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Arsenic inhibits mast cell degranulation via suppression of early tyrosine phosphorylation events

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Abstract

Exposure to arsenic (As) is a global health concern. We previously documented an inhibitory effect of inorganic arsenite on IgE-mediated degranulation of RBL-2H3 mast cells (Hutchinson *et al.*, 2011). Mast cells are tissue-resident cells that are positioned at the host-environment interface, thereby serving vital roles in many physiological processes and disease states, in addition to their well-known roles in allergy and asthma. Upon activation, mast cells secrete several mediators from cytoplasmic granules, in degranulation. The present study is an investigation of arsenite's molecular target(s) in the degranulation pathway. Here, we report that As does not affect degranulation stimulated by either the Ca²⁺-ionophore A23187 or thapsigargin, which both bypass early signaling events. As also does not alter degranulation initiated by another non-IgE-mediated mast cell stimulant, the G-protein activator compound 48/80. However, As inhibits Ca²⁺ influx into antigen-activated mast cells. These results indicate that arsenic's target in the degranulation pathway is upstream of Ca²⁺ influx. Phospho-Syk ELISA and phospho-p85 phosphoinositide 3-kinase ELISA data show that arsenic inhibits early phosphorylation events. Taken together, this evidence indicates that the mechanism underlying arsenic's inhibition of mast cell degranulation occurs at the early tyrosine phosphorylation steps in the degranulation pathway.

Keywords

arsenic; mast cell; degranulation; calcium; Syk; phosphoinositide 3-kinase

Introduction

Exposure to arsenic (As) is a major human health concern according to both the World Health Organization and the Agency for Toxic Substances and Disease Registry (ATSDR, 2007; NRC, 2001). Millions of people worldwide are exposed to high levels of As from a variety of natural and anthropogenic sources, including drinking water, various foods,

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pesticides, mining sites, and toxic waste sites. Many private water wells in the U.S., as well as those around the world (such as South America and Bangladesh) routinely contain levels of As well above 50 ppb (parts per billion; $\mu\text{g/L}$), and sometimes as high as 3000 ppb (ATSDR, 2007).

Chronic exposure to As, in particular via drinking water, has been correlated to many diseases, including severe dermatological disorders (Paul *et al.*, 2013; Smith *et al.*, 2000; Xia *et al.*, 2009), respiratory diseases (Hopenhayn-Rich *et al.*, 1998; Putila and Guo, 2011; Smith *et al.*, 1992), diabetes/obesity (Abernathy *et al.*, 1999; Maull *et al.*, 2012), and cardiovascular disease (Abhyankar *et al.*, 2012; Chen *et al.*, 2009; Das *et al.*, 2012; Huang *et al.*, 2009; Smith and Steinmaus, 2009). As exposure also inhibits hematopoietic and immune systems (NRC, 2001) and causes neurological and cognitive impairments (Chen *et al.*, 2009; Wasserman *et al.*, 2007; Wasserman *et al.*, 2004). Moreover, arsenic is a known human carcinogen, which affects almost all major organs including liver, kidney, bladder, skin and lung (Chiu *et al.*, 2004; Ferreccio *et al.*, 2000; Heck *et al.*, 2009; Liaw *et al.*, 2008; Liu-Mares *et al.*, 2013; Mostafa and Cherry, 2013; Naujokas *et al.*, 2013; Putila and Guo, 2011; Saint-Jacques *et al.*, 2014).

We recently demonstrated that antigen-stimulated degranulation of the mast cell model rat basophilic leukemia cells (RBL-2H3) is inhibited by non-cytotoxic doses of inorganic arsenite (Hutchinson *et al.*, 2011). Mast cells are multi-effector immune cells that participate in the first line of defense against parasites. Mast cells, found in most human tissues (Kuby, 1997), are also major effectors in allergic responses, asthma, other innate immune processes, and carcinogenesis. Mast cells are also involved in neurological conditions such as multiple sclerosis, autism, and anxiety (Silver and Curley, 2013). The RBL-2H3 mast cell model is biologically very similar to human basophils and rodent mucosal mast cells (Fewtrell, 1979; Metzger *et al.*, 1986; Seldin *et al.*, 1985).

Arsenic can be detected in a variety of human samples, and urine and blood are usually the major materials that are monitored for As exposure (Kraus *et al.*, 2000). However, arsenic can be stored and concentrated in the tissues of many organs such as kidney, lung, skin, and liver (Benramdane *et al.*, 1999), and mast cells are found in most human organs, as we discussed in Hutchinson, *et al.* 2011. In fact, the concentrations of arsenic found in tissue tend to be higher (up to ~7-350 fold higher) than in blood (Benramdane *et al.*, 1999). In a previous article (Hutchinson *et al.*, 2011), we noted several publications reporting human tissues containing between 100 and 6000 ppb arsenic. For example, one study examined tissue As concentrations in people who had regularly consumed drinking water containing 220-2000 ppb As and found several hundred to several thousand ppb As (Schroeder *et al.*, 1968). Since mast cells function within tissues, it is necessary to study As concentrations that are found within tissues. Thus, the 100-750 ppb As concentrations employed in this study are biologically relevant.

Highly granulated, mast cells respond to various stimuli by partially or completely releasing the contents of their granules, which include a range of mediators such as histamine and serotonin. The mechanism underlying arsenic's inhibition of the function of mast cell degranulation is not yet known. Given the complexity of the degranulation signal

transduction pathway and the similarities to signaling pathways in other immune cells, such as T cells, there are many potential targets for As inhibition.

Degranulation is a process that classically begins with antigen crosslinking of IgE-bound FcεRI receptors, which abound on the mast cell surface (Kuby, 1997). The physical aggregation of the receptors results in their tyrosine phosphorylation by the protein tyrosine kinase (PTK) Lyn (Kinet, 1999; Xiao *et al.*, 2005). Phosphorylation of the receptor causes the PTK spleen tyrosine kinase (Syk) to be recruited to the receptor (Benhamou *et al.*, 1993; On *et al.*, 2004). The activation of Syk (Minoguchi *et al.*, 1994; Zhang *et al.*, 2000), leads to the activation of phospholipase C (PLC)γ (Beaven and Metzger, 1993; Benhamou *et al.*, 1992) and results in calcium mobilization (Ferris *et al.*, 1989; Millard *et al.*, 1989; Putney *et al.*, 2001), which is essential to degranulation (Zhang *et al.*, 1996).

PLCγ1 is an important player in Ag-receptor signaling: it catalyzes the hydrolysis of the membrane phospholipid PIP₂ to generate second messengers inositol-1,4,5 - trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ initiates the release of calcium from internal stores (i.e., the endoplasmic reticulum [ER]), while DAG is responsible for activation of an assortment of protein kinase C (PKC) isoforms (Kalesnikoff and Galli, 2008). Activated PKC phosphorylates the cytoskeletal protein myosin, important for degranulation (Ludowyke *et al.*, 1989).

