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Lead hampers gill cell volume regulation in marine crabs: Stronger effect in a weak osmoregulator than in an osmoconformer

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ABSTRACT

Hepatus pudibundus is a strictly marine osmoconformer crab, while *Callinectes ornatus* inhabits estuarine areas, behaving as a weak hyper-osmoregulator in diluted seawater. Osmoconformers are expected to have higher capacity for cell volume regulation, but gill cells of a regulator are expected to display ion transporters to a higher degree. The influence of lead nitrate (10 μM) on the ability of isolated gill cells from both species to volume regulate under isosmotic and hyposmotic conditions were here evaluated. Without lead, under a 25% hyposmotic shock, the gill cells of both species were quite capable of cell volume maintenance. Cells of *C. ornatus*, however, had a little swelling (5%) during the hyposmotic shock of greater intensity (50%), while cells of *H. pudibundus* were still capable of volume regulation. In the presence of lead, even under isosmoticity, the gill cells of both species showed about 10% volume reduction, indicating that lead promotes the loss of water by the cells. When lead was associated with 25% and 50% hyposmotic shock, *C. ornatus* cells lost more volume (15%), when compared to isosmotic conditions, while *H. pudibundus* cells showed volume regulation. We then analyzed the possible ways of action of lead on the mechanisms of cell volume regulation in the two species. Verapamil (100 μM) was used to inhibit Ca^{2+} channels, ouabain (100 μM) to inhibit Na^+/K^+-ATPase, and $HgCl_2$ (100 μM) to inhibit aquaporins. Our results suggest that: (1) Ca^{2+} channels are candidates for lead entry into gill cells of *H. pudibundus* and *C. ornatus*, being the target of lead action in these cells; (2) aquaporins are much more relevant for water flux in *H. pudibundus*; and (3) the Na^+/K^+-ATPase is much more relevant for volume regulation in *C. ornatus*. Osmoregulators may be more susceptible to metal contamination than osmoconformers, especially in situations of reduced salinity, for two basic reasons: (1) lower capacity of volume regulation and (2) putative higher uptake of Pb^{2+} through ionic pathways that operate in salt absorption, such as, for example, the Na^+/K^+-ATPase.

1. Introduction

Cell volume regulation is an essential process for maintaining cellular homeostasis. The maintenance of a constant volume in face of osmotic disturbances, both extra- and intracellular, is a critical problem faced by all animal cells. Volume changes are usually grouped into two broad categories: anisosmotic and isosmotic. Anisosmotic volume changes are induced by changes in extracellular osmolality and isosmotic volume changes occur through changes in intracellular solute content (Strange, 2004; Hoffmann et al., 2009).

The function and location of gills leads to the fact that their cells are exposed to changes in the external environment, such as changes in salinity and the presence of contaminants (which most often do not affect the osmolality of the water). Furthermore, besides constitutive metabolic processes common to all cells, the function of transepithelial salt transport will also contribute to volume disturbances of gill cells, even in the absence of changes in the external environment. The volume of gill cells can thus be challenged by these two types of osmotic disturbances. Moreover, the external medium of gills cells, given their polarity and condition of interface epithelium, is represented by two possibly different environments, especially in osmoregulators that keep a gradient between their extracellular medium and the water (Freire et al., 2008a,b). To maintain their vital functions, gill cells must have ability to volume regulate after such changes.

Under normal conditions, the osmolality of the cytoplasm is equal to the osmolality of the extracellular fluid. Changes in solute

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content in any of these compartments (extracellular or intracellular) generate a trans-membrane osmotic gradient (Strange, 2004). Because the plasma membrane is freely permeable to water, any osmotic gradient results in an immediate flow of water into or out of the cell until a new osmotic equilibrium is reached. This flow causes an increase or decrease in cell volume, which means that for the maintenance of cell volume, the effective intracellular osmolality must be equal to the effective extracellular osmolality (Russell, 2000).

After a change in volume, the cell must return to its previous "normal" volume, in order to keep performing its various functions. When the cell, after swelling, uses mechanisms to reduce its volume, this process is called regulatory volume decrease (RVD). Alternatively, mechanisms used to restore the lost volume are called regulatory volume increase (RVI) (Hoffmann and Dunham, 1995; Hoffmann et al., 2009). Both RVD and RVI may be the outcome of the activation of one or more membrane transport systems for inorganic and/or organic solutes (Hallows and Knauf, 1994; Lang et al., 1998; Wehner et al., 2003), solute transport being followed by transport of osmotically obliged water.

Cations such as Na^+, K^+ and Ca^{2+} play a role in cell volume regulatory mechanisms, as well as in signaling involved in cell volume regulation (Lang et al., 1998; Wehner et al., 2003; Strange, 2004). When Pb^{2+} is present in the water or in the extracellular fluid, it may enter the cell because of its similarity to Ca^{2+}, using calcium ion transport pathways (Rainbow, 1995, 1997; Bridges and Zalups, 2005). Pb^{2+} can also reduce unidirectional influx of Na^+ (Wright, 1995; Ahern and Morris, 1998), often correlated with inhibition of the Na^+/K^+-ATPase (Ahern and Morris, 1998), an essential enzyme for the maintenance of cell volume. Thus, inside the cell, Pb^{2+} may affect cell volume (as well as other physiological processes), through its resemblance of calcium, or through its other effects on, e.g., sodium fluxes or enzyme activities.

