Arsenic Inhibits CFTR-Mediated Chloride Secretion by Killifish (*Fundulus heteroclitus*) Opercular Membrane

Caitlin R. Stanton^{1,2*}, Renee Thibodeau^{1,2*}, Alexander Lankowski^{1,2,3}, Joseph R. Shaw^{2,3,4}, Joshua W. Hamilton^{3,5} and Bruce A. Stanton^{1,2,3}

¹Department of Physiology, Dartmouth Medical School, Hanover, NH 03755, ²Mt. Desert Island Biological Laboratory, Salisbury Cove, ME 04672, ³Center for Environmental Health Sciences, Dartmouth Medical School, Hanover NH 03755, ⁴Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, ⁵Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03755, *These authors contributed equally to this manuscript

Key Words

CFTR • Arsenic • Chloride channel • *Fundulus* • Ion transport • Cystic fibrosis • Cl secretion • Environmental toxicant

Abstract

Killifish are euryhaline teleosts that normally experience rapid changes in the salinity of the swim water. Acclimation to seawater is mediated by cortisol, which by activating glucocorticoid receptors, up-regulates CFTR mediated Cl⁻ secretion in the gill and operculum. Arsenic, a toxic metalloid that naturally occurs in the aquatic environment, has been shown to disrupt glucocorticoid hormone-mediated regulation of genes. Because little is known about the effects of environmentally relevant levels of arsenic on ion channels and salt homeostasis, studies were conducted to examine the effects of arsenic on the ability of killifish to acclimate to increased salinity. Arsenic in the swim water or administered by

intraperitoneal injection prevented acclimation. To determine if arsenic blocked acclimation by inhibiting CFTR mediated Cl⁻ secretion (Isc), opercular membranes were isolated and mounted in Ussing chambers and the effects of arsenic on Isc were measured. Arsenic (24 hr exposure) reduced Isc in opercular membranes isolated from salt water acclimated killifish. In addition, arsenic acutely (5-10 minutes) and reversibly inhibited Isc with an IC₅₀ = 4.1 μM (305 ppb) when applied to the apical (seawater) side of the operculum, but not when added to the basolateral side of the operculum. Arsenic (4 μM for 60 minutes) also reduced mitochondrial respiration. Thus, environmentally relevant levels of arsenic block acclimation to seawater in killifish by reversibly inhibiting CFTR-mediated Cl⁻ secretion by the opercular membrane, in part by inhibiting mitochondrial respiration.

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Introduction

Fundulus heteroclitus (i.e., killifish) are euryhaline teleosts that are regularly exposed to a wide range of salinity in the swim water [1-5]. Incredibly, they can rapidly transition from fresh water to seawater, and can even survive in 360‰ seawater [6]. Acclimation to these dramatic changes in salinity depends on the ability of the gill and opercular membrane to adjust NaCl secretion into the swim water to match NaCl intake. Thus, in seawater, the gill and operculum secrete copious amounts of NaCl, and in fresh water these epithelia absorb NaCl. Transepithelial Cl⁻ secretion by the killifish gill and operculum is a two-step process. Chloride uptake across the basolateral membrane is mediated by the 1Na⁺-2Cl⁻-1K⁺ cotransporter, which depends on the operation of the Na⁺-K⁺-ATPase to generate a Na⁺ gradient across the basolateral membrane. The Cl⁻ that enters the cell via the 1Na⁺-2Cl⁻-1K⁺ cotransporter is secreted into seawater via the CFTR Cl⁻ channel located in the apical plasma membrane. Cl⁻ secretion generates an apical-negative, trans-epithelial voltage, which provides the driving force for the paracellular secretion of Na⁺. Long-term acclimation to increased salinity involves upregulation of CFTR gene expression. Increased salinity of the swim water leads to a rise in the plasma levels of cortisol, which via activation of the glucocorticoid receptor results in an increase in functional CFTR at the apical membrane. One mechanism for this increased expression appears to involve a glucocorticoid receptor-mediated transcriptional increase in CFTR mRNA expression [1-4]. However, given the complex nature of CFTR regulation, additional mechanisms are also likely to contribute to this up-regulation, including the release of several other hormones, and possibly an increase in the processing and/or trafficking of intracellular CFTR to the apical membrane [2, 5, 7]. Collectively, the cellular mechanisms underlying acclimation to increased salinity result in an increase in the plasma membrane expression of CFTR and thus result in a profound stimulation of NaCl secretion by the gill and operculum.

