

Low Concentrations of Arsenite Target the Intraluminal Inositol 1, 4, 5-Trisphosphate Receptor/Ryanodine Receptor Crosstalk to Significantly Elevate Intracellular Ca^{2+}

Andrea Guidarelli, Mara Fiorani, and Orazio Cantoni

Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy

Received May 7, 2018; accepted July 27, 2018

ABSTRACT

Arsenite is an established human carcinogen that induces cytotoxic and genotoxic effects through poorly defined mechanisms involving the formation of reactive oxygen species (ROS) and deregulated Ca^{2+} homeostasis. We used variants of the U937 cell line to address the central issue of the mechanism whereby arsenite affects Ca^{2+} homeostasis. We found that 6-hour exposure to the metalloid (2.5 μM), although not associated with an immediate or delayed toxicity, causes a significant increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) through a mechanism characterized by the following components: 1) it was not affected by ROS produced under the same conditions; 2) a small amount of Ca^{2+} was mobilized from the inositol-1,4,5-trisphosphate receptor (IP_3R), and this response was not augmented by greater concentrations of the metalloid; 3) large amounts of Ca^{2+} were instead dose

dependently mobilized from the ryanodine receptor (RyR) in response to IP_3R stimulation; 4) the cells maintained an intact responsiveness to agonist-stimulated Ca^{2+} mobilization from both channels; 5) arsenite, even at 5–10 μM , failed to directly mobilize Ca^{2+} from the RyR; and 6) arsenite failed to enhance Ca^{2+} release from the RyR under conditions in which the $[\text{Ca}^{2+}]_i$ was increased by either RyR agonists or ionophore-stimulated Ca^{2+} uptake. We therefore conclude that arsenite elevates the $[\text{Ca}^{2+}]_i$ by directly targeting the IP_3R and its intraluminal crosstalk with the RyR. This mechanism likely mediates mitochondrial superoxide formation, downstream damage on various biomolecules (including genomic DNA), and mitochondrial dysfunction/apoptosis eventually occurring after longer incubation to, or exposure to greater concentrations of, arsenite.

Introduction

Intracellular mobilization of Ca^{2+} , an event that regulates an array of different physiologic functions, as well as pathologically and toxicologically relevant dysfunctions, is mediated predominantly by two separate, but nevertheless related, Ca^{2+} channels, the inositol 1,4,5-trisphosphate receptor (IP_3R) and the ryanodine receptor (RyR) (Berridge, 2016; Chernorudskiy and Zito, 2017; Meissner, 2017). These Ca^{2+} channels are located on the surface of the endoplasmic reticulum (ER) and share numerous similarities, including a complex regulation mediated by reactive oxygen species (ROS) (Gorlach et al., 2015; Chernorudskiy and Zito, 2017; Csordás et al., 2018). The significance of the IP_3R - and RyR-regulated mechanisms can be easily appreciated in physiologic as well as pathologic conditions (Clapham, 2007; Berridge, 2016; Raffaello et al., 2016; Chernorudskiy and Zito, 2017; Csordás et al., 2018). Toxic treatments often deregulate Ca^{2+} homeostasis, with important consequences on the activity of

enzymatic systems/signal transduction pathways eventually associated with the formation of large amounts of ROS.

Trivalent arsenic, a widely distributed environmental toxic metalloid and human carcinogen (Flora, 2011; Minatel et al., 2018), produces deleterious effects in an array of molecular targets, presumably through its direct binding to protein thiols and indirectly through the intermediate formation of ROS (Flora, 2011). These effects include DNA strand scission, inhibition of DNA repair, altered gene expression, mitochondrial dysfunction and permeability transition, ER stress, autophagy, and apoptosis (Flora, 2011). Not surprisingly, various studies have investigated the effects of trivalent arsenic on Ca^{2+} homeostasis.

This is especially true for As_2O_3 (Hoonjan et al., 2018), a compound widely used as an antileukemic drug. Although the number of studies addressing the effects of sodium arsenite is significantly lower, their outcomes nevertheless provide information for an increased intracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$); however, the increased is mediated by often undefined mechanisms (Chen et al., 2002; Banerjee et al., 2011; Suriyo et al., 2012; Pachauri et al., 2013). Some studies have linked the release of cation to activation of phospholipase C (PLC) and further downstream signaling via inositol 1,4,5-trisphosphate

This work was supported by Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale, 2015, [Grant 2015MJBEM2-003 to O.C.].
<https://doi.org/10.1124/jpet.118.250480>.

ABBREVIATIONS: 2-APB, 2-aminoethoxydiphenyl borate; ANOVA, analysis of variance; $[\text{Ca}^{2+}]_i$, intracellular concentration of Ca^{2+} ; Cf, caffeine; ClCR, Ca^{2+} -induced Ca^{2+} release; CmC, 4-chloro-m-cresol; D cells, differentiated cells; ER, endoplasmic reticulum; IP_3R , inositol 1,4,5-trisphosphate receptor; mitO_2^- , mitochondrial superoxide; PLC, phospholipase C; RD cells, respiration-deficient cells; ROS, reactive oxygen species; RP cells, respiration-proficient cells; Ry, ryanodine; RyR, ryanodine receptor; U-73122, 1-(6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione.

through IP₃Rs (Chen et al., 2002; Suriyo et al., 2012), but unfortunately these studies did not address involvement of the RyR. Finally, other studies have reported effects of arsenite associated with either a decreased Ca^{2+} response of primary chondrocytes to IP₃R agonists (Hsu et al., 2012) or, rather, enhanced Ca^{2+} responses mediated by phenylephrine in vascular smooth muscle cells (Lee et al., 2005).

It therefore appears that arsenite targets the IP₃R, but critical details of the overall scenario are still missing. For example, it is unclear whether the RyR, which presents critical cysteines and releases Ca^{2+} in response to ROS or specific thiol-reactive agents (Chernorudskiy and Zito, 2017; Meissner, 2017), is involved in the Ca^{2+} response elicited by the metalloid. In addition, the RyR could be involved through the so-called Ca^{2+} -induced Ca^{2+} release (CICR) event; however, the major problems for correct interpretation of the available information concern use of different cell types and exposure conditions in the different studies. Most of these studies used high and toxic concentrations of arsenite, possibly triggering multiple mechanisms affecting Ca^{2+} homeostasis and ROS release.

Information is therefore needed to learn more about the mechanism(s) whereby low concentrations of the metalloid affect Ca^{2+} homeostasis. These conditions allow the detection of effects specifically induced by the metalloid in the absence of confounding events resulting from energy depletion and/or toxicity. Understanding the molecular bases of these events is particularly important since deregulated Ca^{2+} homeostasis is a potential trigger for ROS formation and hence for the ensuing formation of potentially mutagenic DNA lesions. As a final note, these same mechanisms are also of potential relevance for the triggering of events promoting mitochondrial permeability transition-dependent apoptosis observed after prolonged exposure to the same arsenite concentrations or after exposure to greater concentrations of the metalloid (Guidarelli et al., 2016a, 2017; Fiorani et al., 2018).

The present study was therefore designed to fill some of the gaps left by previous studies. In particular, using the well characterized and versatile U937 cell line, also used in our previous studies (Guidarelli et al., 2016a, 2017), we found that arsenite elevates the $[Ca^{2+}]_i$ through the sequential mobilization of small and large amounts of the cation from the IP₃R and RyR, respectively. Under these conditions, cells maintained an intact responsiveness to agonist-stimulated Ca^{2+} mobilization from both channels, and the large Ca^{2+} response mediated by the metalloid appeared entirely dependent on direct effects on the intraluminal crosstalk between the two Ca^{2+} channels.

Materials and Methods

Chemicals. Sodium arsenite, 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U-73122), 2-aminoethoxydiphenyl borate (2-APB), ryanodine (Ry), caffeine (Cf), 4-chloro-m-cresol (4-CmC), ATP, histamine, A23187, thapsigargin, rotenone, ascorbic acid (AA), H₂O₂, as well as most of the reagent-grade chemicals, were purchased from Sigma-Aldrich (Milan, Italy). Fluo-4-acetoxymethyl ester was from Molecular Probes (Leiden, The Netherlands).

Cell Culture and Treatment Conditions. U937 human myeloid leukemia cells were cultured in suspension in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Euroclone, Celbio Biotecnologie, Milan, Italy), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Euroclone), at 37°C in T-75 tissue culture

flasks (Corning Inc., Corning, NY) gassed with an atmosphere of 95% air-5% CO₂. These cells were differentiated to monocytes by 4 days of growth in culture medium supplemented with 1.3% dimethylsulfoxide, as previously described (Scotti et al., 2018). U937 cells were made respiration-deficient as indicated in Guidarelli et al. (2016b).