Gill cells from marine/estuarine species of decapod crabs with different osmoregulatory strategies are expected to display (1) different volume regulatory behavior (Foster et al., 2010) and possibly (2) a different response to the presence of lead. *Hepatus pudibundus* is a marine osmoconformer crab, and *Callinectes ornatus* is a marine/estuarine species with some hyper-regulatory capacity (weak osmoregulator) (Freire et al., 2008a, 2011; Foster et al., 2010). This study thus aimed at comparing the interference by lead of gill cell volume regulation in these two species. More specifically, we asked the questions: (1) is there a difference in gill cell volume regulation under hyposmotic shock (i.e., RVD capacity) between the two species? (2) What is the influence of lead on the maintenance of gill cell volume under isosmotic and hyposmotic conditions in the two species? (3) How does lead interact with some membrane transport systems involved in cell volume regulation?

2. Materials and methods

2.1. Animals

The crabs *H. pudibundus* and *C. ornatus* were purchased from fishermen on Ipanema beach, Pontal do Sul, Paraná, Brazil (25°41′S; 48°27′W). Species are sympatric in coastal subtidal marine trawling areas used by shrimp fishermen. They come as a bycatch in shrimp fisheries. The animals collected were transported (~1.5 h drive) in polystyrene boxes to the Laboratory in the Department of Physiology, Federal University of Paraná, in Curitiba. In the laboratory the crabs were kept in a glass stock aquarium (180 L), containing seawater of salinity 33‰ (~1000 mOsm/kg H_2O), under constant aeration, biological filtration, natural photoperiod and room temperature (~20 °C). Crabs were fed with small pieces of fish or shrimp, 2 or 3 times a week.

2.2. Solutions and analytical determinations

The control isosmotic saline osmotically corresponded to both crabs' hemolymph in seawater (in mM): NaCl 470, KCl 8, $CaCl_2$ 15, $MgSO_4$ 10, Hepes 10, and glucose 5, pH 7.6, measured osmolality of 948 mOsm/kg H_2O, Wescor VAPRO 5520. This control saline had lower magnesium levels than those found in the osmoconformer crab *H. pudibundus* (Foster et al., 2010). However, we chose to use a same solution for both species, in order to directly compare the ability of their gill cells to perform cell volume regulation. The hyposmotic shocks of 25 and 50% osmolality reduction were accomplished by simple dilution of the isosmotic saline using distilled water. Measured osmolalities were 706 and 484 mOsm/kg H_2O, respectively.

Lead nitrate (10 mM, $PbNO_3{}^2$) stock solution was prepared in distilled water. This stock solution was diluted in the isosmotic control saline to reach the final nominal concentration of 10 μM lead nitrate. Lead solutions, including the stock solution, were prepared in previously acidified *falcon* tubes. Lead concentrations in the solutions (total and dissolved – filtered through 0.20 μm membranes) were determined through ICP OES – inductively coupled plasma optical emission spectroscopy (PE-LE-032/R01), performed by a certified Laboratory at Federal University of Paraná (CEPPA, http://www.ceppa.ufpr.br/). Lead speciation was determined through the chemical equilibrium model CHEAQS-Pro (*CH*emical *E*quilibria *AQ*uatic *S*ystems), version P2010.3 (Verweij, 2010). Concentrations of the major ions (Na^+, Mg^{2+}, K^+, Ca^{2+}, Cl^-, $NO_3{}^-$, $SO_4{}^{2-}$, H^+) and of lead were introduced in the modeling program and the simulated concentrations of free lead ion as well as lead bound to inorganic ligands were obtained.

The concentration of lead employed here fell within the range of lead levels measured in the hemolymph of other species of crabs (the freshwater *Barytelphusa guerini* and *Cherax destructor*) exposed for up to 30 days to 0.5 and 100 mg L^{-1} of lead nitrate (Tulasi et al., 1987; Ahern and Morris, 1998). This high concentration employed was indeed way higher than environmentally relevant estuarine concentrations reported for dissolved lead (e.g., Severini et al., 2011). However, lead is a potential estuarine pollutant in the Brazilian coast, resulting from mining activities and discharge into rivers that run into the ocean (e.g., Farias et al., 2007). In fact, significant lead contamination was detected in the sediment of Paranaguá Bay – Pb was the third most important toxic metal – an estuarine complex inhabited by the swimming crab *C. ornatus* used in this study (Choueri et al., 2009; Freire et al., 2011). In addition, our purpose here was a mechanistic investigation, rather than environmental assessment of the effects of lead contamination.

2.3. Cell dissociation

Crabs were cryoanesthesized and had their carapace quickly manually opened. The 3 pairs of posterior gills were removed and, using a syringe inserted into the efferent hemolymph vessel, gills were perfused with Ca^{2+}-free PBS (in mM: NaCl 400, Na_2HPO_4 25, KH_2PO_4 3.5, KCl 20, measured osmolality of 940 mOsm/kg H_2O) for the removal of circulating hemocytes. After being perfused, the area around the efferent vessel was removed using scissors, to allow for proper isolation of gill cells. Gills were then transferred to a Petri dish containing PBS plus EDTA (5 mM) and were sliced into small pieces. Mechanical cell dissociation was then facilitated by repetitive movement of the solution with tissue fragments up and down a Pasteur pipette. Once dissociated, cells were filtered through a nylon mesh (pore size of 30 μm) to remove tissue debris; the filtrate was centrifuged at 290 × *g* for 5 min at 20 °C in a refrigerated centrifuge (Eppendorf Centrifuge 5810R, Germany), and cells were then resuspended in PBS.