Arsenic is a toxic metalloid that naturally occurs in the environment. Arsenical compounds are used to treat psoriasis, spirochetal and protozoan diseases, as well as acute promyelocytic leukemia. However, exposure to arsenic in occupational settings, at Superfund sites and other toxic waste sites, and from natural sources in the environment, including well water, is a major health concern [8-13]. Chronic exposure to arsenic contaminated water or air has been linked to cancers of the skin, lung,

bladder, liver and kidney. Chronic arsenic exposure has also been associated with increased risk of type 2 diabetes, vascular disease, cardiovascular disease, and reproductive and developmental disorders [8-10, 12]. Because arsenic is classified as a known human carcinogen the U.S. EPA set a human health criterion for total dissolved arsenic in sea water of 0.0175 ppb (U.S. EPA, 1988, EPA#440,5-80-021) [14]. In clean coastal waters arsenic concentrations range between 1 to 3 ppb (0.013 - 0.039 μM) and in polluted seawater arsenic levels can exceed 100 ppb (1.3 μM), with concentrations over 1000 ppb (13 μM) having been reported [15, 16].

The mechanism(s) by which chronic exposure to low levels of arsenic increases disease risk is not clear. Recent studies have shown that arsenic at low concentrations (e.g., ~10 ppb) is a potent endocrine disrupter, altering hormone-activated gene transcription mediated by the glucocorticoid receptor [17, 18]. Unlike most endocrine disrupters arsenic interferes with the ability of the glucocorticoid receptor to act as a transcription factor once it is bound to DNA in a hormone-activated form [17, 18]. Arsenic also interferes with the function of ion channels and transport proteins. For example, arsenic trioxide at clinically relevant levels (0.1 to 1.5 μM), which is used to treat a number of human diseases, increases cardiac Ca⁺⁺ currents and reduces the number of cardiac K⁺ channels in the plasma membrane [19]. Moreover, arsenic activates the mitochondrial VDAC channel [20] and can either inhibit or facilitate Rb⁺:Rb⁺ exchange by the Na⁺-K⁺-ATPase [21]. Recently, prolonged exposure to arsenic was demonstrated to inhibit the transcription of CFTR in human T84 colon cancer epithelial cells *in vitro* [22]. Because so little is known about the cellular mechanism(s) whereby arsenic causes human disease and affects the function of ion channels and transporters, and because arsenic is a potent endocrine disrupter, studies were conducted to determine if arsenic affects the ability of killifish to acclimate to an increase in salinity.

We report that arsenic prevented killifish from acclimating to an increase in the salinity of swim water, in part by rapidly (minutes) and reversibly inhibiting CFTR-mediated Cl⁻ secretion by the opercular epithelium (IC₅₀ = 4.1 μM; 305 ppb). This rapid effect of arsenic is likely to involve a nongenomic mechanism, and was shown to be due in part to inhibition of mitochondrial respiration. Thus, sub-lethal concentrations of arsenic that are relevant to polluted aquatic environments reversibly inhibit CFTR-mediated Cl⁻ secretion.