A second signaling pathway resulting in IP₃ generation is initiated by PI3K (Phosphoinositide 3-Kinase), which is activated by Syk (Mocsai *et al.*, 2010; Okkenhaug and Vanhaesebroeck, 2003) and which phosphorylates inositol lipids to generate the signaling molecules PIP₂ and PIP₃ (Kitaura *et al.*, 2000). PIP₂ is then used by PLCγ to generate IP₃. PH domains within PLC-γ, Vav, Akt, Btk, and PDK1 mediate binding of these proteins to PIP₂ and to PIP₃, thus drawing these proteins to the plasma membrane for activation (Abramson and Pecht, 2007; Kitaura *et al.*, 2000). For example, PDK1 activation leads to activation of PKCδ, important for degranulation. Overall, PI3K activates Ca²⁺ influx and degranulation of mast cells (Ching *et al.*, 2001)

IP₃ is crucial for degranulation. Activation of IP₃ receptors initiates a biphasic increase in intracellular Ca²⁺ (Berridge, 1993; Taylor and Thorn, 2001). The binding of IP₃ to its receptors in the ER causes Ca²⁺ to be released from the internal ER stores, causing depletion of Ca²⁺ from the ER. IP₃ receptors are ion channels that allow for passive diffusion of calcium from the ER (Scharenberg *et al.*, 2007). Depletion of Ca²⁺ stores results in an influx of Ca²⁺ across the plasma membrane through calcium release-activated calcium (CRAC) channels (Hogan *et al.*, 2010), which is referred to as “store operated calcium entry” (SOCE) (Clapham, 1995; Putney, 1986; Putney, 1990), producing an extracellular calcium influx and I_{CRAC} (calcium-release activated current) (Kraft and Kinet, 2007). Major players in SOCE are STIM-1 (Liou *et al.*, 2005; Zhang *et al.*, 2005), an ER calcium sensor which interacts directly with Orai1 channels (Feske *et al.*, 2006; Vig *et al.*, 2006), the pore subunit of CRAC channels within the plasma membrane, to couple depletion of ER calcium with activation of CRAC channels. Influx of Ca²⁺ across the plasma membrane permits reuptake of Ca²⁺ into the ER through sarco/endoplasmic Ca²⁺-ATPase (SERCA) pumps (Ma and Beaven, 2011), which actively pump Ca²⁺ from the cytosol into the ER to replenish internal stores

(Scharenberg *et al.*, 2007). Depletion of the ER pool of Ca^{2+} by either IP_3 or the compound thapsigargin (selective inhibition of SERCA) (Lytton *et al.*, 1991; Thastrup *et al.*, 1990) leads to entrance of Ca^{2+} into the cell (Ma and Beaven, 2011). Overall, the primary synergistic signals for secretion are an increase in intracellular Ca^{2+} as well as activation of PKC (Ozawa *et al.*, 1993).

Next, increases in intracellular Ca^{2+} and PKC translocation work in tandem to activate phospholipase D (PLD) (Lin and Gilfillan, 1992). In fact, PLD can be activated in RBL-2H3 cells via several means: Fc ϵ RI receptor crosslinkers (Ali *et al.*, 1996; Dinh and Kennerly, 1991), thapsigargin (Cissel *et al.*, 1998), Ca^{2+} ionophore (Lin and Gilfillan, 1992), and compound 48/80 (Chahdi *et al.*, 2000). PLD hydrolyzes phosphatidylcholine into phosphatidic acid (PA), which has been shown to stimulate PLC γ (Nishizuka, 1995). PA is converted to DAG by PA phosphohydrolase, thus causing a secondary rise in intracellular DAG levels (Lin *et al.*, 1992; Nakashima *et al.*, 1991). These increases have been shown to be important for the translocation and activation of the DAG-dependent isoforms of PKC (Lin *et al.*, 1992; Nishizuka, 1995; Peng and Beaven, 2005). PLD1 is involved in granule translocation, and PLD2 is involved in membrane fusion of the granules (Choi *et al.*, 2002). Granules are transported from the cell interior to the plasma membrane with the help of microtubules, where they dock with the help of multiple soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (Baram *et al.*, 1999; Blank *et al.*, 2002; Guo *et al.*, 1998; Logan *et al.*, 2003; Paumet *et al.*, 2000; Puri and Roche, 2008; Tiwari *et al.*, 2008; Woska and Gillespie, 2012). The result of mast cell signaling is fusion of intracellular granules with the plasma membrane, causing the release of histamine, serotonin, leukotrienes, cytokines, β -hexosaminidase, and enzymes that trigger the symptoms of allergy and asthma and that play many other crucial physiological roles (Kope *et al.*, 2006). Simultaneously, F-actin polymerization causes membrane “ruffling.”

Actin cytoskeletal rearrangement is not an essential element of the degranulation pathway (degranulation occurs even when ruffling is stopped by actin inhibitor cytochalasin D (Holowka *et al.*, 2000)), but the two share common events such as activation of PKC and influx of Ca^{2+} (Pfeiffer *et al.*, 1985; Yanase *et al.*, 2011). In a recent paper by Yanase *et al.* (2011), it was shown that the use of inhibitors of PKC β and PKC α inhibit both ruffling and degranulation. PLD2 also is important for membrane ruffle formation.

As part of our investigation of arsenic's mechanism of mast cell inhibition, we utilized another non-IgE-mediated stimulating method, compound 48/80 (c48/80), a polybasic mast cell secretagogue which has potent anaphylactic properties *in vivo* (Chahdi *et al.*, 2000; Lagunoff *et al.*, 1983) and which effects degranulation from rat mast cells (Johnson and Moran, 1969). Compound 48/80 is believed to act by the stimulation of heterotrimeric G-proteins (Chahdi *et al.*, 2000), in particular, via Gi-2 and Gi-3 in RBL-2H3 cells (Senyshyn *et al.*, 1998). Pretreatment of RBL-2H3 cells with the flavonoid quercetin causes an increase in histamine-containing intracellular granules (Trnovsky *et al.*, 1993) and an over-expression of Gi-3 α and Gi-2 α (Chahdi *et al.*, 2000; Senyshyn *et al.*, 1998), so pre-incubation of RBL-2H3 cells with quercetin is required prior to exposure with c48/80 in order to make the cells responsive to c48/80-induced degranulation (Galli *et al.*, 2008). Phospholipase D (PLD) is stimulated by c48/80 in quercetin-treated RBL-2H3 cells (Aridor *et al.*, 1990;

Chahdi *et al.*, 2000; Senyshyn *et al.*, 1998). Compound 48/80 induces a calcium-dependent degranulation response that releases calcium from intracellular pools (Aridor *et al.*, 1990; Hirasawa *et al.*, 1995; Senyshyn *et al.*, 1998), and transiently activates phospholipase C (PLC), possibly through indirect activation of PLD (Ali *et al.*, 1990; Aridor *et al.*, 1990; Park *et al.*, 1991; Senyshyn *et al.*, 1998; Yamada *et al.*, 1992). Therefore, due to c48/80's mechanism of action, we were able to assess the possibility of arsenic's targeting PLC, PLD, and calcium influx.

It is well established that calcium influx is a crucial for mast cell degranulation. To explore whether the level of FcεRI-mediated cytoplasmic Ca²⁺ in RBL-2H3 mast cells is changed by arsenic exposure, we developed a microplate-based method to monitor the level of calcium utilizing the fluorescent calcium indicator. Using this method, we were able to measure the cytosolic calcium levels in real time for over two hours after cells were stimulated and exposed to arsenic.

Finally, we evaluated the effect of As on key phosphorylation events, crucial early signaling events in the mast cell degranulation pathway. It has previously been demonstrated that As affects tyrosine phosphorylation events (Qian *et al.*, 2003), including T cell signal transduction (Soto-Peña and Vega, 2008). Arsenic trioxide inhibits the PI3K/Akt pathway in chronic lymphocytic leukemia cells (Goussetis and Platanias, 2010). PI3K is a heterodimeric enzyme composed of a catalytic subunit (p110) and a regulatory subunit (p85) (Koyasu, 2003). PI3K is activated by Syk kinase (Okkenhaug and Vanhaesebroeck, 2003). Here we have examined the effect of As on PI3K and Syk kinase by employing enzyme-linked immunosorbent assays (ELISA). The global phosphorylated Syk levels in the Ag-activated RBL cells were measured. For PI3K, both the phosphorylated p85 subunit of PI3K and the total PI3K p85 subunit were evaluated.

In this study, we unraveled the molecular mechanism used by arsenite to inhibit mast cell function. We employed multiple experimental approaches in RBL-2H3 cells in order to determine arsenic's mechanism of its inhibition of mast cell degranulation. Here we present mechanistic data resulting from degranulation assays utilizing various stimulants, F-actin imaging, Ca²⁺ measurements, and ELISAs probing early phosphorylation events. We demonstrate that As acts very early in the signaling pathway leading to degranulation, inhibiting the nearly-immediate event of Syk kinase phosphorylation.

