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Interactive effects of pH and metals on mitochondrial functions of intertidal bivalves *Crassostrea virginica* and *Mercenaria mercenaria*

Anna V. Ivanina, Inna M. Sokolova*

Department of Biology, University of North Carolina at Charlotte, Charlotte, NC, USA

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ABSTRACT

Intertidal bivalves experience broad fluctuations of environmental temperature, pH and oxygen content which could change their intracellular pH. They are also exposed to trace metals such as cadmium (Cd) and copper (Cu) that accumulate in their tissues and may negatively affect mitochondrial functions and bioenergetics. We determined the interactive effects of pH and trace metals (25 μM Cd or Cu) on mitochondrial functions (including respiration and membrane potentials in both ADP-stimulated (state 3) and resting (state 4) states) of two common marine bivalves, the hard clams (*Mercenaria mercenaria*) and eastern oysters (*Crassostrea virginica*). In the absence of the trace metals, mitochondrial functions of *C. virginica* and *M. mercenaria* were insensitive to pH in a broad physiologically relevant range (6.6–7.8). Mitochondrial respiration was generally suppressed by 25 μM Cd or Cu (with the stronger effects observed for ADP-stimulated compared to the resting respiration) while the mitochondrial membrane potential was unaffected. pH modulated the effects of Cu and Cd on mitochondrial respiration of the bivalves. In oysters, Cu suppressed ADP-stimulated mitochondrial respiration at high and low pH values (6.6 and 7.8, respectively), but had no effect in the intermediate pH range (7.0–7.4). In clams, the negative effect of Cu on ADP-stimulated respiration was only observed at extremely high pH (7.8). A decrease in pH was also protective against Cd in mitochondria of clams and oysters. In clams, 25 μM Cd suppressed ADP-stimulated respiration at all pH; however, at low pH (6.6–7.0) this suppression was paralleled by a decrease in the rates of proton leak thereby effectively restoring mitochondrial coupling. In oysters, the inhibitory effects of Cd on ADP-stimulated respiration were fully abolished at low pH (6.6–7.0). This indicates that moderate acidosis (such as occurs during exposure to air, extreme salinities or elevated CO_2 levels in the intertidal zone) may have a beneficial side-effect of protecting mitochondria of clams and oysters against the toxic effects of trace metals in polluted estuaries.

1. Introduction

Intertidal zones are among the most stressful marine environments characterized by frequent and large fluctuations of abiotic factors including temperature, salinity, pH, oxygen and carbon dioxide concentrations. In many estuaries and coastal zones worldwide, these natural abiotic stressors are combined with anthropogenic stress such as pollution, eutrophication and formation of "dead zones" (Clark, 1997; Jackson et al., 2001; Howarth et al., 2011). Survival in these environments requires efficient mechanisms of stress tolerance and involves a variety of cellular and physiological mechanisms of stress protection (Hochachka and Somero, 2002; Menge et al., 2002; Somero, 2002). Metabolic regulation plays a key role among those mechanisms allowing intertidal animals to maintain a positive energy balance and survive prolonged periods of extreme stress in the intertidal zone (Sokolova and Pörtner, 2001; Altieri, 2006; Gracey et al., 2008; Sokolova, 2013 and references therein). Several key mechanisms of metabolic adaptations to the intertidal life have been demonstrated including metabolic rate depression, buffering of intracellular pH to prevent extensive acidosis, and evolution of alternative anaerobic pathways that provide better ATP yield than the classical vertebrate glycolysis (review in: Hochachka and Guppy, 1987; Guppy et al., 1994; Willmer et al., 2000). Mitochondrial adaptations of intertidal animals are less well understood but are likely to play a key role in stress tolerance and recovery of these organisms.