Materials and Methods

Animals

All studies described in this manuscript were performed in compliance with Institutional animal care and use guidelines approved by MDIBL (#A3562-01) and Dartmouth Medical School (#A3259-01). Killifish were collected from Northeast Creek (Bar Harbor, ME, USA) and held in aquaria containing running seawater at the MDIBL (Salisbury Cove, Maine) or artificial seawater (Instant Ocean) at the Dartmouth Medical School for at least two weeks to ensure acclimation to seawater (i.e. seawater acclimated). Similar results were obtained at the MDIBL and Dartmouth. Fish were kept in seawater for at least two weeks to acclimate. Acclimation to fresh water was achieved by gradually (~1 hr) reducing the aquaria salinity to 10‰ and maintaining the fish in 10‰ seawater for two weeks. Subsequently, the water was changed gradually (~1 hr) to reconstituted very “soft” fresh water (ASTM, 1985. Annual Book of ASTM Standards. Vol. 11.01, Section 11, Water (I). Philadelphia, PA). Fish were maintained in fresh water for at least two weeks (i.e., fresh water adapted). Studies investigating seawater acclimation involved direct transfer of fresh water acclimated fish from fresh water to 100‰ seawater.

Ussing chamber analysis of CFTR-mediated Cl^- currents

To isolate opercular membranes for Ussing chamber experiments fish were anesthetized in tricaine (0.2 g/l) and then euthanized by double pithing. Opercular membranes were isolated from seawater acclimated fish and mounted in Ussing chambers, and the CFTR mediated Cl^- current (I_{sc}) was measured using a Physiological Instruments Voltage Clamp (Model VCC MC6, San Diego, CA), as previously described in detail [23, 24]. Previous studies have shown that the cAMP-stimulated I_{sc} by opercular membranes is equivalent to CFTR-mediated Cl^- secretion. A modified Cortland's saline solution [1] bathed the apical and basolateral sides of the opercular membranes, and the solutions were continuously stirred by bubbling with 1% CO_2 -air. Isoproterenol (10 μM) was added to the basolateral solution to elevate cAMP levels and stimulate CFTR Cl^- currents [4, 24]. Current output from the voltage clamp was digitized by an analog-to-digital converter (iWORX, Dover, NH). Data collection and analysis were conducted with LabScribe software (iWORX).

Measurements of Mitochondrial Function

To determine if the concentrations of arsenic used in this study reduced I_{sc} across opercular membranes by inhibiting mitochondria function, experiments were conducted to monitor mitochondrial inner membrane potential using MitoTracker Red CM-H2Xros, a fluorescence probe that sequesters in the mitochondrial inner membrane as a function of membrane voltage [25–27]. Opercular membranes attached to the boney operculum were isolated from saltwater acclimated killifish. Each opercular membrane was divided into three pieces. One piece was incubated in modified Cortland's saline solution containing vehicle (H_2O), one piece was incubated in modified Cortland's saline solution containing arsenic (sodium arsenite,

4 μM), and one piece was incubated in modified Cortland's saline solution containing sodium cyanide (1 μM), all for one hour in the presence of MitoTracker Red CM-H2Xros (Invitrogen, 500 nM). Subsequently, Mitotracker Red was removed from the solutions, and MitoTracker Red fluorescence was viewed using a Zeiss Meta 510 LSCM using a 63X plan apo 1.4 NA objective, and the 543 nm laser line. All images were collected and saved using identical pinhole and photomultiplier settings. Preliminary studies revealed that MitoTracker Red (500 nM) achieved steady-state levels in opercular mitochondria-rich cells after 60 minutes. Data were collected from an average of 30 cells from 15 random sections obtained from opercular membranes isolated from three animals (~1,350 cells/treatment). Images presented were not processed and appear exactly as acquired by the Zeiss LSCM. Data are expressed as relative fluorescence units quantified as described [25] using ImageJ version 1.30 (National Institutes of Health, Bethesda, MD.).

Analysis of data

Data are presented as mean $\pm$ SE. Statistical significance of experimental maneuvers was determined by the paired or unpaired Student's *t*-test, or ANOVA and the Tukey-Kramer Multiple Comparison Test, as appropriate, using GraphPad Instat version 3.0a for Macintosh (GraphPad Software, San Diego, California USA). A *P* value <0.05 was considered significant.