Materials and Methods

Chemicals and Reagents

A23187 ionophore, compound 48/80 (c48/80), quercetin, dimethyl sulfoxide (DMSO), sodium azide, and sulfinpyrazone were purchased from Sigma Aldrich (St. Louis, MO, USA). Thapsigargin (Tg) was obtained from Calbiochem/EMD Millipore (Billerica, MA, USA). Tyrodes buffer, BSA-Tyrodes (BT), sodium acetate buffer, and glycine carbonate buffer were prepared as previously described (Hutchinson *et al.*, 2011). A low-calcium Tyrodes (containing 0.9 mM rather than the typical 1.8 mM CaCl₂) was used in all c48/80 experiments. Chemicals of the highest possible purity were used. All buffers and media were

sterile-filtered with VacuCap bottle-top filter devices (0.2 μ M; Pall Life Sciences, Port Washington, NY, USA).

Arsenic (As) was prepared under sterile conditions as 10 mM stocks of inorganic sodium (meta) arsenite (CAS no. 7784-46-5; manufactured by either JT Baker, Phillipsburg, NJ, USA; or Fluka, Seelze, Germany), and dissolved in sterile cell culture water (BioWhittaker Lonza, Walkersville, MD, USA). The stock was 0.2- μ M filtered and stored as previously described by (Hutchinson *et al.*, 2011).

Ca²⁺ ionophore A23187 was prepared by dissolving A23187 powder into 100% DMSO for a final concentration of 2.5 mg mL⁻¹. The solution was transferred to a microcentrifuge tube with a parafilm lid and was wrapped in aluminum foil, for storage at -20 °C. On the day of an experiment, this stock was diluted directly into Tyrodes-BSA solution to create ionophore concentrations of 1.5×10^{-7} M and 2×10^{-7} M (0.004% DMSO).

Thapsigargin (Tg) was prepared as described in (Weatherly *et al.*, 2015). The highest DMSO vehicle concentration used in these experiments was 0.001 % (v/v).

Compound 48/80 was prepared using aseptic conditions as 30 mg mL⁻¹ stocks dissolved in cell culture water, aliquoted into sterile polypropylene microfuge tubes, and frozen at -20 °C until the day of use, so that each individual aliquot underwent only one freeze/thaw cycle. Quercetin was prepared as a 60 mM stock from anhydrous quercetin powder in 100% DMSO. Cells were treated with 20 μ M quercetin in RBL media (Hutchinson *et al.*, 2011), resulting in a DMSO vehicle concentration of 0.033 % (v/v).

Fluo-4/AM (Life technologies, Grand Island, NY, USA) was dissolved in 100% DMSO to prepare a 911 μ M stock and stored at -20 °C until the day of use, so that each individual aliquot underwent only one freeze/thaw cycle.

RBL-2H3 Cell Culture

Cell culture methods were those of (Hutchinson *et al.*, 2011)

Cytotoxicity Assays

Trypan blue exclusion assays were performed as previously described in detail by Hutchinson *et al.* (2011) and Palmer *et al.* (2012). In general the details remained unchanged, with the exception of the use of c48/80, which required a slightly different procedure in order to accommodate a 48-h quercetin pre-treatment as well as the use of a low-calcium Tyrodes buffer. Accordingly, RBL-2H3 cells were plated into sterile tissue culture treated, six-well plates (VWR, Radnor, PA, USA) at a density of 0.85×10^6 cells/well in RBL medium supplemented with 20 μ M quercetin and incubated for 48 h at 37°C, 5% CO₂. After 48 h, spent medium was discarded, and fresh RBL medium was added to the cells for 30 min at 37°C, 5% CO₂. This liquid was then discarded, and low calcium Tyrodes buffer $\pm$ 25 μ g mL⁻¹ c48/80 or vehicle $\pm$ As was added to each well for 15 min at 37°C, 5% CO₂. This medium was removed, and wells were washed with 2 mL low calcium Tyrodes to remove dead cells. Following removal of this wash, wells were rinsed with 1 mL of trypsin. Plates were then incubated with 1 mL fresh trypsin for 10 minutes at 37°C, 5% CO₂ to allow

cells to completely dislodge from the plates. Further removal of cells after this incubation was accomplished by repeated pipetting of the cell-containing trypsin into the wells, removing visible traces of cells from the surface of the well. The cell-containing trypsin was then quenched by addition into 1 mL of RBL media. Equal volumes of this mixture and trypan blue stain (Trypan Blue 0.4% solution in 0.85% NaCl, BioWhittaker Lonza) were then combined, and cell viability was assessed by counting those cells able to exclude the trypan blue stain using a hemocytometer (Bright-line, Hausser Scientific, Horsham, PA, USA). Values of living cells were then normalized to the vehicle values for any given assay before data compilation.

Lactate dehydrogenase (LDH) assay was performed as previously described (Hutchinson *et al.*, 2011; Palmer *et al.*, 2012)

ATP production and cytotoxicity were assayed via Promega (Madison, WI, USA) Toxglokit instructions as described in (Weatherly *et al.*, 2015). Arsenic exposures lasted 1 hour.

Antigen-Mediated Degranulation Assay

Cells were sensitized by IgE, as described in (Weatherly *et al.*, 2013). Immunoglobulin E (IgE)-bound Fc ϵ RI receptors of RBL cells were aggregated by dinitrophenyl (DNP)-BSA multivalent Ag $\pm$ As (for 1 hour). Degranulation (release of β -hexosaminidase) was measured via a fluorescence-based 96-well microplate reader assay, as illustrated in (Weatherly *et al.*, 2013).

Confocal Imaging of Membrane Ruffling

Experiments were performed as in (Palmer *et al.*, 2012). Arsenic was used in place of triclosan; thus, vehicle in control samples was cell culture water.

Ca²⁺ Ionophore-Stimulated Degranulation in RBL-2H3 Cells

This assay was essentially performed as described by (Weatherly *et al.*, 2013), except that two different Ca²⁺ ionophore A23187 doses (1.5×10^{-7} M and 2×10^{-7} M) were used, based on their ability to elicit a low-range and mid-range level of degranulation, respectively, with this lot of A23187. Additionally, arsenic (in place of triclosan) was diluted into A23187/Tyroses-BSA solution at the noted concentrations (0.004% DMSO vehicle in all samples).

Thapsigargin-Induced Degranulation

To test the ability of arsenic to alter an earlier Ca²⁺ signaling event than that induced by A23187 Ca²⁺ ionophore, we used Thapsigargin (Tg), a known inhibitor of sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA) in mammalian cells. Degranulation experiments were conducted similarly to antigen-stimulated RBL-2H3 degranulation experiments (Weatherly *et al.*, 2013), except that there was no IgE pretreatment and that RBL-2H3 cells were treated with 4.6 nM Tg for one hour (37 °C and 5% CO₂) in the presence of 0-750 ppb As. To combine multiple experiments, data were normalized to the response elicited by 4.6 nM Tg in the presence of As vehicle only (cell culture water).

Compound 48/80-Mediated Degranulation

RBL cells were harvested after 2-5 days of growth via trypsinization, and were resuspended at a density of 0.25×10^6 cells mL^{-1} in fresh RBL medium $\pm 20\mu\text{M}$ quercetin. 100 μL of this suspension per well was plated in black flat-bottom, tissue culture-treated, sterile, 96-well plates (Greiner Bio-One, Monroe, NC, USA). After 48 h in a 5% CO_2 incubator at 37°C , spent RBL medium $\pm$ quercetin was removed by decanting. From here, sample wells were washed twice with 0.9 mM Ca^{2+} (low-calcium) Tyrodes, and 100 μL /well fresh RBL medium was added to cells for 30 min (Galli *et al.*, 2008). Stimulated cell samples then received 200 μL aliquots of c48/80, spontaneous release cells received plain low-calcium Tyrodes, and lysed cells received Triton-X 100 diluted to 0.2% (v/v) in low-calcium Tyrodes.