Bivalve mollusks are among the champions of survival in the intertidal zone. They are exposed to broad fluctuations of temperature (exceeding 10–20 °C during the tidal cycle and even more during the seasonal temperature change), oxygen conditions (from fully oxygenated water to deep hypoxia during exposure to air, extreme salinities or in coastal "dead zones") and elevated levels of pollutants (including trace metals such as cadmium (Cd)

* Corresponding author at: Department of Biology, University of North Carolina at Charlotte, Charlotte, NC 28223, USA. Tel.: +1 704 687 8532; fax: +1 704 687 3128.
E-mail address: isokolov@uncc.edu (I.M. Sokolova).

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and copper (Cu) commonly found in estuarine water and sediments). These environmental changes translate into significant shifts in the intracellular milieu of the intertidal bivalves including changes in intracellular pH (pH_i) and metal content. Thus, an increase in environmental temperature leads to a decrease of pH_i in ectotherms including mollusks, at a rate of approximately −0.011 to −0.018 °C^{-1}, depending on the species (Burton, 2002). Environmental anaerobiosis caused by exposure to air or extreme salinity also leads to significant acidification of intracellular milieu in mollusks (Sukhotin and Pörtner, 1999; Sokolova et al., 2000b). Exposure to trace metals through water, sediments and food results in significant accumulation of metal burdens in bivalves, to the levels that exceed the environmental concentrations by orders of magnitude and lead to a significant increase in the intracellular concentration of metals (Roesijadi, 1996; Sokolova et al., 2005; O'Connor and Lauenstein, 2006; Ivanina et al., 2013a). A significant fraction of the intracellular metals is associated with mitochondria (Sokolova et al., 2005) and can therefore interfere with the mitochondrial function leading to a decrease in mitochondrial efficiency, reduced capacity for ATP synthesis and increase in the futile cycling of protons known as proton leak (Sokolova, 2004; Cherkasov et al., 2006a; Garceau et al., 2010; Kurochkin et al., 2011; Lauer et al., 2012). Earlier studies have shown that the effects of trace metals on bivalve mitochondria can be strongly enhanced by temperature (Sokolova, 2004; Cherkasov et al., 2007, 2010; Ivanina et al., 2012); however, the effects of pH on mitochondrial function and sensitivity of mitochondria to trace metals have not been studied in mollusks.

The Eastern oysters, *Crassostrea virginica* (Gmelin, 1791) and the hard clams *Mercenaria mercenaria* (Linnaeus, 1758) are intertidal bivalves widely distributed in estuarine and coastal habitats of the western Atlantic from the Gulf of St. Lawrence in Canada to the Gulf of Mexico, and serve as ecosystem engineers in these habitats. Oysters and clams are exposed to elevated concentrations of Cd and Cu in estuarine waters and sediments and bioaccumulate high levels of metals in their tissues (Wright and Zamuda, 1987; Robinson and Ryan, 1988; Engel, 1999; McIntosh and Robinson, 1999; O'Connor and Lauenstein, 2005; Blanchard et al., 2009). They also experience large fluctuations in temperature, oxygen availability and environmental CO_2 levels that affect their physiology and acid-base regulation (Ringwood and Keppler, 2002; Chapman et al., 2011; Ivanina et al., 2013a). This makes clams and oysters excellent model species to investigate the interactive effects of trace metals and pH on mitochondrial bioenergetics. In this study, we tested a hypothesis that intracellular acidosis (such as observed during environmental hypercapnia, anaerobiosis or extreme warming) may sensitize bivalve mitochondria to the negative effects of trace metals by determining the interactive effects of metals (Cu and Cd) and pH on key bioenergetic parameters including the capacity for ATP synthesis, proton leak and membrane potential in mitochondria of clams and oysters.

2. Materials and methods

2.1. Animal maintenance

Adult *C. virginica* and *M. mercenaria* were obtained from a commercial supplier (Inland Seafood, Charlotte, NC) and placed in tanks with aerated artificial seawater, ASW (Instant Ocean®, Kent Marine, Acworth) at 20 °C and 30‰ salinity. Temperature and salinity were maintained within 1 °C and 1‰ of their respective target values. All animals were allowed to recover for 10 days prior to mitochondrial isolations. Oysters and clams were fed *ad libitum* on alternate days with a commercial algal blend (2 ml per oyster) containing *Nannochloropsis oculata, Phaeodactylum tricornutum* and *Chlorell*a with a cell size of 2–20 μm (DT's Live Marine Phytoplankton, Sycamore, IL, USA). Mortality was less than 5% throughout the experiment.