Results

Arsenic toxicity tests

Initial studies were conducted to determine the toxicity of arsenic in killifish over a range of doses. Range-finder toxicity tests were conducted on fresh water acclimated killifish according to U.S. EPA methods (U. S. Environmental Protection Agency. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to fresh water and marine organisms, 4th edition. EPA/600/4-90/027F. Environmental Monitoring Systems Laboratory, Cincinnati, OH, USA, pp 293) with modifications noted below. Killifish were housed in 10 L glass aquaria, 10 fish per tank, and tanks were randomly assigned a treatment group (three tanks/treatment). Inorganic arsenic was administered by intraperitoneal injection as sodium arsenite (NaAsO_2) at doses of 5, 10, 20, and 50 nmol/g in water. These doses of arsenic were chosen because 1.3 to 13 nmol/g of arsenic has been suggested for use in animal studies [28]. Moreover, the therapeutic index for arsenic when used as a chemotherapeutic agent is ~100 nmol/g [29, 30]. Control animals were either injected with vehicle (water) or were untreated. The highest dose of arsenic (50 nmol/g) was acutely toxic, resulting in 100% mortality within five

minutes (n=30 fish). While no mortality was detected over 96-h at lower doses, or in water injected and untreated controls, a noticeable decrease (>50%) in blood volume was observed at 10 and 20 nmol/g arsenic, but not at 5 nmol/g (n=30 fish/ dose). For these reasons, the 5 nmol/g dose was selected for additional studies.

Arsenic inhibits the ability of killifish to acclimate to increased salinity

Experiments were conducted to investigate the effects of arsenic (5 nmol/g i.p.) on the ability of killifish to acclimate to increased salinity of the swim water. Fresh water acclimated fish were given arsenic or water (vehicle) and returned to either fresh water or to seawater for 24 hours. When fresh water acclimated fish were given arsenic and returned to fresh water all fish survived (n=30). However, when fresh water acclimated fish were given arsenic and transferred to seawater all fish died (n=30). By contrast, when seawater acclimated fish were given arsenic and retained in seawater all fish survived (n=30). Thus, this dose of arsenic (5 nmol/g i.p.) did not affect the survival of fish already acclimated to either fresh water or seawater, but did block the ability of killifish to acclimate to an increase in the salinity of swim water.

Addition of arsenic to the swim water also blocked the ability of killifish to acclimate to increased salinity. Arsenic concentrations as high as 8,000 ppb (48 hours) had no effect on the ability of killifish to acclimate to an increase in the salinity of the swim water. However, 12,000 ppb (i.e., a concentration that was not toxic to either fresh water or seawater acclimated fish) reduced survival by 50% in fish transferred from fresh water to seawater (J. Shaw, J. Hamilton and B. Stanton, unpublished data). These data examining the chronic effects of arsenic in swim water will be published elsewhere. Taken together these experiments demonstrate that arsenic administered by intraperitoneal injection, and by addition to the swim water, blocked the ability of killifish to acclimate to increased salinity.

Arsenic inhibits CFTR-mediated Cl⁻ secretion by the operculum

The next studies were designed to explore the mechanisms by which arsenic blocks the ability of fish to acclimate to an increase in the salinity of swim water. Ussing chamber studies were conducted 24 hours after seawater acclimated fish were given arsenic by intraperitoneal injection to test the hypothesis that arsenic blocked this acclimation, at least in part, by reducing CFTR-mediated Cl⁻ secretion (Isc). Saltwater acclimated

Fig. 1. Arsenic reduced isoproterenol (10 μM) stimulated CFTR-mediated Cl⁻ secretion (Isc) in opercula isolated from saltwater acclimated killifish. Sodium arsenite (5 nmol Na⁺-arsenite/g body weight) or vehicle (water control) alone were injected IP 24 hours before opercula were isolated and mounted in Ussing chambers for measurements of Isc (μA/cm²). Arsenic significantly reduced the Isc (asterisk indicates P<0.05, n=6).

fish were injected with arsenic (Na⁺-arsenite; 5 nmol/g I.P.) or vehicle (H₂O) and 24 hours later the isoproterenol (10 μM) stimulated Isc by the opercular membrane was measured in Ussing chambers. As summarized in Figure 1, arsenic reduced the Isc in salt water acclimated fish from 333 ± 73 μA cm² to 141 ± 60 μA cm² (P<0.05, n=6). These data suggest that arsenic may inhibit the ability of killifish to acclimate to increased salinity of seawater by reducing CFTR mediated Cl⁻ secretion by the operculum, as well as by the gill, which also secretes Cl⁻ via CFTR Cl⁻ channels, and plays a key role in regulating NaCl homeostasis.