Degranulation (release of β -hexosaminidase) was quantified as previously described (Weatherly *et al.*, 2013).

Calcium measurement assay

RBL-2H3 cells (100 μL /well) were plated in a 96-well plate in RBL media at a density of 0.5×10^6 cells mL^{-1} and incubated overnight at 37°C and 5% CO_2 . Also, 2 mM sulfinpyrazone solution in Tyrodes buffer was prepared, vortexed, and rotated at 37°C overnight. On the following day, cells were sensitized with 0.1 $\mu\text{g mL}^{-1}$ anti-DNP IgE (Sigma) (in RBL media; 100 μL /well) for one hour at 37°C and 5% CO_2 . Sulfinpyrazone solution (2mM) was sterilized using syringe filtration (0.2 μm), after its pH was adjusted to 7.4, and then was diluted to 0.6 mM using BT (2 mg/mL BSA in Tyrodes buffer). The stock of 911 μM Fluo-4/AM dye was diluted to 4.55 μM using BT. For DMSO control samples, 0.5% DMSO-BT was also prepared. Some of the 4.55 μM Fluo-4/AM-BT was further diluted to be 2.5 μM using 0.6 mM sulfinpyrazone in BT (Fluo-4/AM-sulfinpyrazone-BT) and 0.27% DMSO-sulfinpyrazone- BT was also prepared as the corresponding vehicle control.

After one hour IgE-sensitization, cells were washed with warm BT (200 μL /well), followed by addition of 100 μL /well of 4.55 μM Fluo-4/AM-BT, 0.5% DMSO-BT, or BT (all in the absence of sulfinpyrazone), to the appropriate wells for 1 minute. Immediately after 1 min, these solutions were discarded, and 200 μL /well of 2.5 μM Fluo-4/AM-sulfinpyrazone- BT, 0.27% DMSO-sulfinpyrazone-BT, or 0.6 mM sulfinpyrazone- BT was added for 30 minutes at 37°C . After 30 minutes of Fluo-4 incubation, cells were washed twice with 0.6 mM sulfinpyrazone - BT (200 μL /well), and then 250 μL /well of 0.6 mM sulfinpyrazone-BT were added for the baseline fluorescence reading at 485 nm excitation and 528 nm emission for 2 minutes (42 second intervals between readings). The sulfinpyrazone-BT solutions were discarded from the plate, and 240 μL /well of 0.6 mM sulfinpyrazone-BT $\pm 0.0002 \mu\text{g/mL}$ antigen ± 750 ppb As were added to the corresponding wells. Fluorescence readings, at 485 nm excitation and 528 nm emission, were taken for 1 hour post- stimulation (42 second intervals between readings).

After the hour, for maximal Ca^{2+} readings, which are a measurement of cell number/well, 10 μL of 10% Triton-X 100 (TX-100) was added to each well. The fluorescence reading was taken after a 10 min incubation (with agitation) at 37°C . TX-100 fluorescent values for all replicates were averaged, and no differences among samples were determined by one-way

ANOVA followed by Tukey's post- test (data not shown), indicating that all wells contained equal numbers of cells.

Finally, 50 μ L of 0.5M EDTA (USB, Cleveland, OH, USA), which chelates calcium and lowers the fluorescent signal to the background level, was added to each well, and the fluorescence reading was taken after a 5 min incubation. Fluorescence levels dropped to no-Fluo-4 background levels, and no statistically significant differences among samples were found (data not shown), indicating that measured fluorescence signals were due to the interaction of Fluo-4 dye and Ca^{2+} .

Phospho-p85 PI3K ELISA

A Fast Activated Cell-based ELISA Kit (Active Motif, Carlsbad, CA, USA) was used, to detect the levels of both phosphorylated and total PI3K p85 in the cell, according to the manufacturer's instructions. RBL-2H3 cells (100 μ L/well) were plated in a clear 96-well plate at a density of 0.5×10^6 cells mL^{-1} and incubated for 12-16 h at 37°C and 5% CO_2 . On the following day, after one hour sensitization with 0.1 $\mu\text{g mL}^{-1}$ anti-DNP IgE (Sigma), cells were stimulated with either 1 $\mu\text{g mL}^{-1}$ DNP-BSA antigen or BT (control) $\pm$ 750 ppb As for 5 minutes at 37°C. Next, cells were fixed with 4% formaldehyde (Sigma) in PBS (Lonza, Rockland, ME, USA) solution at room temperature for 20 minutes. Once the formaldehyde mixture was discarded, cells were washed three times with 1X wash buffer provided in the kit (200 μ L per well per wash; 5 min gentle shaking per wash). The remaining steps were carried out according to the manufacturer's directions. Absorbance was measured (at 450 nm) immediately after the addition of the "stop" solution. Cells were then stained with crystal violet, following the manufacturer's protocol, and absorbance was read at 595 nm. The measured OD_{450} reading was then corrected for cell number by dividing the OD_{450} value by the OD_{595} value for each well.

Phospho-Syk ELISA

PathScan® Phospho-Syk sandwich ELISA kit (Cell Signaling Technologies, Beverly, MA, USA) was used to detect endogenous levels of phosphorylated Syk according to the manufacturer's instructions. This is a pan-tyrosine ELISA, able to detect altered phosphorylation for all Syk's key Tyr residues. For this assay, RBL-2H3 (1.75×10^6 cells per mL) were plated on 10 cm^2 tissue-culture treated, sterile dishes (Cellstar, Greiner Bio-One, Monroe, NC, USA) at a volume of 6.5 mL per well (final cell density of 1.14×10^7 cells per dish), and were incubated overnight (12-16 h) at 5% CO_2 and 37°C. Cells were sensitized overnight by addition of 1 $\mu\text{g mL}^{-1}$ anti-DNP IgE (generously provided by Drs. Barbara Baird and David Holowka, Cornell University) in RBL media or the following day for one hour with 0.1 $\mu\text{g mL}^{-1}$ anti-DNP IgE (Sigma).

Following the overnight incubation, each dish was checked to ensure a cell confluence of 80-90%. After IgE-sensitization (37°C and 5% CO_2), spent IgE-media mixture was discarded, and dishes were washed twice with 5 mL BT to remove any unbound IgE. The 5 mL treatments added next included either BT for the unstimulated controls or DNP-BSA antigen at 1 $\mu\text{g mL}^{-1}$ for stimulated samples; all samples included As or water vehicle (cell culture water, Lonza, Rockland, ME, USA). Dishes were incubated for exactly 5 min in a

bacterial incubator at 37°C. Immediately, dishes were placed on ice, washed once with ice-cold PBS (~5 mL), and lysed with 0.4 mL of ice-cold 1X lysis buffer containing phenylmethanesulfonyl fluoride solution (PMSF) for 5 min. (The 10X lysis buffer provided in the kit was diluted with sterile water before use; 100 mM PMSF [Sigma] stock dissolved in ethanol was added at a concentration of 1 mM immediately prior to buffer addition). Next, cells were harvested by scraping and were placed into pre-cooled 2-mL microcentrifuge tubes. Cell suspensions were sonicated three times on ice by introducing a clean sonicating probe directly into the sample. The sonicator (Branson sonifier, Branson Ultrasonics Corporation, Danbury, CT, USA) was set at output control of 5, duty cycle of 30, Timer at 2, 1 sec in duration, with a 10 sec break on ice in between sonications (these sonicating conditions were found to result in complete cell lysis; data not shown). Finally, the sonicated lysates were spun down at 14,000 × rpm for 10 min at 4°C. A volume of 100 µL of clarified supernatant was transferred to new, pre-cooled tubes containing 100 µL sample diluent (provided in kit). After a brief vortex, 100 µL of each diluted sample was placed into an appropriate ELISA well, sealed with parafilm, and incubated for 16-20 h at 4°C. The remaining steps were carried out according to the manufacturer's directions. Absorbance was measured immediately after the addition of the “stop” solution.

