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ORIGINAL PAPER

Hypoxia attenuate ionic transport in the isolated gill epithelium of *Carcinus maenas*

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Abstract

The gills are osmorespiratory organs of aquatic organisms and the prime target of environmentally induced hypoxia. We have studied the impact of severe hypoxia (0.5 mg O_2/L) on the ionic transport across posterior gills of *Carcinus maenas* acclimated to 12 ppt seawater (DSW). The short-circuit current (Isc) across hemilamellae from gills i.e. active ion transport was studied in micro Ussing chambers. Hypoxia induced by deoxygenation of the basolateral side, and not the apical side, resulted in time-dependent inhibition of Isc and full recovery of Isc after reoxygenation. Exposure of the crabs to severe 7 h hypoxia decreased the specific activity of Na^+,K^+-ATPase in the gills by 36%. Full recovery of enzyme activity occurred in fasted crabs after 3 days of reoxygenation. The intensity of Western blotting bands was not different in the gills of oxygenated, hypoxic and reoxygenated crabs. The reversible, nonspecific blocker of K^+ channels Cs and hypoxia inhibited over 90% of Isc which is after reoxygenation fully recovered. The specific blocker of Cl^- channels NPPB [5-nitro-2-(3-phenylpropylamino) benzoic acid] blocked Isc by 68.5%. Only the rest of not inhibited Isc in aerated saline was blocked by hypoxia and recovered after reoxygenation. The activity of the antioxidant enzyme catalase was not changed during hypoxia and reoxygenation kept the high enzyme activity in the gills at the level of crabs acclimated to DSW. As a response to hypoxia the presence of 2 mM H_2O_2 induce an initial slight transient decrease of Isc followed by a rise and after reoxygenation fully recovered Isc. Incubation of hemilamellae with the antioxidant derivative Trolox did not affect the inhibition of Isc by hypoxia.

Keywords *Carcinus* · Posterior gills · Ussing-chamber · Ion channels · Na^+,K^+-ATPase · Catalase

Introduction

Hypoxia is a severe global phenomenon affecting physiological, morphological and behavioral changes of aquatic organisms with strong implications on the ecosystem (Burnett and Stickle 2001; Diaz and Rosenberg 2008; Riedel et al. 2012). Living in the intertidal zone, *Carcinus maenas* is tolerant to environmental fluctuations, and as invasive intertidal species in world seas widely used as an ecotoxicological model for effects of anthropogenic pollutants (Leignel et al. 2014).

Hypoxia induces ion regulation and acid–base disturbances in *Carcinus maenas* (Johnson and Uglow 1987), and crabs use metabolic depression as a strategy to survive hypoxia (Durand and Regnauld 1998). Decrease in hemolymph Na^+ and Cl^- concentrations in *Carcinus maenas* is evident in hypoxia/heavy metal combination in low salinity exposures (Johnson 1988). Impaired ionic regulation in hypoxia-anoxia has been observed in osmoregulating shrimps *Crangon crangon* and *Palaemon adspersus*. For these species, anaerobic metabolism means a slow breakdown of the main transporting functions and of the tissue osmolarity resulting in cell damage (Hagerman and Uglow 1981). The osmoregulatory capacity of the peneid shrimp *Penaeus vannamei* was depressed by exposure to low oxygen tension and fully recovered after reoxygenation (Charmantier et al. 1994). Combination of temperature and hypoxia during molt stage had a significant effect on the osmoregulatory capacity of the shrimp *Litopenaeus stylirostris*. Low temperature reduced the effect of hypoxia presumably by slowing down the metabolism (Chantal and Claude 2005). In

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the freshwater prawn *Macrobrachium rosenbergii* exposed to hypoxia significant decrease in hemolymph osmolality, Na^+, K^+ and Cl^- concentrations were observed (Cheng et al. 2003). The effect on Na^+ level in hemolymph had its onset in *Hemigrapsus sexdentatus* which is less tolerant to hypoxia than *H. crenilatus* (Falconer et al. 2019).

The posterior gills of euryhaline hyperosmoregulating shore crab *Carcinus maenas* are recognized as highly efficient ion- osmoregulatory system evolved to adapt to changes in external osmolarity (Henry et al. 2012). The gills are the prime organ in crabs and fish to come into contact with gas and ion changes in water and, therefore, the first target in the oxygen depletion and ion fluctuations in water (Claiborne et al. 2002; Henry et al. 2012). Short-circuit current across the one epithelium layer of the posterior *Carcinus* gills was substantially reduced under exposure to 1.6–2.5 mg O_2 hypoxic saline (Lucu and Ziegler 2017). Moreover, in the shore crab increased surface/volume ratio in mitochondrion during hypoxia might be an adaptive mechanism that maximizes the efficiency of O_2 respiration enabling oxidative phosphorylation under restricted oxygen content (Lucu and Ziegler 2017). In the killifish were studied electrogenic and diffusive components of transepithelial potential (TEP) across the gill tissue under hypoxia. TEP was reduced by hypoxia would be deleterious to ionoregulatory balance in SW acclimated killifish (Wood and Grosell 2015).