Arsenic acutely inhibits CFTR-mediated Cl⁻ secretion by the operculum

The Ussing chamber results above suggested that a 24 hr arsenic exposure inhibited Isc by either: (1) An acute, non-genomic effect, and/or (2) Inhibiting the cortisol-glucocorticoid receptor mediated activation of CFTR gene expression. The remainder of this report focuses on studies of the acute, nongenomic effect of arsenic on Isc. Detailed studies on the effects of arsenic on glucocorticoid receptor-mediated regulation of CFTR will be reported elsewhere.

To determine whether arsenic acutely inhibits Isc, opercula were isolated from seawater acclimated killifish, and the opercula were then acutely exposed to arsenic after Isc was stimulated by isoproterenol *ex vivo* (10 μM added to the basolateral membrane). When the Isc

Fig. 2. Representative Ussing chamber experiment illustrating that arsenic (Na^+ -arsenite: $10\ \mu\text{M}$) added to the apical solution following activation of the CFTR Cl^- current by isoproterenol ($10\ \mu\text{M}$; not shown in record) reversibly inhibited the current. The vertical deflections in the current trace every minute were induced by changing the clamp voltage from 0 to +1 mV (1 second) and then to -1 mV (1 second).

Fig. 3. Representative Ussing chamber experiment demonstrating that arsenic inhibits the CFTR mediated Cl^- current. In this experiment the CFTR Cl^- current was activated by isoproterenol ($10\ \mu\text{M}$; not shown in record) and then the CFTR Cl^- current was blocked by the addition of glyburide ($200\ \mu\text{M}$) to the apical solution. Following the decrease in the Isc, arsenic (sodium arsenite: $20\ \mu\text{M}$) was added to the apical solution. The lack of effect of arsenic on the Isc suggests that arsenic reduced the CFTR Cl^- current. The vertical deflections in the current trace every minute were induced by changing the clamp voltage from 0 to +1 mV (1 second) and then to -1 mV (1 second).

reached a new steady state, arsenic (sodium arsenite: $10\ \mu\text{M}$) was added to either the apical solution, the basolateral solution or both solutions. As illustrated in Figure 2, arsenic in the apical solution rapidly, and reversibly, reduced the Isc. The effect of arsenic was evident within 5-10 minutes, and the maximum inhibitory effect ($\sim 50\%$) was achieved after approximately 60 minutes. When the arsenic was washed from the apical solution (See Figure 2), the Isc recovered slowly, and achieved $74.0 \pm 12.1\%$ of its original value after 60 minutes ($n=7$). The Isc recovered to a value that was statistically not different from pre-arsenic levels.

To provide additional support for the conclusion that arsenic inhibits the CFTR mediated Cl^- secretion, studies were conducted with glyburide, an inhibitor of CFTR [5, 31]. Glyburide ($200\ \mu\text{M}$; $n=7$) added to the apical side of opercular membranes reduced the isoproterenol stimulated Isc from 292.3 ± 36.6 to $51.3 \pm 14.4\ \mu\text{A cm}^2$

($P<0.05$). In the presence of glyburide, arsenic had no effect on Isc, which was $51.3 \pm 14.4\ \mu\text{A cm}^2$ before arsenic and $54.0 \pm 17.3\ \mu\text{A cm}^2$ after arsenic was added to the apical bath solution (Figure 3). This experiments provides additional support for our hypothesis that arsenic specifically inhibited the CFTR mediated Cl^- current.

