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The effects of hypoxia on active ionic transport processes in the gill epithelium of hyperregulating crab, *Carcinus maneas*

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ABSTRACT

Effects of hypoxia on the osmorespiratory functions of the posterior gills of the shore crab *Carcinus maenas* acclimated to 12 ppt seawater (DSW) were studied. Short-circuit current (Isc) across the hemilamella (one epithelium layer supported by cuticle) was substantially reduced under exposure to 1.6, 2.0, or 2.5 mg O_2/L hypoxic saline (both sides of epithelium) and fully recovered after reoxygenation. Isc was reduced equally in the epithelium exposed to 1.6 mg O_2/L on both sides and when the apical side was oxygenated and the basolateral side solely exposed to hypoxia. Under 1.6 mg O_2/L, at the level of maximum inhibition of Isc, conductance was decreased from 40.0 mS cm^{-2} to 34.7 mS cm^{-2} and fully recovered after reoxygenation. Isc inhibition under hypoxia and reduced $^{86}Rb^+$ (K^+) fluxes across apically located K^+ channels were caused preferentially by reversible inhibition of basolaterally located and ouabain sensitive Na^+,K^+-ATPase mediated electrogenic transport. Reversible inhibition of Isc is discussed as decline in active transport energy supply down regulating metabolic processes and saving energy during oxygen deprivation.

In response to a 4 day exposure of *Carcinus* to 2.0 mg O_2/L, hemolymph Na^+ and Cl^- concentration decreased, i.e. hyperosmoregulation was weakened. Variations of the oxygen concentration level and exposure time to hypoxia lead to an increase of the surface of mitochondria per epithelium area and might in part compensate for the decrease in oxygen availability under hypoxic conditions.

1. Introduction

The shore crab *Carcinus maenas* Linnaeus, 1758 and its congener *Carcinus aestuarii* Nardo, 1847 are cosmopolitan species and two of the most successful invaders in shores of the world's oceans and seas (Darling et al., 2008). The crab *Carcinus maenas* is a typical inhabitant of coastal and estuarine zones where stress is associated with eutrophication and hypoxia (Weis, 2014).The shore crabs are known for high tolerance to fluctuations of environmental factors i.e., salinities (Siebers et al., 1982; Henry et al., 2002), temperatures (Cohen et al., 1995), and oxygen content in seawater (Taylor et al., 1977). Besides other factors, eutrophication and pollution are the most severe causes of hypoxia, with damaging consequences to aquatic organisms (Gray et al., 2002; Diaz and Rosenberg, 2008). Given the fact that *Carcinus* commonly lives in estuarine habitats, one might expect that they have evolved tissue-specific mechanisms for coping with exposure to environmental hypoxia. Studies of hypoxia at the organismic, tissue and cellular levels are needed to assess the effects of hypoxia on organisms. The gills of Crustacea form an interface between internal milieu and their environment and play a key osmorespiratory role. As a multifunctional organ they serve in: gas exchange (Burnett and Stickle, 2001), osmolyte transport, acid base and volume regulation (Gilles and Péqueux, 1985; Henry et al., 2002, 2003; Weihrauch et al., 2002; Fehsenfeld et al., 2011), immune functions (Burnett and Burnett, 2015) and detoxification (Ahearn et al., 2004).

Carcinus maenas is an osmoconformer in sea water. Under this condition the anterior and posterior gills are highly permeable as required for efficient gas exchange. In DSW (dilute seawater) *Carcinus* is a hyperosmoregulator, their hemolymph osmolarity is about 300 mosmol/L higher then osmolarity in 10 ppt DSW (Pequeux, 1995; Lucu and Flik, 1999). In particular, during environmental changes, the animals change gill morphology in a way that multifunctional processes occur at an optimal level. In most of the hyperosmoregulating crabs, the anterior gill lamellae still function primarily in respiration. Thus, the thin epithelium of pavement cells does not change noticeably when crabs are acclimated to DSW (Pequeux et al., 1988; Compere et al., 1989). However, the ultrastructure of the posterior *Carcinus* gills change considerably after acclimation to low salinity. These changes