Sodium arsenite was prepared as a 1 mM stock solution in saline A (140 mM NaCl, 5 mM KCl, 4 mM NaHCO₃, and 5 mM glucose; pH 7.4) and stored at 4°C. Cells (1.5×10^5 cells/ml) were exposed to arsenite in complete RPMI 1640 culture medium. A 10 mM AA stock solution was prepared in extracellular buffer (15 mM Hepes, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, pH 7.4) immediately before use. Cells (1×10^6 cells/ml) were treated with AA in extracellular buffer supplemented with 0.1 mM dithiothreitol for 15 minutes at 37°C. Stability of AA under these conditions was assessed by monitoring the absorbance at 267 nm for 15 minutes ($\epsilon_{267} = 14,600 \text{ M}^{-1}\text{cm}^{-1}$).

Measurement of Intracellular Free Ca^{2+} Levels. Cells were treated for 20 minutes with 4 μM Fluo-4-acetoxymethyl ester and subsequently exposed for further 10 minutes to selected IP₃R or RyR agonists. In other experiments, cells were exposed for increasing time intervals to arsenite, and Fluo-4-acetoxymethyl ester was added to the culture medium in the last 30 minutes of incubation. After the treatments, cells were washed three times with saline A and immediately analyzed by fluorescent microscopy. Fluorescence images were captured with a BX-51 microscope (Olympus, Milan, Italy), equipped with a SPOT-RT camera unit (Diagnostic Instruments; Delta Sistemi, Rome, Italy) using an Olympus LCAch 40 x/0.55 objective lens. The excitation and emission wavelengths were 488 and 515 nm, respectively, with a 5-nm slit width for both emission and excitation. Images were collected with exposure times of 100–400 milliseconds, digitally acquired and processed for fluorescence determination at the single cell level on a personal computer using NIH ImageJ software. Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/treatment condition in each experiment.

Cytotoxicity Assay. The number of viable cells was estimated with the trypan blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:2 (v/v) with 0.4% trypan blue, and the viable cells (i.e., those excluding trypan blue) were counted using the hemocytometer.

Aconitase Activity. Cells were washed twice with saline A, resuspended in lysis buffer (50 mM Tris-HCl, 2 mM Na-citrate, 0.6 mM MnCl₂, pH 7.4), and finally sonicated three times on ice by using the Sonicator ultrasonic liquid processor XL (Heat System-Ultrasonics, Inc., Jamestown, NY) operating at 20 W (10 seconds). The resulting homogenates were centrifuged for 5 minutes at 18,000g at 4°C. Aconitase activity was determined spectrophotometrically in the supernatants at 340 nm, as described in Gardner (2002).

ATP Determination. Cells were washed twice with saline A and resuspended in ice-cold 5% perchloric acid. After a 10-minute incubation in an ice bath, the samples were centrifuged for 5 minutes at 10,000g. The supernatants were neutralized with 3 M K₂CO₃, and the precipitates were removed by centrifugation. Then 10% (v/v) 1 M KH₂PO₄ (pH 6.5) was added to the nucleotide-containing supernatants. The samples were then filtered through 0.22-μm-pore microfilters and analyzed for ATP content by reversed-phase high-performance liquid chromatography (Stocchi et al., 1985) using a 25 cm × 4.6-mm, 5-μm Supelco Discovery C18 column (Supelco, Bellefonte, PA).

Measurement of Ca^{2+} -ATPase Activity. Cells were washed twice with saline A, resuspended in saline A, and finally sonicated three times on ice, as detailed already. The resulting homogenates were analyzed for Ca^{2+} -ATPase activity by using a commercial Kit (MyBioSource, San Diego, CA).

Statistical Analysis. The results are expressed as means ± S.D. Statistical differences were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparison or two-way ANOVA followed by Bonferroni's test for multiple comparison. $P < 0.05$ was considered significant.

Results

Selection of the Experimental Cell Types. We investigated the effects of arsenite on Ca^{2+} homeostasis and focused our attention on the role of both the IP_3R and RyR . For this purpose, we decided to use U937 cells, which express both channels (Sugiyama et al., 1994; Clementi et al., 1998; Hosoi et al., 2001; Guidarelli et al., 2009) and uniquely generate mitochondrial superoxide (mitoO_2^-) in response to arsenite (Guidarelli et al., 2016a,b, 2017; Fiorani et al., 2018). As we reported elsewhere, mitoO_2^- formation could be selectively prevented with use of complex I inhibitors, by the respiration-deficient phenotype, or by a short-term pre-exposure to a very low AA (Guidarelli et al., 2016a,b, 2017; Fiorani et al., 2018), thereby providing optimal conditions to address the question of whether Ca^{2+} mobilization from intracellular stores is mediated by ROS. There is, however, a final relevant advantage offered by these cells to conveniently address some of the questions asked in the present study. Indeed, U937 cells can be easily differentiated to monocytes, and these conditions are associated with downregulation of the RyR (Guidarelli et al., 2009), thereby providing a useful approach to address the specificity of the effects mediated by RyR inhibitors.

The present study was therefore performed using promonocytic U937 cells, referred to here as respiration-proficient cells (RP cells), respiration-deficient cells (RD cells), and differentiated cells (D cells). RD and D cells were both derived from RP cells and are characterized by similar ATP levels (present study) and antioxidant defense (Guidarelli et al., 2006, 2009). Whereas D cells presented typical features of monocytes, and were therefore unable to proliferate (Guidarelli et al., 2009), RP and RD cells maintained a similar morphology and displayed identical growth kinetics (Guidarelli et al., 2016b).

Preliminary Characterization of the Ca^{2+} Response to Selected Agonists. Experiments were initially performed to characterize the Ca^{2+} response of RP, RD, and D cells to IP_3R and RyR agonists. As indicated in Fig. 1, A–C, a 10-minute exposure to increasing concentrations of ATP caused a comparable, dose-dependent increase in $[\text{Ca}^{2+}]_i$ in the three cell types, suppressed by 2-APB, a specific IP_3R antagonist (Berridge, 2016), or U-73122, a PLC inhibitor (Cardenas et al., 2016), with, however, a different sensitivity to Ry, an RyR inhibitor (Meissner, 2017). Ry marginally reduced the increase in $[\text{Ca}^{2+}]_i$ detected in RP or RD cells, with no apparent effects measured in D cells. Similar results were obtained by replacing ATP with histamine, another IP_3R agonist (Hill et al., 1997) (Fig. 1G). Note that the effects mediated by 100 μM ATP on $[\text{Ca}^{2+}]_i$ were not further increased at up to 10 times greater concentrations (data not shown).

Cf, an RyR agonist (Meissner, 2017), also promoted an increase in $[\text{Ca}^{2+}]_i$ in RP- (Fig. 1D) and RD (Fig. 1E) cells, in both circumstances insensitive to 2-APB, or U-73122, and suppressed by Ry. Cf, however, failed to increase $[\text{Ca}^{2+}]_i$ in D cells (Fig. 1F). Similar results were obtained using 4-CmC, another RyR agonist (Meissner, 2017), in the place of Cf (Fig. 1H). The Ca^{2+} response mediated by Cf, or 4-CmC, was significantly lower than that mediated by either ATP or histamine. Note that the effects mediated by 10 mM Cf on $[\text{Ca}^{2+}]_i$ were not further increased in RP- and RD-cells using up to three times greater concentrations (data not shown).

These results provide a detailed description of the Ca^{2+} responses mediated by IP_3R and RyR agonists in RP, RD, and D cells. This information will be used to interpret the effects mediated by arsenite in the three cell types. These results also provide evidence for the specificity of the effects mediated by Ry, which abolished the response mediated by Cf, with only a small effect on the ATP-dependent stimulation. Likewise, the specificity of the effects of 2-APB is demonstrated by the complete inhibition of the Ca^{2+} response evoked by ATP and by the lack of effect on the response mediated by Cf.

Selection of Appropriate Treatment Conditions. After preliminary characterization of the Ca^{2+} responses mediated by IP_3R and RyR agonists in the three cell types used in this study, we performed experiments to select conditions of arsenite exposure failing to produce detectable signs of cytotoxicity. As shown in Fig. 2, 6-hour exposure to 2.5 μM arsenite failed to promote detectable toxic effects in RP, RD, or D cells. Negative results were obtained using the trypan blue exclusion assay (Fig. 2A) or measuring the release of lactate dehydrogenase (data not shown). In addition, the three cell types maintained normal ATP levels (Fig. 2B) and Ca^{2+} -ATPase activities (Fig. 2C). Note that all the preceding negative results are associated with positive results obtained with other treatments (inset, Fig. 1, A–C).

To provide an additional relevant information documenting that under these conditions arsenite fails to affect the viability of the three cell types, we counted the number of viable cells after increasing the time intervals of post-treatment (6 hours) incubation in fresh culture medium. As indicated in Fig. 2, RP (Fig. 2D) or RD (Fig. 2E) cells treated with arsenite proliferated with kinetics superimposable on those of their untreated counterparts. D cells ceased to proliferate during differentiation and eventually lost this phenotype and proliferated once again after 24–48 hours of incubation in complete culture medium deprived of the differentiating agent. As indicated in Fig. 2F, however, there were no differences in the number of untreated or arsenite-treated D cells after increasing time intervals of incubation in fresh culture medium.