Dose response relationship between arsenic and the CFTR mediated Cl^- current

The dose-dependent effects of arsenic added to the apical solution on the opercular Isc are summarized in Figure 4. Arsenic significantly reduced Isc when added to the apical solution at a concentration of $2.5\ \mu\text{M}$, and maximally inhibited Isc ($\sim 50\%$ inhibition) at a concentration of $10\ \mu\text{M}$. Arsenic in the apical solution (seawater) inhibited the Isc with an IC_{50} of $4.1\ \mu\text{M}$ (305 ppb), a value that is relevant to arsenic levels in contaminated seawater (100 -1,000 ppb) [15, 16].

Fig. 4. Dose response relationship between arsenic (2.5 to 30 μ M; n=6/arsenic concentration) and percent inhibition of CFTR-mediated Cl^- secretion (Isc). Arsenic was added to the apical solution. Data presented as the percent inhibition after 60 minutes. The Isc before the addition of arsenic was $300.8 \pm 43.9 \mu\text{A}/\text{cm}^2$. Data analysis and curve fitting (Boltzmann Sigmoidal gave the best fit) of the data were performed using GraphPad Prism version 4.0c for Macintosh (GraphPad Software, San Diego California USA, www.graphpad.com).

Importantly, arsenic only reduced Isc acutely when added to the apical side (sea water) of the operculum. Arsenic concentrations up to 50 μ M had no effect on Isc when added to the basolateral side of the operculum. The Isc was $295.3 \pm 24.1 \mu\text{A}/\text{cm}^2$ in control untreated opercular membranes and $288.6 \pm 25.6 \mu\text{A}/\text{cm}^2$ 60 minutes after addition of arsenic (50 μ M; n=6) to the basolateral solution. Because the inhibition by arsenic of the Isc was relatively rapid (5-10 minutes to onset) these experiments are most consistent with the conclusion that arsenic acutely inhibits CFTR-mediated Cl^- secretion by a non-genomic mechanism.

Arsenic inhibits mitochondrial respiration in the operculum

The next studies investigated whether arsenic acutely reduces the CFTR mediated Cl^- secretion by inhibiting mitochondrial respiration since it is known that arsenic inhibits cellular respiration as one of its principal mechanisms of toxicity [32, 33] and since CFTR is known to be an ATP-dependent channel [34, 35]. We used a fluorescent dye, MitoTracker Red CM-H2XRos (500

Fig. 5. Representative images of MitoTracker Red fluorescence in mitochondrial rich cells in opercular membranes isolated from Control, Arsenic (4 μ M) and Cyanide (NaCN, 1 μ M) treated tissue. Images are unprocessed and were obtained in each condition using identical settings on the laser scanning confocal microscope.

Fig. 6. Summary of MitoTracker Red experiments on opercular membranes. Data were collected from an average of 30 cells from 15 random sections obtained from opercular membranes isolated from three animals (~1,350 cells/treatment). P values: Control versus Arsenic (4 μ M), $P < 0.001$ (single asterisk), Control versus Cyanide (1 μ M), $P < 0.001$ (Double asterisk), Arsenic (4 μ M) versus Cyanide (1 μ M), $P < 0.01$ (Double asterisk).

nM) that specifically accumulates in active, intact mitochondria, to investigate the effects of arsenic on mitochondrial functional integrity in killifish opercula. Opercular membranes were exposed to no arsenic or 4 μ M arsenic, a concentration that reduced CFTR Cl^- secretion by ~50% and changes in mitochondrial respiration were measured by confocal imaging (Figures

5 and 6). Arsenic decreased mitochondria-specific fluorescence by 61% compared to control. As a positive control, the mitochondrial poison, NaCN (1 μ M), was also used and as expected, this treatment significantly affected opercular mitochondria, decreasing fluorescence by 78% as compared to control. The effect of NaCN on fluorescence was also significantly greater than the effect of arsenic ($P < 0.01$). These data are consistent with the interpretation that arsenic reduced CFTR mediated Cl^- secretion, in part, by inhibiting mitochondrial integrity.