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include an increase in the length of the apical infoldings leading to the development of deep subcuticular channels, thicker gill ionocyte cells due to lengthening of basolateral interdigitations and an increase in number of mitochondria within basal infoldings (Compere et al., 1989; Pequeux, 1995; Freire et al., 2008). Furthermore, a substantial increase in oxygen consumption (Piller et al., 1985; Lucu and Pavičić, 1985) in the posterior gills of the crabs *Callinectes* and *Carcinus* after acclimation to 10–20 ppt DSW indicate an increased gill metabolism. Thus, in these crabs acclimated in DSW activity of Na^+,K^+-ATPase is increased in posterior gills specialized for active ion uptake (Towle et al., 1976; Holliday, 1985; Lucu and Flik, 1999; Lovett et al., 2007; Tsai and Lin, 2007; Henry et al., 2012). Increased diffusion distance may reduce the ability to take up oxygen and reduce loss of ions from the hemolymph and thus the cost of ion regulation. These adaptive changes in gills during acclimation of *Carcinus* in DSW can be regarded as an example of an osmorespiratory compromise. A phenomenon, describing the balance between "need of oxygen" and "need of osmotic regulation" (Nilsson, 1986).

Most of the transport mechanisms of inorganic osmolytes in crustacean gills have been verified by application of perfusion and short-circuit current methods (Henry et al., 2012). The short-circuit current (Isc) represents the rate of active transport across an epithelium bathed on both sides in equal saline and is one of the most powerful methods to measure transepithelial ion transport across a variety of epithelial membranes (Larsen, 2002; Li et al., 2004). The introduction of the short-circuit current method by Hans Ussing in 1955, initiated a novel way to study ion transport across epithelial membranes. The method was applied on numerous mammals epithelia (Clarke, 2009; Hug and Tuemmler, 2004) and fish intestine (Marshall and Grosell, 2005).

Application of the electrophysiological Isc method in transport studies on *Carcinus* gill hemilamella has shown that inward movement of Cl^- is mediated by $Na^+/K^+/2Cl^-$ cotransport (Riestenpatt et al., 1996; Onken et al., 2003; Lucu and Towle, 2010). The main generator of this coupled transport is the basolaterally located Na^+, K^+-ATPase, because specific inhibition of Na^+, K^+-ATPase by applying ouabain to the basolateral side of the posterior *Carcinus* gills inhibits the Isc (Siebers et al., 1985; Onken and Siebers, 1992; Riestenpatt et al., 1996; Lucu and Flik, 1999).

The objective of this study was to determine how hypoxia is associated with electrogenic transport disturbance in the posterior gill preparation isolated from hypoxia-tolerant shore crab *Carcinus*. One of the most fundamental processes for all cells is the maintenance of a high, intracellular content of ATP. Indeed, almost all energy-requiring processes in cells are driven, either directly or indirectly, by hydrolysis of ATP. Differential regulation of ATP in mitochondria and metabolic priorities for Na^+, K^+-ATPase activities depends on tissue oxygenation (Petrushanko et al., 2007).

By using the short-circuit current method we studied the effect of hypoxia on active electrogenic Isc across the hemilamella isolated from posterior gills of the crab acclimated to DSW. Our study is focused particularly on the basolateral side where ouabain-sensitive Na^+,K^+-ATPase is located. In addition, the effect of hypoxia on ^{86}Rb (K^+) fluxes, which reflects at least partially the turnover of K^+ by the Na^+/K^+ pump, was studied. By varying duration and exposure of O_2 saturation in the hemilamella, we tested viability of the epithelium to acute hypoxia. Reversible block of Isc in the present study suggests a modification in which energy consuming processes are down regulated during hypoxia (Boutilier, 2001). Since the crustacean gill has been characterized as a leaky epithelium (Onken and Riestenpatt, 1998)), with a relatively high ionic permeability, we also studied the effect of hypoxia on electrical conductance, across the epithelium.