By all the preceding criteria, we can therefore safely conclude that a 6-hour exposure to 2.5 μM arsenite fails to produce immediate or delayed signs of toxicity in the three cell types used in this study.

Effects of Arsenite on Ca^{2+} Homeostasis Are Not Mediated by ROS. The results illustrated in the previous section indicate that a 6-hour exposure to 2.5 μM arsenite fails to produce obvious signs of toxicity in the three cell types used in this study. This treatment, as we previously determined (Guidarelli et al., 2016b, 2017; Fiorani et al., 2018), is, however, associated with the formation of mitoO_2^- , which can be clearly detected in RP cells in terms of inhibition of aconitase activity (Fig. 3A). Aconitase is an enzyme sensitive to O_2^- -dependent inhibition (Yan et al., 1997; Gardner, 2002) and is largely localized in the mitochondria (Scandroglio et al., 2014). In addition, we recently determined that aconitase activity is only detectable in the mitochondrial fraction of U937 cells (Guidarelli et al., 2014). Figure 3A also shows that mitoO_2^- formation is suppressed by rotenone or AA. As a final note, arsenite failed to inhibit aconitase activity in RD cells, thereby indicating that the effects mediated by the respiratory

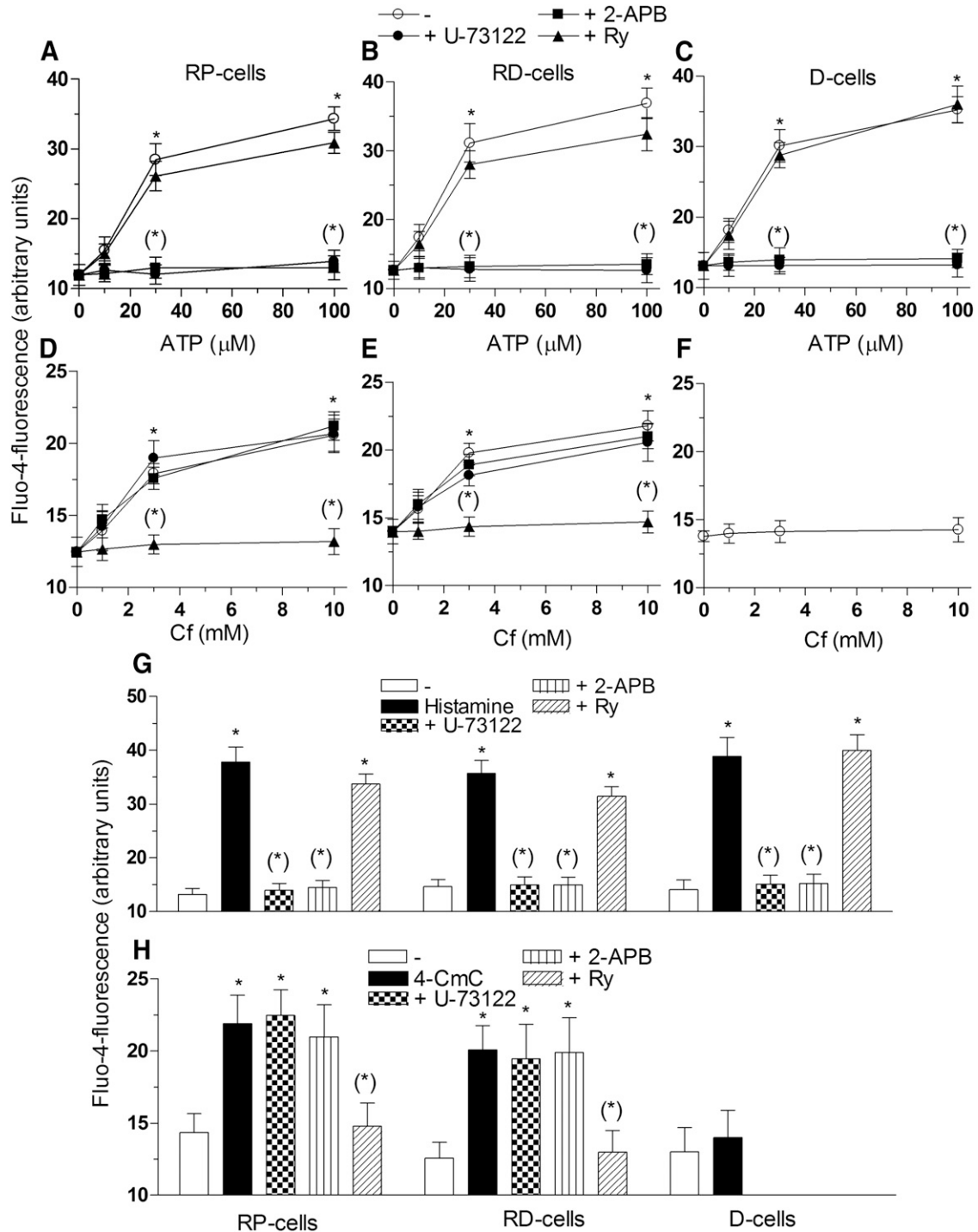


Fig. 1. Characterization of the Ca^{2+} responses mediated by IP₃R and RyR agonists in RP, RD, and D cells. Fluo-4-acetoxymethyl ester-preloaded RP (A and D), RD (B and E), and D cells (C and F) were exposed for 10 minutes to increasing concentrations of ATP (A–C) or Cf (D–F). In some experiments, 2 μ M U-73122, 50 μ M 2-APB, or 20 μ M Ry was added to the cultures 5 minutes before ATP or Cf. Fluo-4-acetoxymethyl ester-preloaded RP, RD, and D cells were exposed for 10 minutes to 10 μ M histamine (G) or 300 μ M 4-CmC (H) in the absence or presence of U-73122, 2-APB, or Ry. Fluo-4-fluorescence was then quantified as detailed in *Materials and Methods*. Results represent the means \pm S.D. calculated from at least three separate experiments. * $P < 0.01$ compared with untreated cells, * $P < 0.01$, or cells treated with agonists (one-way ANOVA followed by Dunnett's test).

chain inhibitor rotenone in RP cells are mimicked by the respiration-deficient phenotype.

These results, although recapitulating our previous findings (Guidarelli et al., 2016a,b, 2017; Fiorani et al., 2018), provide a rationale and a strategy to address the specific question of whether O_2^- and/or its dismutation product, H_2O_2 ,

are causally involved in events associated with the effects of arsenite on Ca^{2+} homeostasis.

We therefore performed experiments in RP cells exposed for 6 hours to 2.5 μ M arsenite to test the effects of rotenone, or AA, in the ensuing increase in $[Ca^{2+}]_i$. As indicated in Fig. 3B, both agents failed to prevent the increase in $[Ca^{2+}]_i$ mediated by