Statistical Analyses

Unless otherwise indicated in individual figures, results from degranulation experiments (using Ag ± A23187 Ca²⁺ ionophore, Tg, or c48/80) are reported as mean ± SEM, with significant differences determined using Graphpad Prism software (San Diego, CA, USA). For multiple-comparison testing, Tukey's post hoc test was used following one-way ANOVA. In several cases, data required normalization in order to control for day-to-day variation of cell culture; in these instances, Tukey's comparisons are reported with regard to a low dose of the agent used that was not significantly different from the 0 control to which all values were normalized. In the event that normalization was not required, significance was directly made to the 0 control.

For calcium measurement assay, the data were analyzed as areas under the curve (AUC), a measure of total calcium response over a 30 or 60 minute period. First, the fluorescence values before Ag stimulation (baseline) were averaged, then subtracted from the corresponding Ag-stimulated samples' fluorescence values. Next, those values were normalized to the last 0 ppb As time point. These normalized values were used to determine AUC via Graphpad Prism software. The AUC values were further normalized to each day's 0 ppb As AUC value, and the data from multiple days of experiments were expressed as a bar graph. The statistical significance was determined via one-way ANOVA with Tukey's post hoc test

For the phospho-p85 PI3K ELISA, the average value from the Ag + 750 ppb As group was normalized to the average value from the Ag + 0 ppb As group from each day before compiling multiple experiments, and significance was determined by one-sample unpaired t-test. For phospho-Syk ELISA, treatment samples (Ag ± 750 ppb As) were expressed as a % increase over spontaneous control (no IgE/Ag) sample, and the statistical analysis was done by unpaired t-test.

Results

Short term exposure of arsenic inhibits antigen-mediated degranulation in RBL-2H3 mast cells without cytotoxicity

We have previously reported the ability of As to inhibit degranulation in RBL-2H3 mast cells following one hour exposure to DNP-BSA antigen (Ag) (Hutchinson *et al.*, 2011) (Figure 1).

We next tested whether degranulation is inhibited within 15-minutes of Ag and As exposure. Based on initial 15-min DNP-BSA Ag dose response data (not shown), $0.0004 \mu\text{g mL}^{-1}$ DNP-BSA Ag was chosen. This Ag dose was chosen because, in the absence of As, it elicited a degranulation value of $9.4\% \pm 0.8\%$ (SEM), which is similar to the response of the 0 ppb As sample in Figure 1 (Hutchinson *et al.*, 2011). Arsenic (750 ppb) causes a $15\% \pm 2\%$ (SEM) inhibition of the 0 ppb control value (Fig. S1A). These data indicate the dampening effect of As on Ag-mediated degranulation begins to occur quite rapidly in RBL mast cells. Arsenic has no significant effect on spontaneous degranulation ($1.4\% \pm 0.1\%$ [SEM]) during this time period (Fig. S1B).

In order to confirm the lack of cytotoxicity of high dose As (up to 1500 ppb) seen in our previous work (Hutchinson *et al.*, 2011), an additional cytotoxicity/mitochondrial function assay that measures both plasma membrane integrity and ATP production was performed. ATP is required for degranulation (Burgoyne and Morgan, 2003). Arsenic (up to 1500 ppb) is not only non-cytotoxic, but also does not disrupt ATP production (Fig. S2), even under buffer and timing conditions that allow for robust As inhibition of Ag-stimulated degranulation (data not shown). Due to mammalian cell generation of ATP via both oxidative phosphorylation and glycolysis, glucose-free, galactose-containing media (“galactose media”) was utilized to avoid ATP production via glycolysis in this experiment (Fig. S2 A-B). Also, neither cytotoxicity nor ATP depletion is observed in experiments utilizing glucose-containing media (Fig. S2 C-D), wherein cells produce sufficient ATP via glycolysis.

Membrane ruffling of F-actin in RBL-2H3 cells is not qualitatively affected by As

RBL-2H3 cells were stained with Alexa 488-conjugated phalloidin to label F-actin and imaged with an Olympus confocal microscope. In the absence of FcεRI engagement (treatment with plain BT only, a “spontaneous release” control), cytoskeletal membrane ruffling is very limited or is not observed in cells (Fig. 2A). As seen in Fig. 2B, the addition of 750 ppb As without an IgE-FcεRI crosslinking agent also does not lead to visible changes in F-actin distribution. Conversely, when IgE-bound FcεRI receptors were crosslinked by $0.00016 \mu\text{g mL}^{-1}$ DNP-BSA Ag, multiple membrane protrusions are observed (Fig. 2C). At this same antigen concentration ($0.00016 \mu\text{g mL}^{-1}$ DNP-BSA) with the addition of 750 ppb As, little or no qualitative difference is observed (Fig. 2D) compared to the no-arsenic control. Similar results are obtained with a mid-range dose of $0.0004 \mu\text{g mL}^{-1}$ Ag in the presence and absence of 750 ppb As (Fig. S3). In summary, co-incubation of RBL-2H3 with DNP-BSA Ag and 750 ppb As does not appear to substantially affect the extent of ruffling.

As does not inhibit degranulation of RBL-2H3 cells when stimulated with A23187 Ca²⁺ ionophore

Stimulation of RBL-2H3 cell degranulation using Ca²⁺ ionophore has been detailed in the scientific literature (Beaven *et al.*, 1984; Hanson and Ziegler, 2002; Siraganian *et al.*, 1975). This method of degranulation bypasses engagement of FcεRI receptors, causing Ca²⁺ influx across the plasma membrane. Use of Ca²⁺ ionophore therefore allows identification of arsenic's cellular targets as being upstream or downstream of Ca²⁺ influx. Doses of A23187 ionophore were selected based on their non-cytotoxicity in a lactate dehydrogenase (LDH) assay (Fig. S4), in the presence of up to 750 ppb As, which did not affect LDH enzyme activity (Fig. S5).

DMSO has been linked to inhibition of IL-8 production (Deforge *et al.*, 1992), decreased NF-κB activation in macrophage cells (Kelly *et al.*, 1994), and has been shown to activate PKC and scavenge free radicals (Ogura *et al.*, 1995). As an oxygen radical scavenger, DMSO has also been shown to inhibit the production of reactive oxygen species (ROS) in experimental studies (Hei *et al.*, 1998; Liu *et al.*, 2001). As ROS production has been shown to be vital to mast cell inflammatory responses (reviewed in (Swindle and Metcalfe, 2007)), and there is a strong relationship between ROS and degranulation in RBL-2H3 (Suzuki *et al.*, 2003), it was important that we ensure the level of DMSO used in these ionophore experiments does not impact degranulation in this way. We demonstrated that degranulation experiments containing 0.004% DMSO retain statistically significant inhibition, due to As, of antigen-stimulated RBL-2H3 cells, and that DMSO at this concentration does not impact the cell's ability to degranulate. A two-way ANOVA in Prism was performed to compare Ag-stimulated degranulation experiments in the presence and absence of 0.004% DMSO: no statistically-significant difference was found (Fig. S6). These control data demonstrate that the degranulation values obtained from the A23187 Ca²⁺ ionophore tests are not impacted by the vehicle conditions.

We used the probe 2',7'-dichlorodihydrofluorescein diacetate (Cell Biolabs; San Diego, CA), which measures various intracellular ROS, in order to directly test whether As modulates ROS under the treatment conditions of the degranulation experiments. Neither 500 nor 750 ppb As affect intracellular ROS production at any time point measured for 1 hour at 1-2 minute intervals (data not shown).