The epithelial cells of posterior crab gills contain numerous mitochondria at the basolateral membrane (Barra et al. 1983; Compere et al. 1989). The mitochondrial respiratory chain requires O_2 and regenerates ATP from ADP and phosphate. This ATP drives numerous cellular processes in crustacean gills, including Na^+,K^+-ATPase, responsible for active salt absorption in hyperosmoregulating *Carcinus* (see Henry et al. 2012). However, the effects of hypoxia on the gill Na^+,K^+-ATPase in crabs was poorly studied. In European flounder, the activity of Na^+,K^+-ATPase in gills is reduced by hypoxia without influence on the extracellular ion concentrations (Lundgreen et al. 2008). Branchial Na^+,K^+-ATPase hydrolytic activity in the gills of fish *Astronotus ocellatus* was depressed by hypoxia (Wood et al. 2007; Richards et al. 2007). Hypoxia induces a decrease of the Na^+,K^+-ATPase activity by promoting the endocytosis of catalytic alpha subunit from the plasma membrane into intracellular pool (Bertorello et al. 1999; Dada et al. 2003). Enhanced resistance to hypoxia might develop if the adapted cells could selectively preserve a limited energy production for indispensable functions necessary for living cells. During metabolic depression, protein synthesis and degradation, and ion-dependent ATPase activity are drastically suppressed (Hochachka et.al. 1996; Boutilier and St Pierre 2000; Hochachka and Somero 2002; Larade and Storey 2002; Gorr et al. 2006). Hypoxia diminishes ATP utilization by downregulating protein translation and the Na^+,K^+-ATPase activity (Storey and Storey 1990; Guppy et al. 1994; Lee and Langhans 2015). Studies in vertebrates epithelia have reported that hypoxia reversibly blocks Na^+,K^+-ATPase activity (Planes et al. 1996; Mairbaurl et al. 2002; Wodopia et al. 2000; Dada et al. 2003).

Hypoxia inhibits background K^+ channels that are open at the resting potential of neuroepithelial cells of the zebrafish gills (Jonz et al. 2004), without the similar effect in hypoxia/anoxia tolerant goldfish (Zacher et al. 2017). Depolarization by hypoxia in some mammal tissues occurs due to inhibition or limitation of K^+ channels activities. Identity of the specific K^+ channel subtypes responsible for chemoreceptor O_2 sensing remains in debate and may vary with species (Lopez-Barneo et al. 1997; Shimoda and Polak 2011). In epithelial cells, K^+ channels are required for the recycling of K^+ taken up by the Na^+-K^+ pump in exchange for Na^+. Therefore, these channels contribute to the negative membrane potential required for the uptake of Na^+ and control transepithelial transport. It is unclear whether these K^+ channels respond to changes in oxygen tension similar to what is found in excitatory cells (Dawson et al. 1980).

Gills exhibit response to oxidative stress and provide a first line of antioxidant defense in crabs and fish. Strategy in the gills of king crab *Lithoder santella* during air exposure is to counteract the overproduction of reactive oxygen species (ROS) to develop a permanently high level of SOD activity favoring the degradation of superoxide radicals which generates H_2O_2 (Schwezov et al. 2017). In the gills of the crab *Chasmagnathus granulata* (Dane 1851; *Neohelice granulata)* daily variations of the antioxidant system should depend on high catalase activity (Maciel et al. 2004). Higher catalase activity was acquired in the crab *Scyilla paramamosian* after induced oxidative stress by lipopolysaccharide (LPS) (Liu et al. 2010). Oxygen sensitive potassium flux in the erythrocytes of rainbow trout is regulated by reactive oxygen species (Berenbrink et al. 2005; Bogdanova and Nikinmaa 2001).

The mechanisms by which oxygen influence ionic membrane transport i.e. short-circuit current in crabs gills are poorly studied. The objective of this study is to determine how stress induced by hypoxia is associated with electrogenic ion transport in the posterior gills of *Carcinus maenas*. Do changes in Isc observed during hypoxia/reoxygenation represent changes in specific transport mechanisms i.e. K^+ and Cl^- channels? Does Na^+,K^+-ATPase specific activity change in posterior gills during short-term hypoxia of the crabs*?* Does antioxidant enzyme catalase change in posterior gills and reactive oxygen species (ROS) scavenger Trolox-C affect Isc inhibition by hypoxia and reoxygenation?

Material and methods

Animal acquisition and holding

Adult shore crabs *Carcinus maenas* were collected from the Wadden Sea coast of Westerland (Sylt) in the period from September to November 2018. In the experiments, only intemoult adult male crabs were used. Animals were fed by bovine heart meat twice a week. Crabs were kept in aquaria with the open circuit of seawater (Institute Alfred Wegener, Sylt). Crabs were acclimated for at least two weeks in 12 ppt seawater prepared by diluting natural seawater by deionized water. The animals were kept in aerated aquaria at 18 ± 2 °C and under natural light condition. The specific activity of the Na^+,K^+-ATPase in the posterior gills was measured in the DSW acclimated crabs exposed to severe hypoxia (0.5 mg O_2/L) for 7 h. During the first hour of hypoxia, crabs were actively trying to escape from the tank. In the second hour of exposure, the crabs were largely inactive, with only oscillatory movement of the antenna and the third maxilliped. After 7 h the hypoxia tone of the claws had decreased, and a few minutes after reoxygenation, the tone was recovered. Survival of crabs under hypoxia necessities a considerable reduction in metabolic rate (Hill et al. 1991).

Electrophysiological studies

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

Short circuit-current (Isc) was measured in the gill epithelium 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. Native hemilamella epithelium 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 so-called dark area of the hemilamella, which is particularly rich in mitochondria rich cells was selected for the Isc measurements. 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 17,100). The measured current across the hemilamella was corrected for each preparation following Ohm's law. In the hypoxia experiments at both sides of epithelium were perfused with identical crab saline, which was circulated by a two-channel Watson-Marlow peristaltic pump (Sci 400) at a flow rate of 0.5 mL/min. Perfusion saline was bubbled with compressed air and N_2 gas to reach the desired O_2 concentration at both sides of epithelia. The O_2 concentration was measured by an oximeter, which electrode was immersed in the perfusion saline during an experiment (Oxytester,WTW ProfiLine oxy 1970, Germany). Oxygen content in normoxic saline ranged from 8.5 to 9.5 mg O_2/L and pH values in the normoxic and groups exposed to hypoxia were 7.7 ± 0.2. The crab saline contained (in mM) to: NaCl, 235; KCl, 5; $MgCl_2$, 4.0; $CaCl_2$ 2.2; $NaHCO_3$, 6; glucose, 10; HEPES, 10. pH of 7.6 was adjusted by TRIS base (see Lucu and Flik 1999).