Discussion

The important finding of this study is that arsenic inhibits the ability of killifish to acclimate to an increase in the salinity of swim water at doses that are not overtly toxic to fish acclimated to fresh or seawater but at levels that are relevant to environmentally polluted waters. Ussing chamber studies demonstrate that this effect is due in part to an acute (minutes), non genomic inhibition by arsenic of CFTR-mediated Cl^- secretion by the opercular membrane. Moreover, since arsenic acutely decreased mitochondrial function and because CFTR is an ATP-gated Cl^- channel, our results are consistent with the hypothesis that the effect of arsenic on CFTR Cl^- secretion is due, at least in part, to a decrease in ATP levels resulting from compromised mitochondrial function.

Arsenic has been shown to affect other ion channels. Arsenic trioxide has been reported to increase cardiac Ca^{++} currents, reduce the number of cardiac K^+ channels in the plasma membrane, activate the mitochondrial VDAC channel, and to either inhibit or facilitate $\text{Rb}^+:\text{Rb}^+$ exchange by the Na^+/K^+ -ATPase [19-21]. Our results suggest that CFTR may also be inhibited by arsenic. However, additional studies are required to show that arsenic directly inhibits CFTR. Arsenic is used therapeutically to treat many diseases, but chronic exposure to arsenic in the environment or in occupational settings is also known to increase the risk of a wide number and diversity of diseases. The mechanisms by which arsenic influences these myriad of physiological and pathophysiological processes remain to be determined. In particular, additional information is needed relating environmentally relevant arsenic exposures to the cellular and molecular effects of arsenic, and then linking these pathways to particular therapeutic or disease processes.

It is likely that arsenic blocks the ability of killifish to acclimate to an increase in the salinity of the swimwater in part by non-genomic mechanisms. In Ussing chamber

experiments arsenic reduced the Isc after 5-10 minutes, an effect that is unlikely to be influenced by longer term changes in gene expression. There are several mechanisms by which arsenic may acutely reduce Isc. Because Cl^- secretion is a two-step process, uptake across the basolateral membrane via the $\text{Na}^+/\text{Cl}^-/\text{K}^+$ cotransporter, which depends on the operation of the Na^+/K^+ -ATPase to generate a Na^+ gradient across the basolateral membrane, and diffusion across the apical membrane via CFTR, it is possible that arsenic may inhibit one or more of these transport proteins and/or signaling pathways regulating these transporters. Because arsenic (2 to 50 μ M) reduced Isc only when added to the apical side of the operculum it is most likely that arsenic reduced Isc by inhibiting CFTR activity and/or by reducing the number of CFTR channels in the apical membrane without a major effect on basolateral transporters. This conclusion is supported by the observation that arsenic has no effect on the $\text{Na}^+/\text{Cl}^-/\text{K}^+$ cotransporter, which is located on the basolateral membrane (personal communication, Dr. Bliss Forbush, III, Yale University School of Medicine). Thus, the most likely mechanism by which arsenic reduced Isc is by inhibiting CFTR. However, additional studies are required to show that arsenic directly inhibits CFTR.

Our experiments with the fluorescent mitochondrial dye, Mitotracker Red, indicate that arsenic (4 μ M) reduced Isc in part by inhibiting mitochondrial function, which in turn presumably led to reduction in ATP and an increase in the AMP/ATP ratio. Because CFTR is an ATP-gated Cl^- channel, our results support the conclusion that arsenic reduced CFTR Cl^- secretion by reducing ATP levels. In addition, because AMP-dependent protein kinase (AMPK) is activated by an increase in the ratio of AMP/ATP and AMPK inhibits CFTR [34], our results suggest that the decrease in mitochondrial function may also inhibit CFTR via activation of AMPK. Arsenic can also bind, as As^{+3} (arsenite), to vicinal dithiols disrupting protein structure and function, and can bind, as As^{+5} (arsenate), to phosphate binding sites on proteins, altering phosphorylation-dependent pathways. The most parsimonious conclusion is that the acute (minutes) effect of arsenic is mediated by reversibly inhibiting mitochondrial function through a direct interaction with one or more arsenic binding sites on proteins, which leads to decreases in ATP levels and increases in the ratio of AMP/ATP, as well as activation of AMPK, both of which inhibit CFTR Cl^- channel activity.