Regarding the use of Ca²⁺ ionophore on RBL-2H3 cells, 1 hr treatment of 1.5×10^{-7} M A23187 elicited an average absolute degranulation response of $11.5\% \pm 2.8\%$ (SEM) in the absence of As (Fig.3A); exposure to 2.0×10^{-7} M A23187 resulted in a degranulation value of $18.1\% \pm 3.2\%$ (SEM) (Fig.3B). Co-exposure to A23187 ionophore and As (0-750 ppb) revealed no arsenic inhibition of degranulation (Fig.3). The lack of an inhibitory response due to As on Ca²⁺ ionophore-mediated degranulation strongly suggests a pathway target upstream of Ca²⁺ influx across the plasma membrane.

As does not inhibit degranulation of RBL-2H3 cells when stimulated with thapsigargin

There is a biphasic calcium modulation in RBL-2H3 cells. Prior to Ca²⁺ being shuttled across the plasma membrane in the degranulation pathway (which we facilitated, as detailed

above, using A23187 ionophore), Ca^{2+} is released from internal ER stores. To investigate this earlier Ca^{2+} signaling event, we utilized the pharmacological agent thapsigargin (Tg). We have found Tg to be extremely vulnerable to oxidation, requiring immediate use within 1-2 days of preparation. In the absence of As, the average % degranulation response for 4.6 nM Tg was $11.9 \pm 3.9\%$; spontaneous release was $2.3 \pm 0.9\%$ (mean $\pm$ SEM). As seen in Fig 4A, no significant difference in Tg-stimulated degranulation is caused by As (Fig. 4A). A series of cytotoxicity experiments were performed to test whether 4.6 nM Tg $\pm$ As is toxic to RBL-2H3 cells. Trypan blue exclusion (Fig. 4B) and LDH (Fig. 4C) data demonstrate no cytotoxicity for Tg and As concentrations tested in degranulation experiments after 1 h exposure. Collectively, these results indicate that arsenic's cellular target is upstream of Ca^{2+} release from internal ER stores.

As does not affect c48/80-mediated degranulation in RBL-2H3 cells

To establish c48/80-mediated degranulation assays as a functional means to measure toxicant effects, it was first determined that 48-h treatment with quercetin (20 μM) followed by 15-min treatment with c48/80 (Senyshyn *et al.*, 1998) induces statistically significant increases in β -hexosaminidase release by RBL-2H3 cells in a dose-dependent manner (data not shown) and non-cytotoxic manner (Fig. 5B). We additionally confirmed that treatment with c48/80 and quercetin has no effect on background fluorescence as measured in the degranulation assay (data not shown). Although we determined that 25 $\mu\text{g mL}^{-1}$ c48/80 induces a non-cytotoxic increase in β -hexosaminidase release in the absence of quercetin pre-treatment, this modest response is enhanced by 48-h quercetin treatment (data not shown), allowing for greater dynamic range in which to study potential effects due to As treatment.

In Fig. 5A, it is clear that As does not affect c48/80-mediated degranulation responses, despite similar absolute levels of degranulation induced by c48/80 ($7.7\% \pm 0.6\%$ [SEM]) to the Ag used in the 15 min assay in Fig S1A. The lack of inhibition is not due to synergistic cell membrane damage between compound 48/80 and As, since the combination of c48/80 and As does not cause cytotoxicity (Fig. 5B). While 50 $\mu\text{g/mL}$ c48/80 is cytotoxic (Fig. 5B), this concentration was not used in the degranulation experiments (Fig. 5A), which employed the non-cytotoxic dose of 25 $\mu\text{g/mL}$ c48/80.

As inhibits Ag-stimulated Ca^{2+} influx

Calcium influx is a key event in the signal transduction pathway leading to mast cell degranulation. To directly measure cytosolic Ca^{2+} levels, RBL-2H3 cells were incubated with fluo 4/AM fluorescent dye for 30 minutes, to allow dye uptake, and then were exposed to 0.0002 $\mu\text{g mL}^{-1}$ DNP-BSA Ag $\pm$ 750ppb As. Next, fluorescence indicating levels of Ca^{2+} inside the cell were measured in a plate reader for 1 hour post stimulation. Our data indicate that As dampens Ca^{2+} influx into Ag-activated mast cells (Fig. 6). These results suggest that arsenic's target lies upstream of the Ca^{2+} influx event.

As inhibits phosphorylation of Phosphoinositide 3-kinase (PI3K)

Phosphoinositide 3-kinase (PI3K) is involved in a variety of cell functions because it catalyzes the production of many crucial lipid signaling molecules, including

phosphatidylinositol 4,5-bisphosphate(PIP₂). PIP₂ enables recruitment of a wide range of proteins containing Pleckstrin homology domains, thus drawing these proteins to the plasma membrane for activation (Abramson and Pecht, 2007). PIP₂ is used by phospholipase C γ to generate inositol-1,4,5-trisphosphate (IP₃), an important player in Ca²⁺ mobilization (Kalesnikoff and Galli, 2008). Overall, PI3K activation supports Ca²⁺ mobilization and degranulation. Therefore, we examined whether PI3K is affected by As exposure. We utilized a commercially available Fast Activated Cell-based ELISA kit, which quantifies activated PI3K and/or total PI3K. Two primary antibodies provided from this ELISA kit recognize either phosphorylated p85, the regulatory subunit of PI3K or total PI3K p85 levels, respectively.

Using this ELISA, we detected a significant decrease in phosphorylation of the p85 subunit of PI3K due to 750 ppb As exposure, compared to the 0 ppb As group (Fig. 7A). Because PI3K is constitutively associated with the Kit receptor and because RBL-2H3 cells' Kit receptor is known to be constitutively active, there is a high level of phosphorylated PI3K even in the absence of Ag stimulation in RBL-2H3 cells (Tsujimura *et al.*, 1995). Thus, the data were normalized to the Ag-stimulated, 0 ppb As samples, not to the unstimulated, 0 ppb As sample. The level of total p85 protein was unchanged regardless of As treatment (Fig. 7B). These results suggest that As interferes with Ag-activated phosphorylation of PI3K.

As inhibits phosphorylation of Syk kinase

Previous studies report that the regulatory subunit (p85) of phosphoinositide 3-kinase (PI3K) is one of the direct binding partners of Syk protein (Mocsai *et al.*, 2010; Okkenhaug and Vanhaesebroeck, 2003). Since we observed the reduction of phosphorylation/activation level of PI3K (Figure 7), we sought to investigate As effects on phosphorylated Syk protein using a commercially available pan-tyrosine phosphorylated Syk ELISA. Using this ELISA, we detected significant changes in phosphorylated Syk protein between Ag-stimulated and spontaneous-release samples. Figure 8 compares Ag-stimulated samples $\pm$ 750 ppb As and shows a significant decrease in phosphorylation of Syk protein due to As treatment. Phospho-Syk ELISA data provide evidence that arsenic is interfering with early tyrosine phosphorylation events in the degranulation pathway in mast cells.

Discussion

Previously, we determined that arsenic's inhibition of mast degranulation, an important event in many physiological states and diseases, is not caused by its direct interference with the crosslinker-IgE interaction at the IgE-bound Fc ϵ RI receptors at the plasma membrane: the level of As inhibition of crosslinker-stimulated degranulation was equivalent, regardless of the type of crosslinker used (i.e., DNP-BSA antigen or anti-IgE IgG; as long as the absolute level of degranulation triggered in the absence of As was held constant) (Hutchinson *et al.*, 2011). Additionally, As showed no induction of degranulation response and no inhibition of spontaneous granule release in the absence of degranulation-triggering treatments such as IgE receptor crosslinking (Hutchinson *et al.*, 2011). These data indicated that crosslinking of the Fc ϵ RI receptors is a necessary co-treatment for arsenic's inhibition of degranulation.