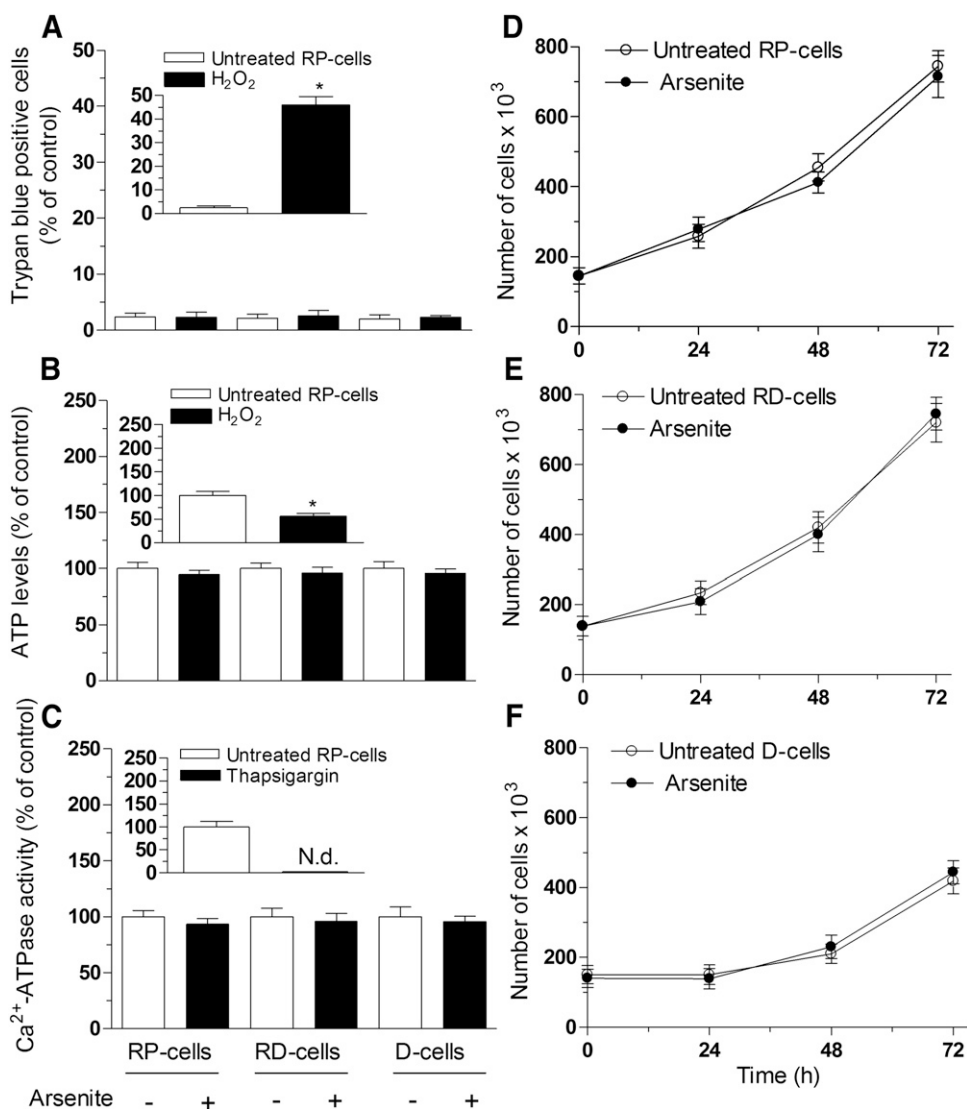


Fig. 2. The effects of arsenite on cell viability, energy status, and Ca²⁺-ATPase activity. RP, RD, and D cells were exposed for 6 hours to 2.5 μ M arsenite and then analyzed by counting the number of trypan blue-positive cells (A) and measuring their ATP levels (B) and Ca²⁺-ATPase activities (C). The positive controls were obtained using RP cells exposed for 120 (A, inset) or 30 (B, inset) minutes to 150 μ M H₂O₂. Cells were exposed for 10 minutes to 1 μ M thapsigargin (C, inset; n.d., not detectable). In other experiments, RP (D), RD (E), and D cells (F) were treated with arsenite as indicated in (A), washed, resuspended in fresh culture medium, and grown for up to 72 hours. The cell number was determined using a hemocytometer 24–72 hours after 6-hour exposure to the metalloid. Results are expressed as percentages of the untreated control and represent the means \pm S.D. calculated from at least three separate experiments. * $P < 0.01$ compared with untreated cells (one-way ANOVA followed by Dunnett's test).

the metalloid. Along the same lines, identical time-dependent increases in [Ca²⁺]_i were observed in RP and RD cells exposed for increasing time intervals to arsenite (Fig. 3C).

These results rule out the possibility that mitoO₂⁻/H₂O₂ contributes to events associated with altered Ca²⁺ homeostasis.

Arsenite Promotes the Sequential Release of Ca²⁺ from the IP₃R and the RyR. We performed experiments to determine the source of the Ca²⁺ mobilized by arsenite in RP cells exposed to arsenite. As indicated in Fig. 4A, the time-dependent increase in [Ca²⁺]_i observed in these cells was prevented by 2-APB and significantly blunted, but not abolished, by Ry. To obtain further information allowing interpretation of these results, we performed similar experiments in D cells. These cells responded to arsenite with a significantly lower time-dependent increase in [Ca²⁺]_i (Fig. 4B) compared with RP cells. In addition, the Ca²⁺ response detected in D cells was suppressed by 2-APB and unaffected by Ry. Note that similar low [Ca²⁺]_i are detected in D cells exposed to arsenite and in RP cells exposed to arsenite in combination with Ry. We could not test the effect of U-73122 on the increase in [Ca²⁺]_i mediated by arsenite because of its intrinsic toxicity. Loss of viability was detected in each of the three cell types

after 6 hours' incubation with U-73122 or even after a 10-minute exposure to the inhibitor before the subsequent 6-hour incubation in fresh medium. The susceptibility of tumor cells to PLC inhibitors has been reported elsewhere (Cardenas et al., 2016).

These results therefore indicate that arsenite induces a time-dependent increase in [Ca²⁺]_i likely attributable to mobilization of the cation from both the IP₃R and RyR. The outcome of inhibitor studies in RP cells, combined with that from experiments using D cells, which fail to express the RyR, suggest that the initial low Ca²⁺ response mediated by stimulation of the IP₃R is functional to recruitment of the RyR to contribute to a significantly greater increase in [Ca²⁺]_i.

Characterization of the Effect of Arsenite on the IP₃R and RyR. The results summarized in the previous section suggest that a 6-hour exposure to 2.5 μ M arsenite causes a weak IP₃R response. The simplest explanation obviously would be based on the concentration dependence of the effects mediated by arsenite, although alternative explanations are based on the possibility that the metalloid inhibits critical steps of the machinery involved in Ca²⁺ release by the IP₃R.

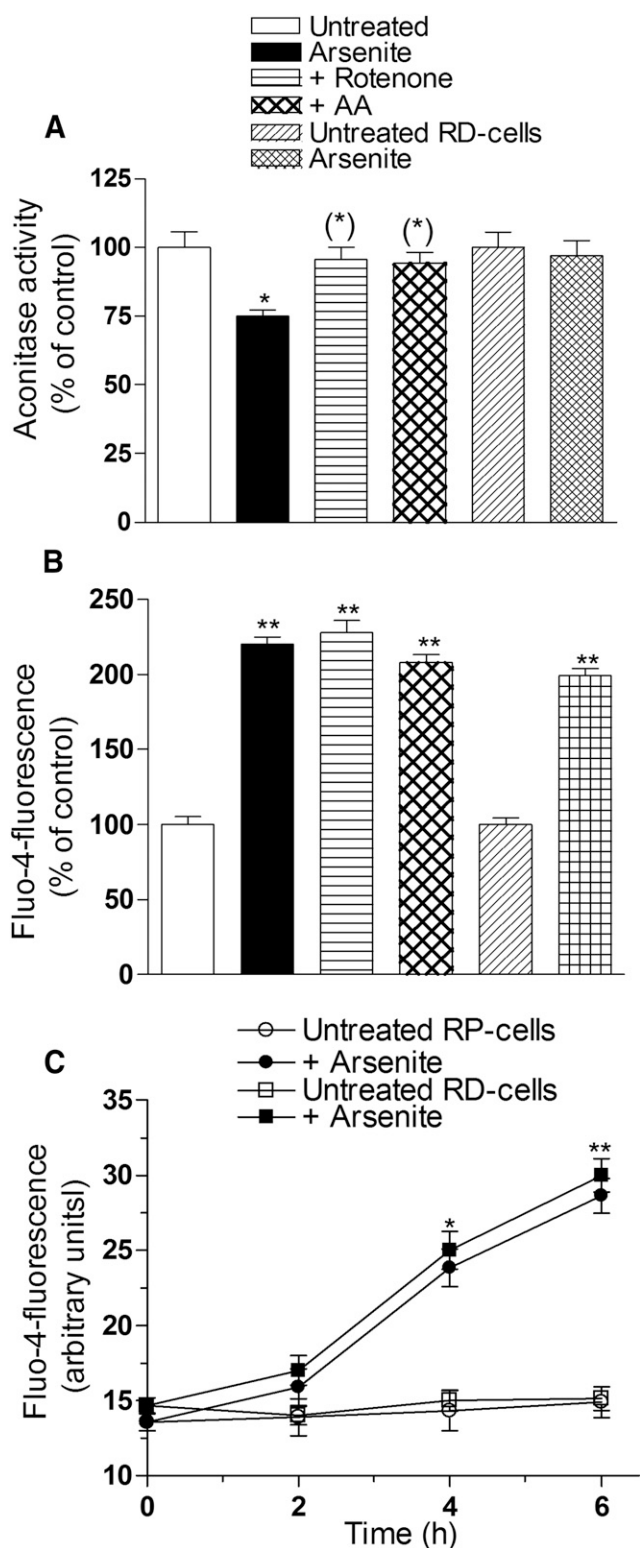


Fig. 3. The effects of arsenite on Ca^{2+} homeostasis are not mediated by ROS. Cells were exposed for 6 hours to 2.5 μ M arsenite in the absence or presence of either 0.5 μ M rotenone or 10 μ M AA and then analyzed for aconitase activity (A) or Fluo-4-fluorescence (B). Selected experiments were performed using RD cells either untreated or supplemented with arsenite. In other experiments, Fluo-4-acetoxymethyl ester-preloaded RP and RD cells were incubated for increasing time intervals with 2.5 μ M arsenite and then processed as indicated (C). Results represent the means \pm S.D. calculated from at least three separate experiments. * P < 0.05; ** P < 0.01 compared with untreated cells. * P < 0.05 compared with cells treated with arsenite (two-way ANOVA followed by Bonferroni's test).