Na^+,K^+-ATPase determination

After dissection, posterior gill pairs were slightly dried on filter paper. Posterior pooled gills were homogenized in a ice-cold hypotonic buffer (10 mL/g fresh weight) in Dounced homogenizer by 50 strokes. The homogenate was filtered through 0.1 mm nylon mesh and centrifuged at 450× *g* for 20 min to remove the nuclear fraction and cellular debris. Homogenization medium contained (in mmol/L): 12.5 NaCl, 1 HEPES, 1 DTT (dithiotreitol), 0.5 EDTA, pH 8–8.1 by TRISMA base, and the protease inhibitor aprotinin (0.1 mL/100 mL;Sigma).

Na^+,K^+-ATPase activity was determined by a slightly modified methodology described in detail earlier (Lucu and Flik 1999). Na^+,K^+-ATPase activity was assayed in triplicate by incubating 50 µL of homogenate with 500 µL assay solution A or E. A contains (in mmol/L): 100 NaCl, 5.0 $MgCl_2$, 0.1 EDTA, 15 imidazol/histidine (pH 7.5), 3 Na_2 ATP and 12.5 KCl. E is composed as A but KCl is omitted and ouabain (1 mmol/L) is added. The difference in phosphate released from ATP in solutions A and E reflects the ouabain -sensitive K dependent Na^+,K^+-ATPase. After incubation in thermostatted bath at 37 °C, for 15 min, the reaction was stopped by addition of 1 ml ice-cold 8.6% trichloroacetic

acid. Liberated phosphate was quantified colorimetrically by addition of 1 ml of 1.14% (wt/vol) ammonium heptamolybdate in sulfuric acid (36.3 ml concentrated sulfuric acid/L distilled water), supplemented immediately before use with 4.5 g/50 ml $FeSO_4 \times 7\ H_2O$ (9.0 g/100 mL) After about 20 min incubation the change in absorbance at 700 nm was recorded. Sigma Phosphate standard 0.65 mmol/L (Sigma) was used for expression of enzyme activity in Pi units.

Western blotting

Posterior gills were homogenized using a Dounce homogenization device (30 strokes). The homogenate was filtered over cheese cloth (100 µm mesh). Filtrate was spin for 5 min at 400 *g* to remove nuclei and cellular debris. The membranes were collected by centrifugation for 40 min at 50.000 *g*. The pelletetized membranes were resuspended in a small volume of buffer containing (in mmol/L) 300 sucrose, 10 HEPES/Tris (pH 7.4), 2 DTT, 0.5 EDTA and 10 U/ml aprotinin. Protein was measured, and the suspension was brought to 2 mg/mL with the same buffer. Just before electrophoresis, 5 µL membrane suspension was mixed with 10 µL sample buffer (2 mM DTT, 0.5% bromophenol blue, 30% glycerol, 20 mM Tris HCl, pH, 6.8) microwave treated (2 × 1 min at 500 W), this treatment was critical to extract a single immunoreactive species from the membrane preparation. Samples were run on 10% polyacrylamide slab gel; kaleidoscope prestained markers (Biorad No. 161 0324) were used as a reference. After SDS-PAGE, proteins were electroblotted to nitrocellulose membranes (pore size 0.45 µm; Schleicher and Schuell, code 401196). After blocking the membranes with 3% low-fat coffee creamer plus 1% BSA and 0.1% gelatin, the proteins were probed with a mouse monoclonal antibody against the -subunit of an avian Na^+,K^+-ATPase (Developmental Study Hybridoma Bank, The University Iowa) for 1 h at room temperature and subsequently for 16 h at 4 °C. Goat anti-mouse IgG-peroxidase conjugate was used to visualize the Na^+,K^+-ATPase epitope. Preparation was made of posterior gills obtained from crabs acclimated in DSW i.e. aerated, exposed to hypoxia (0.5 mg O_2/L) during 7 h and those reoxygenated 3 days after exposure to hypoxia.

Catalase

Catalase activity was studied by measuring the rate of degradation of H_2O_2 according to the method described by Claiborne (1985). Posterior gills 7 and 8 pairs were homogenized (1: 25 w/v) in the buffer containing 20 mM HEPES, 1 mM EDTA and 0.1% Triton X adjusted to pH 7.2. Homogenate was centrifuged at 10.000 *g* for 20 min at 4 °C. The decrease in absorbance of H_2O_2 at a wavelength of 240 nm was recorded for a time period of 30 s, taking measurements at 5 s intervals at 20 °C. The values are expressed as U/mg protein where U is µmol/min.

Protein determination

Protein content of homogenate and membrane vesicles was measured by Coomassie Brilliant Blue G-250 shifts from 465 to 595 nm when binding to protein occurs. Bradford procedure (BioRad catalog no 500–0002) using BSA as a reference. Protein BSA standards 1.29 mg BSA/mL.

Statistics

Data have been expressed as mean values ± SEM. Statistical analyses were performed with SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA). A one-way ANOVA was applied with a Tukey's post-hoc test. Significance for all statistical tests was accepted at $p < 0.05$. Quantitation of Western blots was carried out determining the "adjusted volume" of immunoreactive protein bands (i.e. optical density multiplied with the area, OD × mm^2) using molecular Analysis Software (Biorad, Hercules, CA).

Chemicals

Dimethylsulfoxide (DMSO), and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB soluble in DMSO) were obtained from Sigma-Aldrich. CsCl dissolved in a crab saline was used as a unselective inhibitor of K^+ channels (Van Driesche and Zeiske 1985). Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid soluble in water and H_2O_2 were obtained from Sigma-Aldrich.