The main finding of the current study is that the cellular target for As inhibition of mast cell degranulation lies early in the signaling pathway, likely involving altered phosphorylation of tyrosine kinase Syk (Fig. 8) and subsequently affecting the activation of downstream target proteins of Syk, such as PI3K (Fig. 7). Syk and PI3K are both activated rapidly (within minutes) after antigen exposure. In our previously published study (Hutchinson *et al.*, 2011), degranulation was inhibited by As in a one-hour treatment with DNP-BSA antigen. When we examined the same DNP-BSA antigen treatment in a shorter period (15 minutes), we found that As inhibits degranulation at this earlier time point as well, indicating that its effects are acute and rapid (Fig. S1A). We also performed experiments with chronic As exposure of mast cells and found that longer exposure of As did not alter the extent of arsenic's inhibition of degranulation (data not shown).

Acute As poisoning, at very high As concentrations, can lead to inhibition of glycolysis and cellular ATP production, due to As substitution for phosphate (DeMaster and Mitchell, 1973). While we earlier showed, via multiple methods, that As is not cytotoxic to RBL-2H3 mast cells at the concentrations used in this study (Hutchinson *et al.*, 2011), we had not yet determined whether As affects ATP production under these experimental conditions. Here we show that As doses up to 1500 ppb do not affect ATP production (Fig S2), suggesting that inhibition of catabolism and widespread phosphate substitution is not occurring. Under the same glucose-free conditions, the canonical mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone, which also inhibits degranulation of RBL-2H3 cells, decreases ATP production, with an EC₅₀ of 0.8 μM – 1.6 μM (95% CI), with no cytotoxicity (Weatherly *et al.*, 2015).

To narrow down arsenic's target(s) in the mast cell signaling pathway, we investigated whether As affects the F-actin rearrangement or “membrane ruffling” in RBL-2H3 cells, another phenotype of activated mast cells. This strategy tests for potential cellular targets for As that are common to both the degranulation and membrane ruffling pathways, such as PKC (Pfeiffer *et al.*, 1985; Yanase *et al.*, 2011). Our finding of no or a small As effect on membrane ruffling (Fig 2) supports the conclusion that cellular targets that are downstream of Ca²⁺ influx and that are common to both degranulation and ruffling are unaffected by As.

Ca²⁺ ionophore A23187 was utilized to bypass FcεRI and the early tyrosine phosphorylation events and to stimulate the release of granules through the influx of Ca²⁺ alone into the cell (Siraganian *et al.*, 1975). In experiments utilizing A23187, we determined that As does not inhibit degranulation (Fig 3), indicating that arsenic's pathway target is upstream of Ca²⁺ influx across the plasma membrane.

We also used the pharmacological agent thapsigargin (Tg), which is a potent, cell permeable, IP₃- independent intracellular calcium releaser/SERCA pump blocker that acts a mast cell secretagogue (Patkar *et al.*, 1979; Rasmussen and Christensen, 1978). Like A23187 Ca²⁺ ionophore, Tg stimulates mast cell degranulation while by passing FcεRI crosslinking and early tyrosine phosphorylation events. However, unlike with A23187-induced cell activation, Tg stimulation does involve STIM-1 in the ER and the plasma membrane CRAC channels. Our data indicate that, with the use of the non-cytotoxic dose of 4.6 nM Tg to elicit degranulation, As does not inhibit degranulation (Fig 4). This concentration of Tg is within

the documented IC₅₀ for inhibiting SERCA (4-13 nM) (Calbiochem production information). Thus, these data show that arsenic's cellular target not only lies upstream of Ca²⁺ influx across the plasma membrane, but also is not STIM-1 or other players in endoplasmic reticulum Ca²⁺ mobilization.

In view of the fact that c48/80 activates degranulation via PLD in a Ca²⁺- dependent manner, c48/80-mediated degranulation assays can be used to measure toxicant effects on these portions of the degranulation pathway. Because there is no arsenic effect on cells stimulated to degranulate with c48/80 (Fig.5), arsenic's inhibitory actions on the degranulation pathway are likely on some portion of the pathway that lies upstream of PLD and Ca²⁺ influx. Combined, the A23187 (Fig.3), Tg (Fig.4), and c48/80 (Fig.5) data strongly suggest that arsenic's inhibitory action lies upstream of all calcium signaling in the degranulation pathway. Also, these data indicate that PKC, PLD and other enzymes activated following Ca²⁺ mobilization are not arsenic's direct targets in RBL-2H3 mast cells.

To investigate As effects on events upstream of Ca²⁺ influx, we developed a microplate-based assay to measure the level of cytoplasmic Ca²⁺ after Ag exposure, ± As, in RBL-2H3 mast cells. We found a strong inhibition of the Ag-stimulated Ca²⁺ level in the cells due to As exposure after both 30 min and 60 minutes of exposure (Fig 6). The dampened Ca²⁺ influx into As-exposed cells is another indicator that arsenic's target lies upstream of Ca²⁺ influx. Therefore, we investigated the effects of As on early tyrosine phosphorylation events, which occur immediately after FcεRI receptor activation.

Previous researchers have reported that arsenic may interfere with phosphorylation events during signal transduction. For example, Cheng et al. (2004) found that sodium arsenite inhibits the Janus kinase signal transducer and activator of transcription (JAK-STAT) cascade, via inhibition of phosphorylation of STAT3 specifically at tyrosine residues (Cheng *et al.*, 2004). Moreover, Soto-Peña et al. (2008) demonstrated that As can interfere with phosphorylation of the kinases Lck and Fyn during T-cell signal transduction (Soto-Peña and Vega, 2008). In this study, we have found that As inhibits phosphorylation of both PI3K and Syk kinases in Ag-activated RBL-2H3 cells (Fig. 7 and 8), thus providing a mechanism for As inhibition of mast cell degranulation.

Arsenite (As³⁺) has been shown in certain cases to interact with sulfhydryl groups of kinases and to form reactive oxygen species (ROS), which affect kinase activity (Liu *et al.*, 2001). In this study, we have found that As does not affect ROS levels under the treatment conditions of these experiments. However, As can directly bind to sulfhydryl groups of certain proteins, causing detrimental structural modification or denaturation (Chang *et al.*, 2012). Syk kinase (both human and rat) contains numerous cysteine residues, five of which are located specifically in its kinase domain. Therefore, derangement of normal phosphorylation activity of Syk, via arsenic-sulfhydryl interactions, may be a mechanism underlying the inhibition of both Ag-stimulated Syk phosphorylation and degranulation of mast cells.

The implications of this mechanistic study can assist researchers to better understand a mode of As toxicity that is likely to not be organism-specific or even mast-cell specific. Classic immunoreceptors, such as B cell receptors, T cell receptors, and Fc receptors, signal via

similar mechanisms, and the signal transduction pathways present in mast cells share many similarities with those of T and B cells. Arsenic's inhibition of Syk activity (and, subsequently, PI3K) in mast cells suggests that As inhibits Syk and similar proteins in the same manner in numerous cell types. Syk has garnered attention as a potentially novel target for treatment of allergic and inflammatory disorders (Bajpai *et al.*, 2008; Ulanova *et al.*, 2005; Wong *et al.*, 2004). Because As has now been shown to inhibit this vital process in allergy and asthma development, mast cell degranulation, our data strongly suggest that Syk could be a drug target to treat these diseases.

In fact, arsenic has been employed in Chinese medicine to treat asthma (ATSDR, 2007). While arsenic inhibition of mast cells *in vivo* has not yet been directly demonstrated, arsenic has been shown to block anaphylactic responses in guinea pigs (Poriadin *et al.*, 1977) and to alleviate asthma responses in mice (Chu *et al.*, 2010; Zhou *et al.*, 2006). Also, arsenic-exposed, parasite-infected children in Bangladesh were found to be thinner than controls also exposed to parasites but not to arsenic (Minamoto *et al.*, 2005), a result that suggests that arsenic exposure could inhibit mast cells *in vivo*, thus increasing helminth burden and wasting. Inhibition of mast cell function by arsenic was not offered as an explanation for these findings but is a possible mechanism of the *in vivo* effects described in these studies.