We therefore performed experiments to address these questions and initially determined the $[Ca^{2+}]_i$ resulting from a 6-hour exposure of RP cells to 2.5–10 μ M arsenite. The results illustrated in Fig. 4C indicate that, under these conditions, the metalloid promotes a remarkable and progressive increase in $[Ca^{2+}]_i$. Ry significantly blunted this Ca^{2+} response and abolished the concentration dependence of the effects of arsenite. The outcome of experiments using D cells indicated that 5 or 10 μ M arsenite fails to promote greater effects than 2.5 μ M arsenite (Fig. 4D). The curve obtained in these conditions was virtually identical to that obtained using RP cells supplemented with Ry (note the different ordinate axis). Interestingly, 2-APB also abolished the Ca^{2+} response mediated by the high concentrations of arsenite, thereby indicating that the metalloid, even under harsh conditions, fails to directly mobilize the cation from the RyR.

We then asked whether treatment with arsenite is associated with specific effects on the IP₃R structure and function, thereby limiting the Ca^{2+} response mediated by its stimulation. For this purpose, we tested the effect of the IP₃R agonist ATP under nonpermissive conditions for further Ca^{2+} release by the RyR. In these experiments, RP cells were treated for 6 hours with 2.5 μ M arsenite in the presence of Ry (Fig. 5A) and D cells with the metalloid alone (Fig. 5B) before 10-minute exposure to increasing concentrations of ATP/Ry (RP cells) or ATP alone (D cells). We found that the Fluo-4-dependent fluorescence responses mediated by ATP in arsenite-supplemented RP and D cells, although originating by the ordinate axis with an initial value corresponding to the fraction of the cation mobilized by the metalloid, rapidly converged with the dose-response curve obtained in naïve RP and D cells. As expected, under all the conditions, the Ca^{2+} responses were suppressed by 2-APB (inset, Fig. 5).

Collectively, the results obtained in these experiments indicate that arsenite is a poor activator of the signaling leading to Ca^{2+} mobilization from the IP₃R and that this response is maximally stimulated at 2.5 μ M. Furthermore, arsenite does not limit the responsiveness of the IP₃R to agonist stimulation. An additional conclusion derived from these experiments is that arsenite, even at very high concentrations, does not directly mobilize Ca^{2+} from the RyR.

Arsenite Fails to Affect the CICR Event Induced by Local Increases in $[Ca^{2+}]_i$ in Close Proximity of the RyR or by More General Increases of the Cytosolic $[Ca^{2+}]_i$ Induced by a Ca^{2+} Ionophore. The results thus far presented provide evidence for specific and concentration-dependent effects of arsenite resulting in enhanced Ca^{2+} release from the RyR, under conditions in which the $[Ca^{2+}]_i$ derived from the IP₃R is weakly stimulated. Hence, a possible explanation would be that arsenite binds to specific thiols of the RyR, thereby causing its concentration-dependent sensitization to the increased $[Ca^{2+}]_i$. We therefore used 2-APB to prevent Ca^{2+} release from the IP₃R, and the ensuing events and investigated the effect of arsenite on agonist-induced stimulation of Ca^{2+} release by the RyR. With this experiment, we were testing whether a local increase in $[Ca^{2+}]_i$ was able to trigger a greater release of the cation from the RyR in arsenite-supplemented cells.

RP cells were therefore exposed for 6 hours to 2.5 μ M arsenite in the presence of 2-APB, before 10 minutes' exposure to increasing concentrations of Cf (Fig. 6A). Interestingly, the Fluo-4-dependent fluorescence response observed under

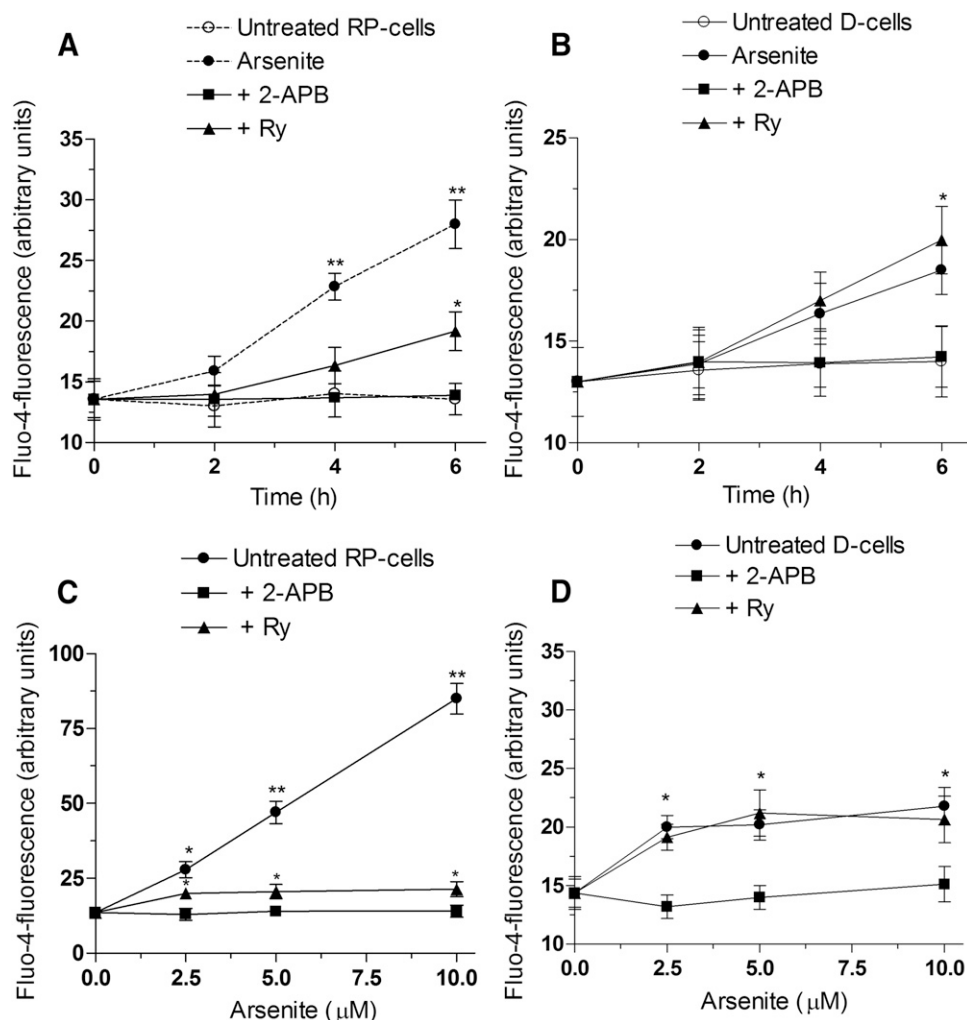


Fig. 4. Arsenite promotes a sustained increase in the $[Ca^{2+}]_i$ through a mechanism mediated by an initial small effect on the IP_3R and a subsequent remarkable effect on the RyR. Fluo-4-acetoxymethyl ester-preloaded RP (A) and D cells (B) were incubated for increasing time intervals with $2.5 \mu M$ arsenite in the absence or presence of 2-APB or Ry. Fluo-4-acetoxymethyl ester-preloaded RP (C) and D cells (D) were incubated for 6 hours with increasing concentrations of arsenite in the absence or presence of 2-APB or Ry. After treatments, the cells were analyzed for Fluo-4-fluorescence. Results represent the means \pm S.D. calculated from at least three separate experiments. * $P < 0.05$; ** $P < 0.01$ compared with untreated cells (two-way ANOVA followed by Bonferroni's test).

these conditions appeared identical to that mediated by Cf in cells pretreated with 2-APB alone. Ry abolished the increase in $[Ca^{2+}]_i$ detected under both conditions (inset, Fig. 6A). These results indicate that arsenite fails to enhance the Ca^{2+} response mediated by a RyR agonist, which obviously increases the $[Ca^{2+}]_i$ in the same microdomains in which the RyR is located.

We therefore asked the question of whether the same is true under conditions in which the $[Ca^{2+}]_i$ is elevated with the use of A23187, a Ca^{2+} ionophore (Peiretti et al., 1996). In these experiments, RP cells were treated with arsenite/2-APB, as indicated, subsequently stimulated with increasing concentrations of A23187 and finally analyzed to determine the Fluo-4-fluorescence response. The increase in $[Ca^{2+}]_i$ detected under these conditions was virtually identical to that detected in cells pre-exposed to 2-APB alone and treated with the ionophore (Fig. 6B). Ry induced a significant and similar inhibition of both Ca^{2+} responses (inset, Fig. 6B). Hence, a general increase in $[Ca^{2+}]_i$ resulting from ionophore stimulation fails to trigger an enhanced CICR event in cells exposed to arsenite.

These results indicate that arsenite does not sensitize the RyR to the CICR event mediated by an increased $[Ca^{2+}]_i$ resulting from exposure to either a RyR agonist or a Ca^{2+} ionophore. In this perspective, the possibility of a general

sensitizing effect to the $[Ca^{2+}]_i$, even when the cation is released by the IP_3R , appears unlikely. These results are therefore indicative of specific intraluminal effects of arsenite on the crosstalk between the IP_3R and RyR, finally leading to a remarkable release of the cation in the cytosol.