Results

Once the hemilamella was mounted in the Ussing-type microchamber, the short-circuit current (Isc) reached baseline value in approximately 30 min. Deoxygenation at the apical side of *Carcinus* hemilamella (0.5 mg O_2/L) and oxygenation of the basolateral side maintained Isc in the same range as the aerated control (-279.4 ± 19.2 cm^{-2}; Fig. 1). Within 20 min hypoxia at the basolateral side suppressed Isc 90%, to -28.2 ± 4.0 A cm^{-2}. High viability of the epithelium preparation was evidenced based on the recovery of Isc to $-254.6 \pm$ A cm^{-2} (Fig. 1). The Isc reflect the quantity of active ionic charge transfer (i.e. electrogenic transport) generated by Na^+,K^+-ATPase activity. The specific activity of the Na^+,K^+-ATPase in the posterior gills was measured in the crabs exposed to hypoxia (0.5 mg O_2/L) for 7 h. Under these conditions, the activity of the enzyme was decreased 36%, from 7.61 ± 0.49 (air; $n = 8$) to 4.86 ± 0.60 µM Pi/mg protein/h (hypoxia;

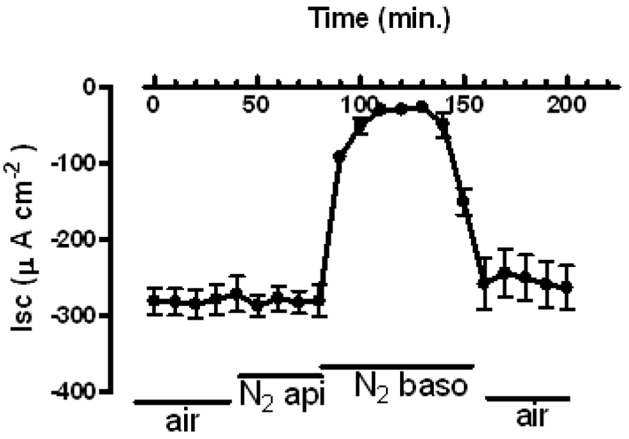

Fig.1 Time-course of the short-circuit current (Isc) across the hemilamella of the posterior gill exposed apically and basolaterally to deoxygenation (0.5 mg O_2L^{-1}). Both sides of the epithelia were perfused initially with aerated saline. During 40 min. deoxygenation of the apical side (basolateral side was aerated) Isc was not changed. When the basolateral side was deoxygenated (apical side was aerated) Isc was almost fully reduced. Isc was recovered during 30 min reoxygenation at both sides of epithelia. Mean values ± S.E.M. per time point are given for four individual samples

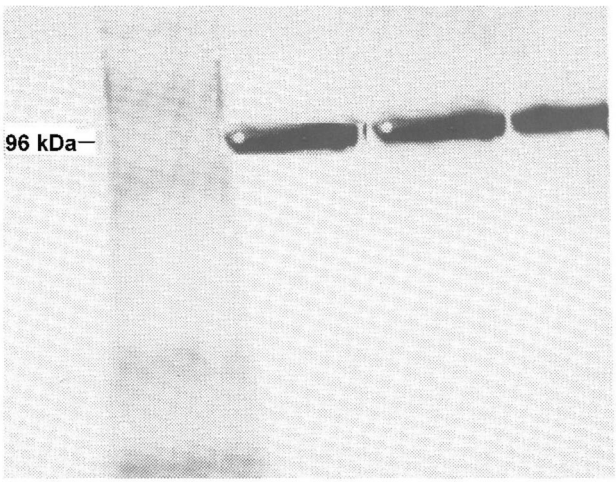

Fig.3 Western blot of Na^+,K^+-ATPase extracted from a homogenate of the posterior gills in the control aerated saline (line 1) and after 7 h (line 2) and 3 days deoxygenation (line 3) reoxygenation. Blots were probed with mouse monoclonal antibody against chicken Na^+,K^+-ATPase (cytosolic epitope of -subunit kDa recognizing a 100.4 kDa protein at the prominent molecular species

$p < 0.01$; $n = 6$). Gradual increase of oxygen during 20 h reoxygenation increased the Na^+,K^+-ATPase activity to 6.33 ± 0.37 M Pi/mg protein/h ($p < 0.05$; $n = 6$) and after 3 days of reoxygenation of fasted crabs, the specific activity of the enzyme had fully recovered (7.39 ± 0.51 M Pi/mg protein/h; $p > 0.05$; $n = 6$; Fig. 2). Western blot analysis of the posterior gill membrane preparation revealed that the $Na^{+'}$,K^+-ATPase protein-subunit expression in membranes of deoxygenated and reoxygenated crabs did not differ from the control normoxic group (Fig. 3).

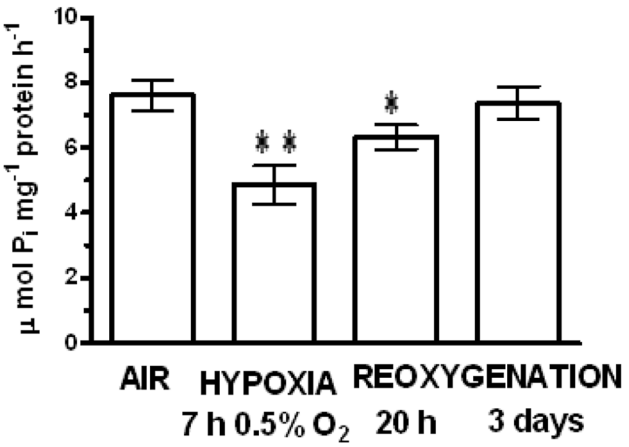

Fig.2 Na^+,K^+-ATPase activity in crude homogenates of the posterior gills in the control group compared with that exposed to hypoxia (7 h) and reoxygenated for 20 h and 3 days. Data are expressed as means of 5–8 individual samples ± SEM. Statistical significance was calculated using one-way ANOVA and the Tukey's multiple comparision test. No significant differences were found between control (air) and group after 3 days reoxygenation. Significant difference was found between control and 7 h hypoxia ($p < 0.01$), and between control and 20 h reoxygenation ($p < 0.05$). *$p < 0.05$; **$p < 0.01$

The effects of non-specific inhibitors of K^+ channels (10 mM CsCl) and Cl^- channels (1×10^{-4} M NPPB) on the ion transport-related Isc at the basolateral side of the hemilamella was studied to gain insight into the possible participation of ion channels in hypoxia.