Our Ussing chamber studies also demonstrated that chronic arsenic (24 hrs) exposure in intact animals reduced Isc, an effect that may be mediated in part by down-

regulating CFTR gene expression. Chronic exposure to arsenic reduces CFTR mRNA levels in human colon T84 cells *in vitro* [22], most likely by interfering with the ability of the glucocorticoid receptor to act as a transcription factor once it is bound to DNA in a hormone-activated form [17, 18]. The chronic inhibitory effect of arsenic on CFTR mediated Cl⁻ secretion observed in our Ussing chamber experiments may be caused by inhibition of mitochondrial function and by an inhibitory effect on CFTR gene expression. Increased salinity increases plasma cortisol in killifish, which via activation of the glucocorticoid receptor is thought to stimulate the transcription of CFTR [1-4]. This, in turn, increases the expression of CFTR Cl⁻ channels, and hence NaCl secretion by the gill and operculum. Thus, it is possible that arsenic chronically reduced CFTR Cl⁻ secretion by the operculum by inhibiting CFTR gene expression. The chronic effects of arsenic on CFTR gene expression are beyond the scope of the present manuscript.

It is not clear why arsenic reduced *I*_{sc} acutely when added to the apical side (seawater) and not to the basolateral side of opercular membranes. It is possible that arsenic could not diffuse across the connective tissue covering the basolateral side of the opercular membrane. Because arsenic is transported into cells by phosphate transporters as well as by the aquaporins, AQP7 and AQP9 [36, 37], it is tempting to speculate that arsenic transporters may be expressed in the apical but not the basolateral membrane. Arsenic uptake across the apical membrane could result in a microdomain below the apical membrane and in the vicinity of CFTR channels such that arsenic levels would be higher than in the cytosol and near basolateral membrane transporters. Additional studies are required to examine these possibilities.

These results are relevant for several reasons. First, these effects may directly relate to effects of arsenic on killifish and other natural species in aquatic environments. The levels of arsenic that inhibit *I*_{sc} in killifish operculum are in the range of 2.5 -10 μM. In clean coastal waters, arsenic concentrations range between 1 and 3 ppb (0.013 - 0.039 μM), but in polluted sea water arsenic levels can exceed 1000 ppb (13 μM) [15, 16]. Thus, arsenic-contaminated waters might be expected to have similar pathophysiological effects on aquatic species in these environments. Second, these effects may directly relate to pathophysiological effects of arsenic in humans. In particular, it will be interesting to determine whether such

effects on CFTR mediated Cl⁻ secretion occur in humans expressing wt-CFTR and/or in cystic fibrosis (CF) patients who have mutations in the CFTR gene that lead to altered CFTR expression and function. In this regard it is interesting to note that arsenic reduces wt-CFTR gene expression in a human colonic epithelial cell line [22]. The effects of arsenic on CF cells have not been reported. Third, the effects of arsenic on CFTR mediated Cl⁻ secretion in killifish provide an interesting model in which to investigate the mechanisms of action of arsenic. Arsenic has many cellular targets and initiates many different effects, some of which are dose-, time- and cell type-specific [17-22]. However, the specific effects of arsenic on CFTR mediated Cl⁻ secretion, at non-overtly toxic doses, as well as the profound pathophysiological consequences, represent a unique opportunity to investigate specific mechanisms of action distinct from toxicity and linked to an individual pathway that is directly relevant to both environmentally relevant outcomes and to human health.

In conclusion, we demonstrate that arsenic at environmentally relevant levels inhibits the ability of killifish to acclimate to an increase in the salinity of swim water at doses that do not affect acclimated fish. The inhibitory effect of arsenic is due, in part, to an acute, non genomic inhibition of CFTR-mediated Cl⁻ secretion by the opercular membrane. Arsenic acutely decreased mitochondrial function; since CFTR is an ATP-gated Cl⁻ channel and is regulated by AMPK, our results are most consistent with the interpretation that the effect of arsenic on CFTR mediated Cl⁻ secretion is a result of decreases in ATP levels.

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