Discussion

We have investigated the effect of a nontoxic concentration of arsenite on Ca^{2+} homeostasis with the aim of understanding the specific mechanism involved in this response, in the absence of confounding factors associated with the parallel triggering of toxic events. Using a well defined, versatile cellular system, we have demonstrated that the metalloid targets the IP_3R to release low levels of Ca^{2+} , an event associated with an ensuing much larger response mediated by the RyR. We also determined the specific effects of the metalloid on each of the two Ca^{2+} pools to conclude finally that the increased $[Ca^{2+}]_i$ takes place as a consequence of ROS-independent, direct effects of arsenite on the intraluminal crosstalk between the two Ca^{2+} channels.

Our studies on the effects of arsenite on Ca^{2+} homeostasis were preceded by an initial characterization of the Ca^{2+} responses evoked by selected IP_3R and RyR agonists in the three cell types used in this study. These experiments provided

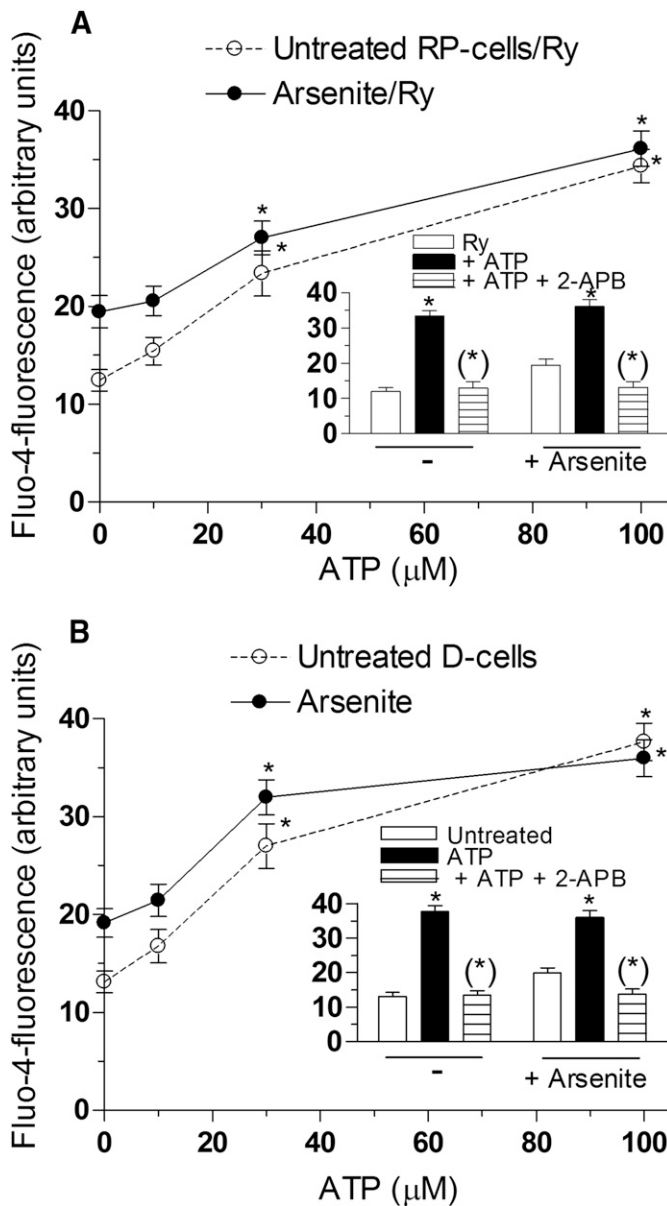


Fig. 5. Arsenite fails to affect the Ca^{2+} response mediated by the IP_3R agonist ATP. Fluo-4-acetoxymethyl ester-preloaded RP (A) and D cells (B) were incubated for 6 hours with $2.5 \mu M$ arsenite in the presence (A) or absence of Ry (B), washed, resuspended in fresh culture medium, and finally exposed for 10 minutes to increasing concentrations of ATP/Ry (A) or ATP alone (B). Also shown in the insets (A and B) are the effects of 2-APB on the observed Fluo-4-fluorescence. Results represent the means \pm S.D. calculated from at least three separate experiments. $*P < 0.01$ compared treated cells with Ry or arsenite/Ry treated cells. $*P < 0.01$ compared with cells treated with Ry/ATP or arsenite/Ry/2-APB (one-way ANOVA followed by Dunnett's test).

results consistent with the well established notion that ATP, or histamine, causes a significant elevation in $[Ca^{2+}]_i$ by signaling through PLC and the IP_3R . The contribution of the Ca^{2+} released by the RyR in response to the increase in $[Ca^{2+}]_i$ instead appeared quite limited. We also noted that the effects mediated by ATP, or histamine, are identical in RP, RD, and D cells, thereby implying that the IP_3R signaling is independent on the respiratory and differentiation status of the parental cell line. Additional information derived from this preliminary investigation is that stimulation of the RyR with Cf (or

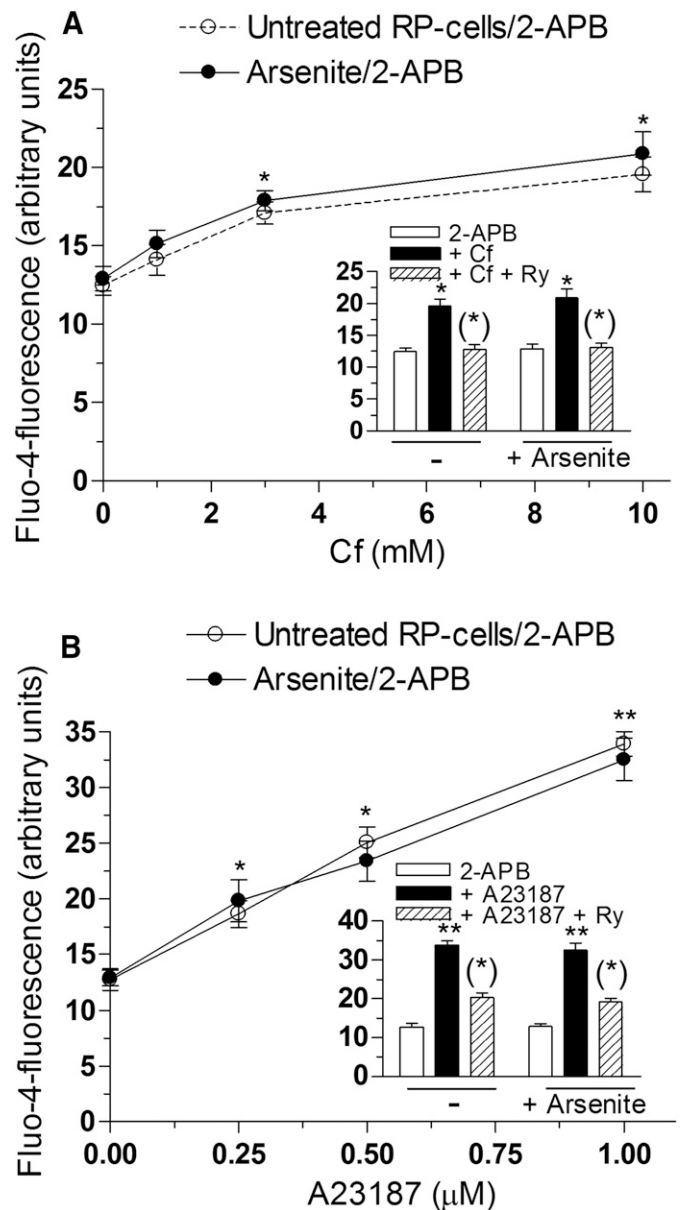


Fig. 6. Arsenite fails to affect the CICR event induced by local increases in Ca^{2+} concentration in the close proximity of the RyR (i.e., induced by Cf) or by more general increases of $[Ca^{2+}]_i$ (i.e., induced by a Ca^{2+} ionophore). Fluo-4-acetoxymethyl ester-preloaded RP cells were incubated for 6 hours with $2.5 \mu M$ arsenite and 2-APB, washed, resuspended in fresh culture medium, and subsequently exposed for 10 minutes to increasing concentrations of Cf (A) or A23187 (B). Also shown in the insets (A and B) are the effect of Ry on Fluo-4-fluorescence responses detected in the two conditions. Results represent the means \pm S.D. calculated from at least three separate experiments. $*P < 0.05$; $**P < 0.01$ compared treated cells with 2-APB or arsenite/2-APB. $*P < 0.05$ compared with cells treated with 2-APB/Cf or arsenite/2-APB/Cf (A). $*P < 0.05$ compared with cells treated with 2-APB/A23187 or arsenite/2-APB/A23187 (B) (one-way ANOVA followed by Dunnett's test).