Hypoxic saline and CsCl containing aerated and hypoxic saline, respectively, inhibited more than 90% of Isc in comparison to the control aerated saline ($p < 0.001$). The non-inhibited portion of Isc remained at − 8 to − 13 µA cm^{-2} (Fig. 4). No significant difference between the non-inhibited portion of Isc in hypoxic versus CsCl saline, in the absence and presence of hypoxia, was found ($p > 0.05$). At the end of the experiments, Isc was fully recovered by reoxygenation (Fig. 4). The results suggest that hypoxia as well as CsCl-sensitive non-specific K^+ channels almost completely inhibits Isc.

In the NPPB—containing aerated saline, non-inhibited portions of Isc remained at 31.5%, and in hypoxic saline, in the absence and presence of NPPB, Isc remained at 10.5% and 5.5%, respectively, compared with the control group ($p < 0.001$; Fig. 5.). There was no significant difference in Isc between the hypoxic groups in the presence and absence of NPPB ($p > 0.05$). During reoxygenation, only a portion of the Isc inhibited by hypoxia was recovered. The remainder was the non-recoverable portion of NPPB-sensitive Cl^- channels.

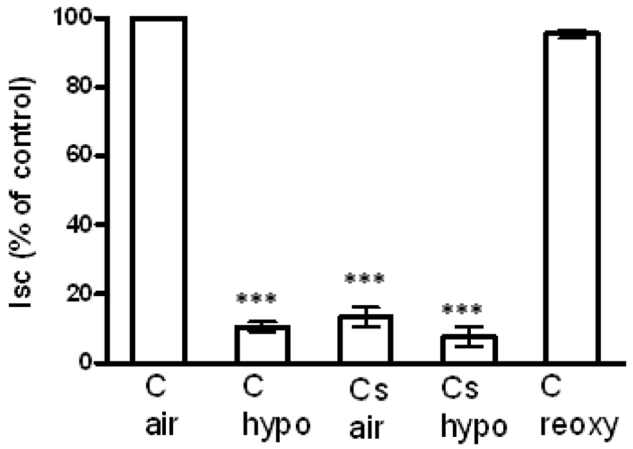

Fig. 4 Combined effects of hypoxia and the nonspecific inhibitor of K^+ channels Cs (10 mM CsCl) on Isc in the hemilamella (basolateral side).We have measured consecutively Isc over 2.5 h period, and presented at the reached equilibrium relative to each group (means ± SEM; $n = 4$). C air vs C hypo ($p < 0.001$); C air vs Cs air ($p < 0.001$) and vs Cs hypo ($p < 0.001$); C hypo vs Cs air ($p > 0.05$) and vs Cs hypo ($p > 0.05$). By reoxygenation Isc was recovered to the baseline value ($p > 0.05$); ***$p < 0.001$

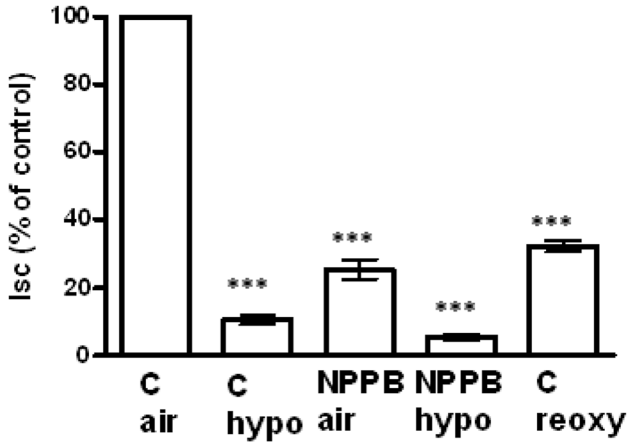

Fig. 5 Combined effects of hypoxia and the nonspecific blocker of Cl^- channels NPPB (1×10^{-4} M) on Isc in the hemilamella (basolateral side) of the crabs posterior gills. We have measured consecutively Isc over 2.5 h period and presented at the reached equilibrium relative to each group (means ± SEM; $n = 4$). C air vs C hypo ($p < 0.001$); C air vs NPPB air ($p < 0.001$) and vs NPPB hypo ($p < 0.001$). C hypo vs NPPB hypo ($p > 0.05$). NPPB air vs C reoxy ($p > 0.05$) C air vs C reoxy ($p < 0.001$). After reoxygenation portion of Isc inhibited by NPPB was not recovered; ***$p < 0.001$

The antioxidant catalase activity in posterior gills exposed to severe hypoxia (113.6 ± 24.1 U/mg protein; $n = 5$) for 7 h was not significantly different from the enzyme activity in the aerated control group (126.0 ± 15.0 U/mg protein; $p > 0.05$; $n = 5$) and that after reoxygenation for 20 h (131.0 ± 28.1 U/mg protein; $p > 0.05$; $n = 5$; Fig. 6).

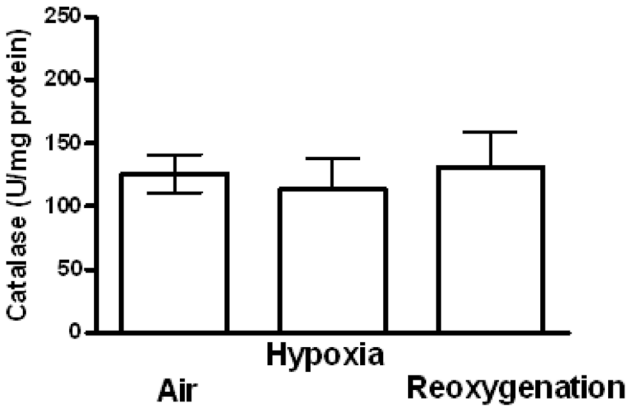

Fig.6 Catalase activity (U/mg protein) was measured in the posterior gills of crabs exposed to hypoxia for 7 h and afterwards maintained in normoxia for 20 h. Data are expressed as means ± SEM of 5–6 samples per treatment. Statistical significance was calculated using one-way ANOVA and the Tukey's multiple comparision test. No significant differences were found between control and treated groups