Oxygen limitation is generally considered an impairment of mitochondrial respiration and thus ATP synthesis. Therefore, we examined if the volume and surface area of mitochondria change when the posterior gill cells of the crab are exposed to hypoxia. To our knowledge this is the first study to report on the effects of hypoxia on active ion transport in a crustacean isolated gill epithelium.

2. Material and methods

2.1. Animal, exposure

Shore crabs, *Carcinus maenas* weighing 30–50 g, were collected from the North Sea coast of Westerland (Sylt) in the period September–November 2015, and only intermoult male crabs were used. Before the experiments, crabs were fed 2 times weekly with chopped bovine heart meat. Crabs were kept in aquaria with an open circuit of seawater (Institute Alfred Wegener, Sylt). Crabs were acclimated for at least three weeks in 12 ppt dilute seawater (DSW) prepared by diluting natural seawater with deionized water. The animals were kept in aerated aquaria at 15 °C and under natural light condition. The dissolved oxygen concentration in normoxic DSW was controlled daily and ranged from 8 to 9 mg/L.

The oxygen level was reduced by bubbling nitrogen to obtain the desired oxygen saturation. DSW was flowing through an open aeration column supplied with polypropylene spheres and then through a column where nitrogen flow was adjusted to maintain the oxygen level on the set point. An oxygen controller actuated the valves connecting to the nitrogen gas tank and air pump to maintain the desired oxygen level by delivering either nitrogen or air into the experimental tank (Bennett and Beitinger, 1995). Oxygen content in DSW and incubation medium was measured by an oximeter with automatic calibration (accuracy 0.5% of value; Oxytester, WTW ProfiLine Oxy 1970, Germany).

2.2. Electrophysiological studies

After destroying the ventral ganglion, the carapace was lifted and then posterior pairs of gills were cut at the base by scissors and removed. We choose the 7th or 8th posterior gill for our studies because previous studies on *Carcinus* and some other Crustacea have measured significantly higher specific activities of the Na^+,K^+-ATPase in these gills than in anterior gills, leading to the suggestion that the posterior gills are mostly specialized for osmoregulation (Neufeld et al., 1980; Siebers et al., 1985).

Short circuit-current (Isc) and conductance (G) were measured in the gill epithelia as described by Onken and Siebers, 1992; Lucu and Flik, 1999. Hemilamella consisting of a single epithelial layer supported by an apical layer of cuticle were prepared by splitting the gill lamella in half longitudinally. Hemilamella isolated from crabs acclimated in normoxic DSW were used for measuring effects of hypoxia on Isc. This preparation was mounted in a modified Ussing micro-chamber with a circular aperture of 1.25 mm in diameter. The epithelium was positioned onto the aperture, which rim area was slightly greased to minimize edge damage. The criterion for the validity of the preparation was a stable Isc (for > 3 h) when control physiological saline was applied. The electrical parameters of this preparation were measured using an automatic voltage clamp 558C-5 amplifier (Bioengineering, The University of Iowa, USA). The transepithelial potential was controlled by mercury reference electrodes (Broadley James Corporation; USA). Voltage pulses of 1.0 mV (duration 1 s; 500 s. interval between pulses) were applied by a pulse generator to measure epithelial conductance. The outputs from the voltage clamp were visualized using a pen recorder (Linseis Ly 17100). The total resistance measured by voltage pulses was corrected for chamber resistance by subtracting the resistance measured in the saline filled chamber after the installed tissue had been pierced with a needle on completion of each experiment. The measured current across the hemilamella was corrected for each preparation following Ohm's law. In the hypoxia experiments apical and basolateral sides were perfused with identical crab saline, which were circulated by a two-channel Watson-Marlow peristaltic pump (Sci 400) at a flow rate of 0.5 ml/min. The crab saline contained (in mM) to: NaCl, 235; KCl, 5; $MgCl_2$,4.0; $CaCl_2$ 2.2; $NaHCO_3$, 6;