10µM Zn resulted in delayed and reduced increases in [Ca2+]i as arsenic concentration was increased (Fig. 9B). Quantitative analyses showed that 88.8 ± 3.2% (n = 4) of cells in arsenic-free solutions responded to 10µM Zn with a [Ca2+]i of at least 200nM (Fig. 9C). The response to Zn was significantly reduced when the cells were incubated for 24 h in 0.8µM arsenic (69.0 ± 2.1%, n = 4) or 3.9µM arsenic (49.5 ± 7.2%, n = 4). These changes represent a dose-dependent reduction in P2X receptor function in response to 24-h exposure to submicromolar and micromolar arsenic. This type of reduction in Ca2+ influx would likely result in a faster recovery of [Ca2+]i to baseline following Ca2+ release (e.g., Fig. 3B).

To further explore arsenic effects on P2X receptor Ca2+ signaling, we used real-time RT-PCR to evaluate changes in mRNA expression of individual P2X receptors reported to be expressed in airway epithelial cells (Liang et al., 2005; Ma et al., 2006; Zsembery et al., 2004). Of the four P2X receptors identified as expressed in airway epithelial cells (P2X4, P2X5, P2X6, and P2X7), P2X4 receptor displayed a robust expression under our growth conditions. P2X5 receptor displayed measurable mRNA at a much lower expression level, and P2X6 and P2X7 receptors were not expressed (not shown). In response to 24-h incubation with 3.9µM arsenic, P2X4 mRNA expression was significantly reduced, whereas P2X5 remained unaffected (Fig. 9D). Examination of protein expression using immunoblot showed that P2X4 was expressed in arsenic-free and arsenic-exposed cells but not significantly altered (Fig. 9E). The lack of a dose-dependent change in mRNA and/or protein expression suggests that arsenic may physically block or functionally alter P2X4 receptor activity to effectively reduce the ATP-induced Ca2+ signal.

**DISCUSSION**

Arsenic is unique in that it has been shown to be a lung toxicant both via inhalation and via ingestion. In the case of arsenic-induced lung cancer, both inhalation and ingestion have similar dose effects (Smith et al., 2009). Chronic arsenic exposure through contaminated drinking water represents one of the most widespread environmental exposures of toxicants worldwide. Such exposure has detrimental effects on human health, including a variety of lung diseases (Kapaj et al., 2006; Mazumder, 2007; Schuhmacher-Wolz et al., 2009), most notably bronchiectasis (Mazumder et al., 2005; Smith et al., 2006). Bronchiectasis is characterized by repeat airway infections, a loss of airway innate immune function and a compromised airway epithelial barrier. Recent studies in mouse models have linked submicromolar and micromolar arsenic exposure to increased susceptibility to viral infection of the airway epithelium (Kozul et al., 2009a), alteration of a number of lung innate immune response genes (Kozul et al., 2009b) and alteration of innate immune response proteins collected from bronchiolar lavage (Lantz et al., 2007). In both the transcriptional (Kozul et al., 2009b) and translational (Lantz et al., 2007) profiling studies, a common theme of reduced wound repair in the airway was uncovered. These studies are supported by limited human drinking water studies where wound repair proteins were altered in sputum of humans exposed to chronic submicromolar (~20 ppb) arsenic (Josyula et al., 2006) or increased in in utero exposure of arsenic (as measured from urine samples of pregnant mothers) leading to reductions in innate immunity (Raqib et al., 2009).

In this report, we used an acute (i.e., 24 h) exposure of submicromolar (0.8µM) and micromolar (3.9µM) levels of arsenic that was directly applied to cell monolayers as Na-arsenite supplemented medium to evaluate arsenic effects on airway epithelial innate immunity via paracrine signaling. Our range of arsenic concentrations is well below levels associated with inhalation and chronic drinking water studies where arsenic is correlated with lung disease (e.g., Kapaj et al., 2006; Mazumder, 2007; Schuhmacher-Wolz et al., 2009; Smith et al.,
ARSENIC ALTERS AIRWAY ATP-CA\(^{2+}\) SIGNALING

203

2009). It is easy to assume that inhalation exposures would produce relatively high arsenic doses at the lung epithelium, but it is less obvious how much arsenic would reach the lung following ingestion. Oral administration of arsenic in mouse models have shown accumulation of ingested arsenic into the lung within 1–4 h of a single administration, and arsenic concentration was doubled if administration was repeated over 9 days (Hughes et al., 2003; Kenyon et al., 2005). Despite results from these studies, the concentration of arsenic in human lung epithelium under conditions of chronic exposure to contaminated drinking water remains elusive. Acute exposure models as used in this study typically require higher concentrations of toxin/toxicant to model chronic interactions, and preliminary results from our laboratory using chronic lower level exposure models (data not shown) are consistent with this finding. We believe that the cell culture arsenic exposure model used herein provides an opportunity to delineate mechanistic insight into arsenic toxicity in the lung epithelium under conditions that are prevalent in real-world exposures.