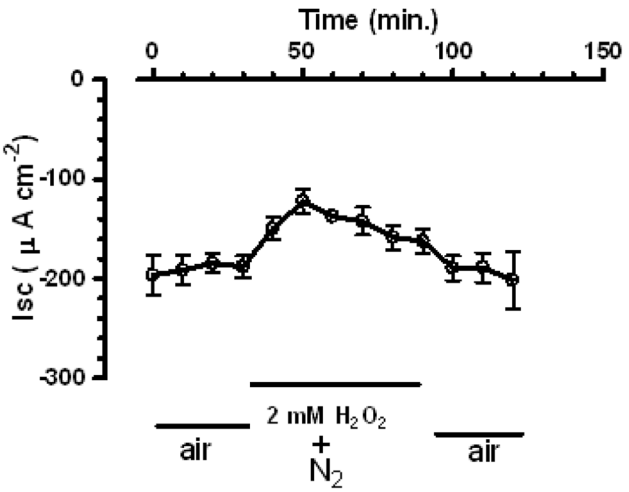

Fig.7 After reached steady-state, time-course of Isc in the hemilamella basolaterally incubated with deoxygenated saline containing 2 mM hydrogen peroxide was studied. Isc transiently dropped by 33% and afterwards in the next 40 min steadily increase. During reoxygenation Isc was fully recovered to the value of the control saline. Mean values ± S.E.M. per time point are given for four individual samples

Incubation of the basolateral side of the epithelium with 2 mM H_2O_2 in deoxygenated saline induced transient blockage of Isc, changing it from − 190.0 ± 16.2 μA cm^{-2} to − 126.0 ± 11.5 μA cm^{-2} at 30 min of exposure, but stimulated it during a further 40 min to − 160.4 ± 12.3 μA cm^{-2}. At 30 min after reoxygenation, the Isc was recovered (− 193.2 ± 18.6 μA cm^{-2}; Fig. 7).

Figure 8 (lower figure) shows the effect of hemilamella incubation with the antioxidant Trolox C (200 μM) on Isc during deoxygenation and reoxygenation, and in comparison with the not incubated group (upper figure). During

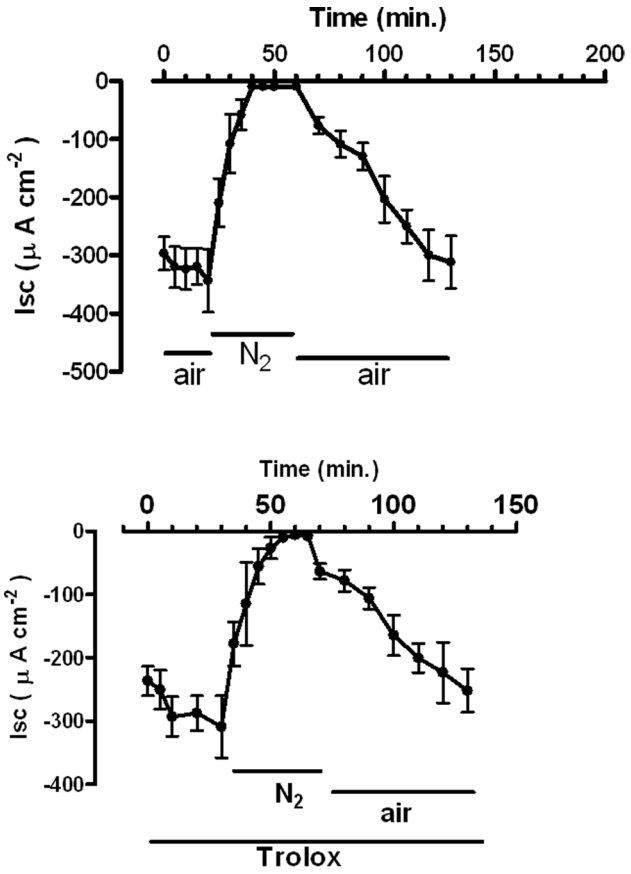

Fig.8 Time-course of the short-circuit current (Isc) in the hemilamella of the posterior gill of shore crab *Carcinus maenas* in the control group compared with this treated by Trolox C. Both sides of the epithelia were perfused initially and during the phase of reoxygenation with aerated saline. Upon hypoxia of the both sides of epithelia Isc was reduced 97% during 20 min. In the next 40 min reoxigenation Isc value was almost fully recovered. Values are means ± S.E.M. for four individual samples (upper figure). Both sides of hemilamella were incubated with 200 μM Trolox. After reached steady-state, upon hypoxia Isc was reduced for 98% in 20 min. In the next 40 min. Isc was recovered. Mean values for three individual samples ± S.E.M. per time point are given

incubation of both sides of the hemilamella with Trolox C Isc decreased from − 275.12 ± 33.2 A cm^{-2} (normoxia) to − 7.9 ± 3.5 A cm^{-2} (hypoxia), 97% inhibition in 20 min, consistent with the results of the control group. At 40 min. reoxygenation the Isc was recovered in both groups (Fig. 8, upper and lower figure).

Discussion

We examined the energetic consequences of hypoxia in the posterior gill epithelium of the hyperosmoregulating crab on the active ionic transport rate measured by Isc, specifically, the Na^+,K^+-ATPase activity and K^+ and Cl^- channels. Based on present data Isc could be inhibited by deoxygenation of the basolateral side but not the apical side. During reoxygenation phase, Isc was fully recovered. The chitinous cuticle barrier present at the apical side hinders O_2 diffusion (Taylor and Butler 1978). Higher basolateral than apical oxygen consumption is present because densely-packed mitochondria are particularly there expressed in DSW-acclimated crabs (Compere et al. 1989; Lucu and Ziegler 2017). The mitochondrial respiratory chain uses oxygen to generate ATP which is consumed mainly by Na^+,K^+-ATPase for the functioning of primary and secondary active ionic transport processes (Henry et al. 2012).