In conclusion, we have shown that arsenic inhibits mast cell degranulation at environmentally-relevant, non-cytotoxic doses, via inhibition of early tyrosine phosphorylation events. These data provide a molecular mechanism for arsenic's effects on this ubiquitous cell type and suggest that As will have similar molecular targets in many additional cell types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ag	Antigen
As	Arsenic
AUC	Area under the curve
BT	BSA-Tyrodes
c48/80	Compound 48/80
CRAC	Calcium release-activated calcium
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DNP	Dinitrophenyl
ELISA	Enzyme-linked immunosorbent assays
IgE	Immunoglobulin E
IP₃	Inositol-1,4,5-trisphosphate
LDH	Lactate dehydrogenase
PA	Phosphatidic acid
PI3K	Phosphoinositide 3-kinase
PIP₂	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PMSF	Phenylmethanesulfonyl fluoride
ppb	parts per billion
RBL-2H3	Rat basophilic leukemia subclone 2H3 cells
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate

SEM	Standard error of the mean
SERCA	Sarco/endoplasmic Ca^{2+} -ATPase
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
Syk	Spleen tyrosine kinase
SOCE	Store operated calcium entry
Tg	Thapsigargin
TX-100	Triton-X 100

Figure 1.

Arsenic inhibits RBL-2H3 cell degranulation following 1 hour exposure to DNP-BSA antigen (Ag). Effects of various concentrations of As on 1 hour, $0.00016 \mu\text{g mL}^{-1}$ Ag-mediated degranulation were assessed as described (Hutchinson *et al.*, 2011). In the absence of As, this antigen dose elicited an average absolute degranulation response of $12 \pm 2\%$ (SEM; $\sim 23\%$ of the maximal response). Values represent means $\pm$ SEM for five independent experiments, where three replicates per dose were performed for each experiment. One-way ANOVA with Tukey's post-tests (compared to the 1 ppb sample) were performed using Graphpad Prism software; * $p < 0.05$, *** $p < 0.001$. This figure is reprinted from (Hutchinson *et al.*, 2011) (Copyright 2010 John Wiley & Sons, Ltd.)

Figure 2.

F-actin ruffling during antigen-stimulated degranulation of RBL-2H3 cells. F-actin was visualized using Alexa Fluor 488 conjugated phalloidin. RBL-2H3 cells were exposed to (A) no Ag; (B) no Ag, plus 750 ppb As; (C) 0.00016 $\mu\text{g mL}^{-1}$ Ag; (D) 0.00016 $\mu\text{g mL}^{-1}$ Ag, plus 750 ppb As for one hour before being fixed. A representative set of images is shown.

Figure 3.

Arsenic does not inhibit A23187 Ca²⁺ ionophore-stimulated degranulation in RBL-2H3 cells. RBL-2H3 cells were harvested and plated as described in the “Methods” section. Cells were stimulated to degranulate for 1 h with either (A) 1.5 × 10⁻⁷ M (which caused an average absolute degranulation response of 11.5% ± 2.8% [SEM]) or (B) 2.0 × 10⁻⁷ M (which caused an average absolute degranulation response of 18.1% ± 3.2% [SEM]) A23187 Ca²⁺ ionophore. Resultant β-hexosaminidase was measured as described in “Methods.” Values are means ± SEM from seven independent experiments per A23187 dose, each with three replicates per experiment. No statistical significance was determined by one-way ANOVA followed by Tukey's post- test, where multiple comparisons were made to the 100 ppb sample.

Figure 4.

Arsenic does not inhibit thapsigargin (Tg) -induced degranulation. **(A)** Relative degranulation response of RBL-2H3 cells treated for 1hr with 4.6 nM Tg (which caused an average absolute degranulation response of $11.9 \pm 3.9\%$ [SEM]), $\pm$ As. Values represent mean $\pm$ SEM of 3-5 experiments of triplicate samples per dose per experiment. A spontaneous release measurement (no Tg or As present) is depicted for reference. No significant difference was determined by one-way ANOVA. In **(B)** trypan blue exclusion assays, the percentage of living cells present in the 0 nM Tg control is plotted against concentration of Tg (1.5 and 4.6 nM); all samples contain 750 ppb As. Values are means of two experiments, each with triplicate samples, where data were normalized to the 0 control (with no Tg and no As, but containing Tg vehicle of 0.001% DMSO). No significant difference was determined by one-way ANOVA. **(C)** Short-term cytotoxicity was determined using an LDH cytotoxicity detection kit; concentration of Tg is plotted against the percentage LDH released in the absence or presence of 750 ppb As; values represent individual wells ($n = 6-9$), and error bars are SD. No significant difference was determined by one-way ANOVA.

Figure 5.

Arsenic does not affect c48/80-induced degranulation of RBL-2H3 cells. **(A)** Effect of As on c48/80-mediated degranulation. Cells were pre-treated with quercetin and then co-exposed to $25 \mu\text{g mL}^{-1}$ c48/80 (which caused an average absolute degranulation response of $7.7\% \pm 0.6\%$ [SEM]) and varying concentrations of As (0-750 ppb) for 15 minutes, followed by β -hexosaminidase quantification as described in "Methods." Data were normalized to the 0 ppb As response value of each experiment ($n = 5$). **(B)** LDH cytotoxicity assay indicating non-cytotoxicity of $25 \mu\text{g mL}^{-1}$ c48/80 treatment ($n = 3$). Cells were treated as in (A), and LDH release was measured as described in "Methods." In (A-B), values represent means $\pm$ SEM; statistical significance was determined by one-way ANOVA followed by Tukey's post test (in A, comparison made to 100 ppb; in B, direct comparison is made to $0 \mu\text{g mL}^{-1}$ c48/80); * $p < 0.05$, *** $p < 0.001$.

Figure 6.

Arsenic dampens Ca^{2+} influx into antigen-activated RBL-2H3 cells. Fluo-4/AM was used to measure Ca^{2+} levels within RBL-2H3 cells which had been sensitized with anti-DNP IgE ($0.1 \mu\text{g mL}^{-1}$) for 1 hr before exposure to $\pm 0.0002 \mu\text{g mL}^{-1}$ DNP-BSA Ag $\pm 750 \text{ ppb As}$, at 30 minutes (**A**) or 1 hour (**B**) post-Ag stimulation. Control samples received no As or Ag. Values represent mean (normalized to 0 ppb As) $\pm$ SEM of four experiments, with 8-16 replicates per dose per experiment. Statistical significance, as compared to the 0 ppb As sample, was determined by one-way ANOVA followed by Tukey's post test; *** $p < 0.001$

Figure 7.

Arsenic inhibits phosphorylation of phosphoinositide 3-kinase (PI3K) in RBL-2H3 cells.

RBL-2H3 cells were sensitized with anti-DNP IgE ($0.1\mu\text{g mL}^{-1}$) for 1 hr before being

treated with DNP-BSA Ag ($1\mu\text{g mL}^{-1}$) $\pm$ 750ppb As for 5 min. Levels of (A)

phosphorylation of the p85 subunit of PI3K and (B) total p85 subunit of PI3K were

measured with a Fast Activated Cell-based ELISA kit. Data were plotted after correction for

cell number (by use of crystal violet staining). Values represent mean (normalized to 0 ppb

As) $\pm$ SEM of 3-4 experiments of triplicate samples. Statistical significance was determined

by one sample t-test; ** $p < 0.01$.

Figure 8.

Arsenic inhibits phosphorylation of Syk kinase. RBL-2H3 cells were sensitized with anti-DNP IgE ($0.1\mu\text{g mL}^{-1}$) for 1 hr before being treated with DNP-BSA Ag ($1\mu\text{g mL}^{-1}$) $\pm$ 750 ppb As for 5 min. Phosphorylation of Syk was measured with a PathScan® Phospho-Syk (panTyr) Sandwich ELISA Kit. Values are expressed as % increase over spontaneous (no Ag) control samples and represent mean $\pm$ SEM of four experiments, each of triplicate samples. Statistical significance was determined by one sample t-test; * $p < 0.05$