ATP is an important paracrine signal that helps to coordinate cellular physiology in the airway epithelium (Buchheimer and Linden, 2004; Lazarowski and Boucher, 2009; Lieb et al., 2002; Schwiebert and Zsembery, 2003; van der Vliet, 2008). Release of ATP following wounding and subsequent activation of P2Y\(_{2}\) receptors contributes to proper wound response in airway and other epithelial cell types (Klepeis et al., 2004; Wesley et al., 2007). Although many factors released from a wounded cell may initiate intercellular Ca\(^{2+}\) signaling following wounding, our localized wound experiments suggest that extracellular ATP is the dominant primary messenger for coordinated changes in [Ca\(^{2+}\)]. The question remains if release of ATP under \textit{in vitro} experiments can model paracrine signaling \textit{in vivo} (reviewed in Schwiebert and Zsembery, 2003). Direct measurements of ATP at the airway epithelial apical surface shows a resting concentration of between 1 and 10nM and a signaling-induced ATP concentration of 1–1.2\(\mu\)M ATP (Okada et al., 2006). The arsenic-induced loss of ATP Ca\(^{2+}\) signaling we report herein occurs within the projected signaling range and not at levels of ATP considered to be physiologically high for the airway (e.g., 10\(\mu\)M or greater).

Important early response in airway epithelial wound healing is comprised of changes in the interaction between cells and the basal lamina that include attachment, cell spreading, and cell migration (reviewed in Crosby and Waters, 2010; Puchelle et al., 2006). These changes are followed by proliferation and differentiation that help to restore the epithelium. The immortalized 16HBE14o- cell line has provided a model for understanding some of the early events associated with repair (Crosby and Waters, 2010). It has been proposed that ATP release by wounded cells is an early and important local signal for the initiation of wound repair (Wesley et al., 2007). We explored the effects of extracellular ATP on cellular physiological response through use of the high-throughput RTCA assay (Fig. 6). This assay allowed us to look at the longer term (4 h) response of 16HBE14o- cells to ATP following arsenic exposure as opposed to the immediate Ca\(^{2+}\) signaling response also demonstrated in this report. Significantly, pretreatment with arsenic altered the physiologic response to ATP throughout the 4-h experiment. These data indicate a role for arsenic-altered ATP activated pathways downstream of Ca\(^{2+}\) signaling consistent with decreased cellular adhesion and/or cell spreading.

The localized wound model used in this report allowed for quantification of intercellular Ca\(^{2+}\) signaling and the ability to assess arsenic-induced effects on the intracellular dynamics of Ca\(^{2+}\) signaling in cells immediately neighboring the wounded cells. Purines such as ATP can bind to purine receptors to activate cell signaling (Burnstock, 2007; Erb et al., 2006; Schwiebert and Zsembery, 2003). The P2X family of ligand-gated ion channels and the P2Y family of G protein–coupled receptors can both contribute to Ca\(^{2+}\) signaling in the cell. Several subtypes in these families have been reported as expressed in conducting airway epithelial cells and thus make likely targets for dysregulation of Ca\(^{2+}\) signaling following arsenic exposure (Communi et al., 1999; Liang et al., 2005; Ma et al., 2006; Wong et al., 2009; Zsembery et al., 2003, 2004). From the experiments presented herein, we propose that localized wounding results in the release of a bolus of ATP sufficient to initiate a coordinated increase in [Ca\(^{2+}\)]\(_{i}\) among neighboring cells. Exposure to lower levels of arsenic (e.g., 0.8\(\mu\)M) is sufficient to reduce the extent of this intercellular Ca\(^{2+}\) wave through reduction in P2Y receptor (more specifically P2Y\(_{2}\) receptor) function in airway epithelial cells but not sufficient to reduce the P2Y-Ca\(^{2+}\) responses in cells closest to the wound. This occurs because ATP concentration is naturally reduced by diffusion and ectonuclease breakdown and thus tends to be quickly lowered as the distance from the wound is increased. This interpretation is supported by the bath-applied experiments that demonstrate an ATP concentration-dependent Ca\(^{2+}\) response. At higher arsenic concentrations (e.g., 3.9\(\mu\)M), P2Y function is sufficiently reduced to limit P2Y-dependent ATP responses even at the high concentrations endogenously released following a localized wound. Evaluation of P2Y\(_{2}\) using real-time RT-PCR showed a dose-dependent decrease in mRNA following 24-h arsenic exposure. Consistent with a dose-dependent reduction, but not elimination of P2Y\(_{2}\) mRNA, P2Y\(_{2}\) protein could be detected in arsenic-free and arsenic-treated cultures. Although P2Y\(_{2}\) receptor turnover has been shown to be on the order of 4 h (Garrad et al., 1998), we cannot conclude that arsenic effectively lowers the P2Y\(_{2}\) proteins available at the plasma membrane. Alternative explanations for a reduction in P2Y\(_{2}\) receptor function are via direct or indirect interactions between arsenic and the receptor; further studies are required to elucidate these mechanisms.