4-CmC) provides a lower Ca^{2+} response with respect to that mediated by the IP_3R agonists, restricted to the proliferating cells and insensitive to their respiratory status. Differentiation of RP cells to monocytes, as we reported several years ago (Guidarelli et al., 2009), instead leads to RyR downregulation and loss of responsiveness to RyR agonists.

Our studies on the effects of arsenite on Ca^{2+} homeostasis were also preceded by the demonstration that the experimental

conditions used were not associated with detectable signs of toxicity or even with effects on the activity of the Ca^{2+} -ATPases or the energy status of RP, RD, or D cells. This preliminary characterization is essential for correct interpretation of the significance of the results of our experiments investigating the impact of arsenite on Ca^{2+} homeostasis with use of U937 cells treated, or manipulated, to provide detailed information in this direction.

With this background information, we investigated the effects of arsenite on Ca^{2+} homeostasis in both RP and RD cells. As indicated in the *Introduction*, exposure to 2.5 μM arsenite selectively promotes the formation of mitoO_2^- in RP-cells (Guidarelli et al., 2016a,b, 2017; Fiorani et al., 2018). This notion was further established in this study by monitoring the inhibition of the activity of a mitoO_2^- -sensitive enzyme, aconitase, taking place via a mechanism suppressed by mitochondrial AA (Fiorani et al., 2015; Cantoni et al., 2017) rotenone or by the respiration-deficient phenotype; that is, RD cells failed to produce ROS in response to arsenite. It was then interesting to observe that all these treatments, or manipulations, suppressing mitoO_2^- formation failed to produce parallel effects on Ca^{2+} homeostasis. It follows that the metalloid directly promotes effects associated with an increased $[\text{Ca}^{2+}]_i$, in the absence of an intermediate role mediated by mitoO_2^- and/or its dismutation product H_2O_2 . These effects are therefore most likely dependent on the direct binding of arsenite to specific protein-SH located in critical proteins regulating Ca^{2+} release from the ER.

This conclusion is particularly interesting since ROS mediate most of the toxic effects of arsenite, and their formation is most likely associated to Ca^{2+} -dependent mechanisms (Flora, 2011). Under the specific conditions used in this study, the increased $[\text{Ca}^{2+}]_i$ may therefore result in enhanced mitochondrial Ca^{2+} accumulation, thereby fostering the formation of mitoO_2^- . This notion is currently being tested, but the preceding findings nevertheless lead us to propose that the direct effects of arsenite in the ER might represent an early and critical effect of the metalloid, likely responsible for the triggering of downstream genotoxicity and cytotoxicity.

Subsequent studies using various inhibitors and RP as well as D cells provided interesting information about the mechanism leading to the increase in $[\text{Ca}^{2+}]_i$ -mediated by arsenite. Although it appears difficult to compare an acute agonist-induced Ca^{2+} response measured over a 10-minute time frame with the time-dependent sustained response mediated by arsenite over a 6-hour period, we nevertheless noted remarkable differences in these conditions.

Indeed, we previously discussed that RyR agonists release much less Ca^{2+} than IP_3R agonists and that the CICR response provides a minor contribution to the large Ca^{2+} response mediated by the IP_3R agonists. The scenario was completely different after exposure to arsenite. The overall information provided by the results presented in this study demonstrates the existence of a dichotomy between the persistently low Ca^{2+} response derived from IP_3R and the remarkable and progressive increase in the amount of Ca^{2+} subsequently released by the RyR.

The first observation was integrated by the information deriving from two separate approaches. The first was the demonstration that the IP_3R -mediated response (i.e., the Ry-insensitive elevation of Ca^{2+} detected in RP cells or the overall response detected in D cells is not further increased

by arsenite concentrations up to 10 μM . The second approach instead produced results arguing against the possibility that the saturable mechanism is dependent on putative inhibitory effects of arsenite on Ca^{2+} release by the IP_3R . Using the same conditions described in the preceding section to prevent the release of Ca^{2+} from the RyR, we indeed demonstrated that arsenite does not affect the Ca^{2+} response mediated by the IP_3R agonist ATP.

Collectively, the results obtained in these experiments indicate that arsenite is a poor activator of the signaling leading to Ca^{2+} mobilization from the IP_3R and that this response is maximally stimulated at 2.5 μM . The effects of arsenite were instead concentration-dependent under conditions permissive for the involvement of the RyR in the overall Ca^{2+} response. This remarkable response was not due to direct stimulation of Ca^{2+} release by the RyR since high concentrations of arsenite failed to increase the $[\text{Ca}^{2+}]_i$ under conditions preventing the mobilization of the cation from the IP_3R .

Collectively, the results thus far discussed indicate that the Ca^{2+} released by the IP_3R , remaining low and constant at increasing arsenite concentrations, leads to further release of the cation from the RyR, which instead becomes progressively more elevated at increasing concentrations of the metalloid. Hence, these results imply that arsenite sensitizes the RyR to events other than then the increase in $[\text{Ca}^{2+}]_i$, likely dependent on direct effects on the intraluminal crosstalk between the IP_3R and RyR.

This notion is also consistent with other findings obtained in experiments using RyR agonist- or A23187 ionophore-induced stimulation. Our results, indeed, argued against a general sensitizing effect of arsenite on the RyR. It rather appeared that the sensitization, clearly appreciable after the IP_3R stimulation mediated by the metalloid, was not apparent under conditions in which the cation was released by agonist-mediated stimulation of the RyR itself or eventually derived from the external milieu.

We are therefore left with the only possibility that the remarkable release of RyR-derived Ca^{2+} detected in response to arsenite is largely mediated by mechanisms regulated within the ER.

In conclusion, using well defined cellular systems and conditions, we investigated the mechanism whereby nontoxic concentrations of arsenite, which nevertheless significantly and selectively stimulate mitoO_2^- formation, lead to an increased $[\text{Ca}^{2+}]_i$. The metalloid promoted a sustained increase in $[\text{Ca}^{2+}]_i$ through a mechanism independent on the effect of these radicals and associated with an initial mobilization of low levels of the cation from the IP_3R . This event, although limited and saturable, was critical for the triggering of further Ca^{2+} release from the RyR that was, however, concentration dependently modulated by further effects of arsenite on the intraluminal crosstalk between the IP_3R and the RyR (Fig. 7). Our results indicate that this response accounts for the very large proportion of the overall Ca^{2+} response evoked by arsenite under the conditions used in the present investigation.

The present study therefore provides critical information for understanding the mechanism whereby arsenite promotes its deleterious effects in mammalian cells. We have identified important direct effects mediated by the metalloid in the ER, critically contributing to the increase in $[\text{Ca}^{2+}]_i$, the likely *primum movens* of the overall cellular response to arsenite. It

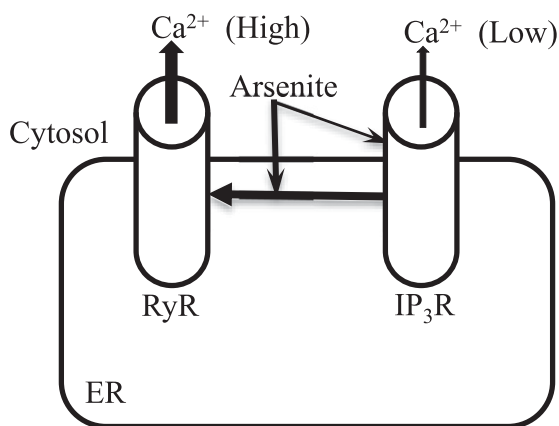


Fig. 7. Mechanism whereby a low concentration of arsenite mobilizes Ca^{2+} from the ER. Arsenite elevates the $[Ca^{2+}]_i$ by directly targeting the IP₃R and its intraluminal crosstalk with the RyR.

is easy to predict that these events will also regulate accumulation of the cation in specific compartments, such as the mitochondria. Mitochondrial Ca^{2+} would then critically regulate processes leading to $mitoO_2^-$ formation, thereby promoting the ensuing downstream effects, as the strand scission of genomic DNA, even at low and nontoxic arsenite concentrations. Mitochondrial Ca^{2+} , however, also mediates processes leading to cell death, such as mitochondrial dysfunction and permeability transition (Gorlach et al., 2015; Csordás et al., 2018). With this perspective, the mechanisms identified in this study should be considered relevant also for the induction of apoptosis caused by a more prolonged exposure to or exposure to greater concentrations of arsenite.

Authorship Contributions

Participated in research design: Guidarelli, Fiorani, Cantoni.
Conducted experiments: Guidarelli, Fiorani.
Performed data analysis: Guidarelli, Fiorani, Cantoni.
Wrote or contributed to the writing of the manuscript: Cantoni, Guidarelli, Fiorani.