Reversible inhibition of Isc in *Carcinus* hemilamella that is, a decline in active ion transport energy supply, downregulates metabolic processes to save energy during oxygen deprivation. Inhibition of Isc in *Carcinus* hemilamella was caused preferentially by inhibition of basolaterally located ouabain-sensitive Na^+,K^+-ATPase (Lucu and Ziegler 2017). In the mucosa-submucosa preparations from human colon, the Isc and transepithelial resistance under hypoxia were transiently decreased (Carra et al. 2011, 2013). If hypoxia in rat colonic epithelium is shifted enough to prevent damage to the epithelium or pump, Isc is fully recovered after reoxygenation (Saravi et al. 2017).

In the present study was found a significant decrease in the Na^+,K^+-ATPase specific activity in the posterior gills of DSW- acclimated crabs *Carcinus* exposed to severe hypoxia. When *Carcinus* was acclimated in dilute seawater, Na^+,K^+-ATPase specific activity had increased in the posterior gills, and in line with these results, Western blotting showed an increased amount of the Na^+,K^+-ATPase protein (Lucu and Flik 1999). The data presented here revealed that the intensity of the Western blotting band representing the catalytic subunit of Na^+,K^+-ATPase remained high and was not changed in oxygenated, hypoxic and reoxygenated posterior gills. Highly expressed alpha catalytic subunits during short-term hypoxia could be beneficial for reversible enzyme recovery during reoxygenation. Similarly, in the gills and kidney of the Amazonian cichlid fish *Astronotus ocellatus* exposed to hypoxia, a downregulation of Na^+,K^+-ATPase was found, and the catalytic alpha subunit protein abundance was not changed suggesting that the rapid modulation of the enzyme responding to hypoxia is due to post-translational modification (Richards et al. 2007). Suppression of Na^+,K^+-ATPase during aestivation of the land snail *Otala lactea* was linked to posttranslational modification of the enzyme (Ramnanan and Storey 2006). The activity of Na^+,K^+-ATPase in trout hepatocytes was decreased by hypoxia. Authors speculated that decrease in Na^+,K^+-ATPase activity in response to hypoxia may be accomplished by changes in ROS levels, but no precise mechanism was given (Bogdanova et al. 2005). The present study demonstrates that fasted crabs recovered the

Na^+,K^+-ATPase activity by using internal metabolic sources following 3 days reoxygenation. Hypoxia tolerant brackish water Crustacea are faced with changing of multiple environmental factors—they have the ability of conservation of glycogen as a raw material for ATP production. Stores of glycogen in the hepatopancreas, muscle, and other tissues allow crabs to survive hypoxia (Storey and Storey 2004; Marqueze et al. 2011). *Carcinus maenas* tolerate up to 10 ppt salinity and oxygen content of ambient water of 1.4 mg L^{-1} for 24 h, without switching to anaerobic metabolism (Legeay and Massabuau 2000).

Inhibition of the gill Na^+,K^+-ATPase specific activity under hypoxia and recovery of the enzyme activity after reoxygenation corroborate by studies on the electrophysiology of isolated gill hemilamella. Isc ion transport activity i.e. electrogenic ion transport fueled predominantly by the Na,K-ATPase activity (Onken and Siebers 1992; Riestenpatt et al. 1996; Lucu and Flik 1999). Our results show inhibition of Na^+,K^+-ATPase generated transport at the basolateral side by hypoxia and recovery after reoxygenation. When exposed to hypoxia cells develop adaptive strategies to maintain appropriate level of ATP. These strategies include decreasing energy-consuming processes such as Na^+,K^+-ATPase activity. The ability to suppress cellular ATP demand to match the limited capacity for O_2-independent ATP production has emerged as the unifying adaptive strategy ensuring hypoxia survival (Hochachka et al. 1996). In the lung alveolar cells Na^+ pump activity is regulated through the endocytosis / exocytosis of the enzyme molecules between the plasma membrane and intracellular compartments (Bertorello et al. 1999; Dada et al. 2003).

To study whether hypoxia affects the K^+ and Cl^- channels in the *Carcinus* gill preparation, we used in the present study CsCl (Gögelein 1990) and NPPB non-specific blockers of K^+ and Cl^- channels, respectively, in hyperosmoregulating crabs (Onken et al. 1992, 2003; Riestenpatt et al. 1996; Lucu and Towle 2010). Blocking of the K^+ channels by Cs^+ tended not to change Isc at the onset of hypoxia. Hypoxia has the same degree of effect on Is as that caused by CsCl. Whether these K^+ channels are affected directly by the reduced O_2 level or by the another factor that is generated as a result of hypoxia remains to be determined. The effect of inhibition of the basolateral K^+ channels abolishes K^+ recycling to the lumen which is essential for salt absorption (Riestenpatt et al. 1996; Onken et al. 2003; Lucu and Towle 2010). Cs^+ -sensitive K^+ channels located in the basolateral membrane translocate K^+ and NH_4 ions from the hemolymph of *Carcinus* into the basolateral cytoplasm of the gill epithelium (Weihrauch et al. 2004). One of the primary functions of the basolateral K^+ channels is to recycle K^+ across the basolateral membrane for the proper functioning of the Na^+,K^+-ATPase (Hamilton and Devor 2012). In the crab gills, the basolaterally located potassium channel is a major determinant of the negative cell membrane potential. In the hyperosmoregulating crab gills apical K^+ channels have a role to enable K^+ for $Na^+/K^+/2Cl^-$ cotransporter function. The cotransporter loads Cl^- into the cell and leaving the cell across basolateral Cl^- channel (Riestenpatt et al. 1996). In transcriptome studies hyperpolarization-activated cyclic nucleotide-gated potassium channel of the posterior gill epithelium of *Carcinus* is down-regulated by short-term hypercapnia (Fehsenfeld et al. 2011). The activity of this K^+ channel is significantly decreased in response to respiratory-metabolic acidosis during high environmental P_{CO2} (Fehsenfeld and Weihrauch 2016). In the isolated rat distal colon, the pivotal role of K^+ channels was underlined by the action of Ba^{2+} which suppressed the peak in the Isc during hypoxia (Schindele et al. 2016).