The experiments carried out in Na\(^{+}\)-free media and Ca\(^{2+}\)-free media helped to elucidate the contribution by P2X receptors to the arsenic-altered Ca\(^{2+}\) response. First, the direct
activation experiments with Zn in Na+-free media demonstrated a clear dose-dependent loss of Ca²⁺ influx with increased arsenic concentrations. Second, the loss of the change in shape of the Ca²⁺ recovery between arsenic-free and 0.8μM arsenic exposure in the Ca²⁺-free medium suggested a need for Ca²⁺ influx in this event. Taken together, these data suggest that P2X receptors extend the Ca²⁺ signal within the cell by allowing for Ca²⁺ influx, and the arsenic block on P2X receptors limits the full Ca²⁺ response to ATP. The lack of any changes in P2X mRNA or protein expression upon arsenic exposure that caused the initial Ca²⁺ influx differences (i.e., 0.8μM) supports an arsenic-induced posttranslational modification of P2X receptors. Because arsenic has been shown previously to interact with free thiol groups and such groups are abundant on the surface of P2X receptors (North, 2002; Schiewbert et al., 2005), these sites should be considered potential targets for arsenic. More specifically, it has been proposed that histidines may cluster with cysteines in the extracellular domain of P2X receptors to form a binding motif reminiscent of a zinc (Zn) finger (Schiewbert et al., 2005). Consistent with arsenic interruption at this site, arsenic has been shown to bind to Zn finger domains on the estrogen receptor-α (Kitchin and Wallace, 2005) and to compete for binding with Zn on a zinc finger domain of the DNA repair protein poly(ADP-ribose) polymerase-1 (PARP-1; Ding et al., 2009). In effect, the extracellular surface of P2X receptors provides ideal binding sites for arsenic to directly alter P2X receptor function.

Although P2X and P2Y were the primary molecular targets examined in this study, we cannot rule out other components of the Ca²⁺ signaling apparatus that could be changed by arsenic and contribute to the altered Ca²⁺ footprints presented. Additional potential targets for arsenic disruption are the channels and pumps related to Ca²⁺ release (ryanodine receptor and inositol triphosphate receptor) and recovery (plasma membrane calcium ATPase and the sarcoplasmic and endoplasmic reticulum calcium ATPase: SERCA). In particular, SERCAs have been shown to be denatured following arsenic exposure and lead to a cellular stress response (Senisterra et al., 1997). Further examination of arsenic effects on the Ca²⁺ signaling pathway is needed to fully define the mechanisms involved in its toxicity.

Purinergic signaling contributes to cellular airway innate immunity in a variety of ways, from coordinated ciliary beat to release of potent factors that communicate cell recruitment or themselves have antimicrobial function and to protection from oxidative stress (Ahmad et al., 2006; Boots et al., 2009; Lazarowski and Boucher, 2009; Lieb et al., 2002). Notably included in this response system is the ability of the epithelium to close a barrier breach in a sufficient and timely manner. ATP released by scrape wounding airway epithelial cells has been shown to act via Ca²⁺ signaling to regulate MMP-9 and contribute to proper wound repair (Wesley et al., 2007). Our previous report with the 16HBE14o- model showed a loss of wound repair in airway epithelial cells via an MMP-9 regulation dysfunction (Olsen et al., 2008); we now show purinergic signaling as a second cellular target of arsenic in this model system. Alterations in airway ATP signaling have been correlated with chronic lung diseases including asthma and COPD (Bratke et al., 2008; Idzko et al., 2007; Lommatzsch et al., 2010). In mouse model experiments focused on emphysema, changes in lung innate immune cell recruitment was associated with both P2Y₉ and P2X₇ ATP receptors (Cicko et al., 2010; Lucattelli et al., 2010). These experiments highlight the importance of P2 receptors in nonepithelial-based lung signaling and their contributions to chronic airway disease. It remains of interest to evaluate if purinergic signaling pathways may be a target for reduced innate immune function to treat arsenic-associated lung diseases. Finally, paracrine-mediated Ca²⁺ signaling is important in multiple tissues affected by systemic arsenic exposure, and thus, disease implications reported here may not be restricted to the lung.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci. oxfordjournals.org/.

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