References

- Banerjee C, Goswami R, Datta S, Rajagopal R, and Mazumder S (2011) Arsenic-induced alteration in intracellular calcium homeostasis induces head kidney macrophage apoptosis involving the activation of calpain-2 and ERK in *Clarias batrachus*. *Toxicol Appl Pharmacol* **256**:44–51.
- Berridge MJ (2016) The inositol trisphosphate/calcium signaling pathway in health and disease. *Physiol Rev* **96**:1261–1296.
- Cantoni O, Guidarelli A, and Fiorani M (2017) Mitochondrial uptake and accumulation of vitamin C: what can we learn from cell culture studies? *Antioxid Redox Signal* DOI: 10.1089/ars.2017.7253 [published ahead of print].
- Cárdenas C, Müller M, McNeal A, Lovy A, Jaña F, Bustos G, Urrea F, Smith N, Molgó J, Diehl JA, et al. (2016) Selective vulnerability of cancer cells by inhibition of Ca^{2+} transfer from endoplasmic reticulum to mitochondria [published correction appears in *Cell Rep* (2016) 15:219–220]. *Cell Rep* **14**:2313–2324.
- Chen A, Cao EH, Zhang TC, and Qin JF (2002) Arsenite-induced reactive oxygen species and the repression of alpha-tocopherol in the MGC-803 cells. *Eur J Pharmacol* **448**:11–18.
- Chernorudskiy AL and Zito E (2017) Regulation of calcium homeostasis by ER redox: a close-up of the ER/mitochondria connection. *J Mol Biol* **429**:620–632.
- Clapham DE (2007) Calcium signaling. *Cell* **131**:1047–1058.
- Clementi E, Guidarelli A, and Cantoni O (1998) The inositol 1,4,5-trisphosphate-generating agonist ATP enhances DNA cleavage induced by tert-butylhydroperoxide. *Exp Cell Res* **239**:175–178.
- Csordás G, Weaver D, and Hajnóczky G (2018) Endoplasmic reticulum-mitochondrial contactology: structure and signaling functions. *Trends Cell Biol* **28**:523–540 DOI: 10.1016/j.tcb.2018.02.009.
- Fiorani M, Azzolini C, Cerioni L, Scotti M, Guidarelli A, Ciacchi C, and Cantoni O (2015) The mitochondrial transporter of ascorbic acid functions with high affinity

- in the presence of low millimolar concentrations of sodium and in the absence of calcium and magnesium. *Biochim Biophys Acta* **1848**:1393–1401.
- Fiorani M, Guidarelli A, Capellacci V, Cerioni L, Crinelli R, and Cantoni O (2018) The dual role of mitochondrial superoxide in arsenite toxicity: signaling at the boundary between apoptotic commitment and cytoprotection. *Toxicol Appl Pharmacol* **345**:26–35.
- Flora SJ (2011) Arsenic-induced oxidative stress and its reversibility. *Free Radic Biol Med* **51**:257–281.
- Gardner PR (2002) Aconitase: sensitive target and measure of superoxide. *Methods Enzymol* **349**:9–23.
- Görlich A, Bertram K, Hudcová S, and Krizanová O (2015) Calcium and ROS: a mutual interplay. *Redox Biol* **6**:260–271.
- Guidarelli A, Carloni S, Balduini W, Fiorani M, and Cantoni O (2016a) Mitochondrial ascorbic acid prevents mitochondrial O_2^- formation, an event critical for U937 cell apoptosis induced by arsenite through both autophagic-dependent and independent mechanisms. *Biofactors* **42**:190–200.
- Guidarelli A, Cerioni L, Fiorani M, Azzolini C, and Cantoni O (2014) Mitochondrial ascorbic acid is responsible for enhanced susceptibility of U937 cells to the toxic effects of peroxynitrite. *Biofactors* **40**:236–246.
- Guidarelli A, Cerioni L, Fiorani M, and Cantoni O (2009) Differentiation-associated loss of ryanodine receptors: a strategy adopted by monocytes/macrophages to prevent the DNA single-strand breakage induced by peroxynitrite. *J Immunol* **183**:4449–4457.
- Guidarelli A, Fiorani M, Carloni S, Cerioni L, Balduini W, and Cantoni O (2016b) The study of the mechanism of arsenite toxicity in respiration-deficient cells reveals that NADPH oxidase-derived superoxide promotes the same downstream events mediated by mitochondrial superoxide in respiration-proficient cells. *Toxicol Appl Pharmacol* **307**:35–44.
- Guidarelli A, Fiorani M, Cerioni L, Scotti M, and Cantoni O (2017) Arsenite induces DNA damage via mitochondrial ROS and induction of mitochondrial permeability transition. *Biofactors* **43**:673–684.
- Guidarelli A, Fiorani M, Tommasini I, Cerioni L, and Cantoni O (2006) Reduced mitochondrial formation of H_2O_2 is responsible for resistance of dimethyl sulfoxide differentiated U937 cells to peroxynitrite. *Int J Biochem Cell Biol* **38**:56–68.
- Hill SJ, Ganellin CR, Timmerman H, Schwartz JC, Shankley NP, Young JM, Schunack W, Levi R, and Haas HL (1997) International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacol Rev* **49**:253–278.
- Hoonjan M, Jadhav V, and Bhatt P (2018) Arsenic trioxide: insights into its evolution to an anticancer agent. *J Biol Inorg Chem* **23**:313–329 DOI:10.1007/s00775-018-1537-9.
- Hosoi E, Nishizaki C, Gallagher KL, Wyre HW, Matsuo Y, and Sei Y (2001) Expression of the ryanodine receptor isoforms in immune cells. *J Immunol* **167**:4887–4894.
- Hsu WL, Tsai MH, Lin MW, Chiu YC, Lu JH, Chang CH, Yu HS, and Yoshioka T (2012) Differential effects of arsenic on calcium signaling in primary keratinocytes and malignant (HSC-1) cells. *Cell Calcium* **52**:161–169.
- Lee MY, Lee YH, Lim KM, Chung SM, Bae ON, Kim H, Lee CR, Park JD, and Chung JH (2005) Inorganic arsenite potentiates vasoconstriction through calcium sensitization in vascular smooth muscle. *Environ Health Perspect* **113**:1330–1335.
- Meissner G (2017) The structural basis of ryanodine receptor ion channel function. *J Gen Physiol* **149**:1065–1089.
- Minatel BC, Sage AP, Anderson C, Hubaux R, Marshall EA, Lam WL, and Martinez VD (2018) Environmental arsenic exposure: from genetic susceptibility to pathogenesis. *Environ Int* **112**:183–197.
- Pachauri V, Mehta A, Mishra D, and Flora SJ (2013) Arsenic induced neuronal apoptosis in Guinea pigs is Ca^{2+} dependent and abrogated by chelation therapy: role of voltage gated calcium channels. *Neurotoxicology* **35**:137–145.
- Peiretti F, Fossat C, Anfossio F, Alessi MC, Henry M, Juhan-Vague I, and Nalbone G (1996) Increase in cytosolic calcium upregulates the synthesis of type 1 plasminogen activator inhibitor in the human histiocytic cell line U937. *Blood* **87**:162–173.
- Raffaello A, Mammucari C, Gherardi G, and Rizzuto R (2016) Calcium at the center of cell signaling: interplay between endoplasmic reticulum, mitochondria, and lysosomes. *Trends Biochem Sci* **41**:1035–1049.
- Scandroglio F, Tórtora V, Radi R, and Castro L (2014) Metabolic control analysis of mitochondrial aconitase: influence over respiration and mitochondrial superoxide and hydrogen peroxide production. *Free Radic Res* **48**:684–693.
- Scotti M, Fiorani M, Guidarelli A, and Cantoni O (2018) Differentiation of promonocytic U937 Cells to monocytes is associated with reduced mitochondrial transport of ascorbic acid. *Oxid Med Cell Longev* **2018**:4194502.
- Stocchi V, Cucchiari L, Magnani M, Chiarantini L, Palma P, and Crescentini G (1985) Simultaneous extraction and reverse-phase high-performance liquid chromatographic determination of adenine and pyridine nucleotides in human red blood cells. *Anal Biochem* **146**:118–124.
- Sugiyama T, Yamamoto-Hino M, Miyawaki A, Furuichi T, Mikoshiba K, and Hasegawa M (1994) Subtypes of inositol 1,4,5-trisphosphate receptor in human hematopoietic cell lines: dynamic aspects of their cell-type specific expression. *FEBS Lett* **349**:191–196.
- Suriyo T, Watcharasi P, Thiantanawat A, and Satayavivad J (2012) Arsenite promotes apoptosis and dysfunction in microvascular endothelial cells via an alteration of intracellular calcium homeostasis. *Toxicol In Vitro* **26**:386–395.
- Yan LJ, Levine RL, and Sohal RS (1997) Oxidative damage during aging targets mitochondrial aconitase. *Proc Natl Acad Sci USA* **94**:11168–11172.

Address correspondence to: Orazio Cantoni, Dipartimento di Scienze Biomolecolari, Sezione di Farmacologia e Farmacognosia, Università degli Studi di Urbino, Urbino (PU), Italy. E-mail: orazio.cantoni@uniurb.it