2.2. Chemicals

Chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA) unless otherwise stated, and were of analytical grade or higher.

2.3. Mitochondrial isolations

Mitochondria were isolated from gills of clams and oysters using a method modified from Sokolova (Sokolova, 2004). Briefly, 2–4 g of gills were placed in an ice-cold buffer containing 100 mM sucrose, 200 mM KCl, 100 mM NaCl, 8 mM ethylene glycol tetraacetic acid (EGTA), and 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, and homogenized with several passes of a Potter-Elvenhjem homogenizer and a loosely fitting Teflon pestle at 200 rpm. The homogenate was centrifuged at 2000 × *g* for 8 min to remove cell debris, and the supernatant was centrifuged at 8500 × *g* for 8 min to obtain a mitochondrial pellet. The mitochondrial pellet was resuspended in homogenization buffer without EGTA, centrifuged again at 8500 × *g* for 8 min and resuspended in 1 ml of ice-cold assay medium consisting of 150 mM sucrose, 250 mM KCl, 10 mM glucose, 10 mM KH_2PO_4, 1% bovine serum albumin (fatty acid free), and 30 mM HEPES adjusted to one of five different pH (6.6, 7.0, 7.4 or 7.8). Protein concentrations in mitochondrial isolates were measured using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) in the presence of 0.1% Triton X-100 used to solubilize mitochondrial membranes. Each biological replicate represented an individual mitochondrial isolate obtained from pooled tissues of 2–3 animals.

2.4. Measurements of mitochondrial respiration and membrane potential

Mitochondrial respiration and membrane potential were measured simultaneously in a water-jacketed, temperature-stabilized four-port chamber (World Precision Instruments, Sarasota, FL) at 20 °C in the absence of metals or in the presence of 25 μM Cd or 25 μM Cu added as $CdCl_2$ or $CuCl_2$, respectively. Assay media were adjusted to the same pH as the mitochondrial suspension (6.6, 7.0, 7.4 or 7.8). Oxygen concentrations were determined using a fiber optic oxygen sensor connected to the Microx TX3 oxygen monitor with temperature correction (Precision Sensing, Dusseldorf, Germany) and OxyMicro ver. 2.00 software (World Precision Instruments, Sarasota, FL). A two-point calibration (0% and 100% of air saturation) was performed prior to each measurement with saturated Na_2SO_3 solution and air-saturated assay media serving as 0% and 100% calibration points, respectively. Mitochondrial membrane potential ($\Delta\psi$) was determined by $\Delta\psi$-dependent mitochondrial accumulation of tetraphenyl phosphonium (TPP^+) ions using a TPP^+-selective electrode (KWIKTPP-2) and a Super Dri-Ref reference electrode (World Precision Instruments, Sarasota, FL) connected to a pH meter (model 1671; Jenco Instruments, San Diego, CA) as described elsewhere (Kurochkin et al., 2011). The TPP^+ electrode was filled with a solution containing 10 mM TPP^+ and 10 mM NaCl, pH 7.2 and calibrated before each measurement using stepwise additions of TPP^+ (2–10 μM). It is worth noting that mitochondrial protonmotive force (Δp) consists of the electrical membrane potential ($\Delta\psi$) and the pH gradient (ΔpH) across the inner mitochondrial membrane. A standard procedure to determine Δp in mitochondria involves clamping of ΔpH by adding a H^+/K^+ exchanger (such as nigericin) and converting all Δp to $\Delta\psi$ (Brand, 1995; Kurochkin et al., 2011; Ivanina et al., 2012). However, this procedure acidifies the mitochondrial matrix and thus can interfere with the determination of the effects of pH on mitochondria. Therefore, in this study we measured only $\Delta\psi$ which is the main contributor to the