The present study examined the effect of hypoxia on Isc in combination with Cl^- channel blocker NPPB. Combined effects inhibits more than 90% of Isc. Only hypoxia inducible portion of Isc was recovered during reoxygenation. The Cl^- channels are involved in numerous functions in transepithelial transport, cell volume regulation, stabilizing membrane potential, synaptic inhibition and extracellular acidification (Jentsch et al. 2005). In the rat's distal colon, NPPB suppressed the Isc induced by hypoxia and reduced the secondary rise of the Isc during reoxygenation (Schindele et al. 2016). Based on the transcriptome analyses that showed changes in the gill epithelium cells of *C. maenas* after one week exposure to 400 Pa pCO_2, significant changes in a Ca^{2+}—activated Cl^- channel, a hyperpolarization-activated nucleotide-gated K^+ channels and a Cl^-/HCO_3^- exchanger were suggested (Fehsenfeld et al. 2011).

We found that the elevated level of catalase in the gills of crabs acclimated to DSW remain up-regulated during hypoxia and reoxygenation. A significant increase in catalase and superoxide dismutase under hyposmotic stress condition was observed in posterior gills of *Carcinus aestuarii* (Rivera-Ingraham et al. 2016). Moreover, assuming an antioxidant role of metallothyonein (MT) associated with intracellular partitioning, the observed MT induction in posterior gills of *Carcinus aestuarii* may be considered as an adaptive response to hyposmotic stress (Lucu et al. 2008). Posterior gills showed a higher antioxidant enzyme activity than the anterior gills (de Oliveira et al. 2005).No increase in the activities of the antioxidant enzyme in the posterior gills of *Callinectes danae* and *C. ornatus* was noticed upon hypo- salinity stress (Freire et al. 2011). No change in expression of catalase during exposure to hypoxia was found in muscle of the pelagic crab *Pleuroncodes planipes* (Seibel et al. 2018). In the posterior gills of the crab *Chasmagnathus granulata (Neohelice granulata),* the activity of catalase and glutathione S-transferase were increased mostly when the crab was exposed to anoxia (de Oliveira et al. 2005). During exposure of the crab *Neohelice granulata*

to hypoxia, the locomotor muscle reactive oxygen species (ROS) and lipid peroxidase activity were briefly increased during reoxygenation and quickly recovered (Geihs et al. 2013). Over-expression of the antioxidant enzyme catalase acts against free radicals, preventing the hypoxia-mediated Na^+,K^+-ATPase degradation in air-breathing fish and trout hepatocytes (Huang et al. 2015).

In the presence of N_2, exposure of H_2O_2 produced an initial slight decrease of Isc with subsequent increase. It seems that H_2O_2. does not participate in ROS signaling, it is more likely blocking detection of hypoxia, by supplying O_2 through its decomposition by catalase and/or peroxidases. The initial decrease in the Isc was probably affected by hypoxia and/or the relatively high initial H_2O_2 concentration penetrating the epithelium during the first few minutes and partially blocking the Isc. After H_2O_2 partial decomposition to O_2 and $2H_2O$ by catalase and peroxidase activities in the epithelium (Lyons et al. 2014), the liberated oxygen in the saline may affect the rise of the Isc. During reoxygenation, the Isc continued to increase to baseline level. The result exhibit high tolerance of *Carcinus* to cope with highly toxic hydrogen peroxide which does not participate at concentration 2 mM in ROS signaling. Exogenous exposure of one of the most toxic oxygen species, hydrogen peroxide, depresses the Na^+,K^+-ATPase activity in vertrebrates (Clerici et al. 1992; Wong et al. 2014). Brain Na^+,K^+-ATPase has been found to be more sensitive than kidney enzyme to inhibition by H_2O_2 in mammals (Kurella et al. 1999). It has been suggested that H_2O_2 is a signaling messenger that causes the -catalytic subunits of Na^+,K^+-ATPase to become sensitive to modification by hypoxia by targeting thiol oxygen groups of these subunits (Bogdanova et al. 2016). It is hypothesised that H_2O_2 action affects ATP, subsequently inhibiting apical membrane Cl^- conductance or altering action of transporters directly (Du Vall et al. 1998).

To study whether radicals, which are known to act as intracellular signaling molecules (Dröge 2003), play a role in the current induced by hypoxia/reoxygenation, hemilamella was treated with a ROS scavenger, the antioxidant Trolox C a water soluble analogue of vitamin E. Trolox C was previously used to reduce oxidative stress damage in vertebrate epithelia (Vergauwen et al. 2015; Schindele et al. 2016). No difference in the level and time-dependent Isc inhibition by hypoxia and reoxygenation of the gill hemilamella between the group treated with Trolox C and the control group was found. This lack of change in the Isc after incubation of gill hemilamella with Trolox C during hypoxia and reoxygenation phases suggests the relatively high capacity of the epithelium to deal with free radical production or that the hypoxia signal is not transduced by ROS signaling during hypoxia and reoxygenation. In the hypoxic rat colonic epithelium, the secondary decrease in the Isc was significantly reduced by Trolox (Schindele et al. 2016).

In summary, our study revealed that in the posterior gill epithelium layer mounted in a Ussing-type chamber, hypoxia induced basolateral inhibition of the Isc. In crabs exposed to acute severe hypoxia, the posterior gills showed decreased Na^+,K^+-ATPase specific activity without changes in the Na^+,K^+-ATPase alpha subunit protein abundance. Fasted crabs recovered Na^+,K^+-ATPase activity by using internal metabolic sources following 3 days reoxygenation. To keep ATP homeostasis, cells can decrease ATP demands via inhibition of ATP-consuming enzymes i.e. by reducing specific activity of Na^+,K^+-ATPase. In the gill epithelium layer of hyperosmoregulating *C. maenas,* a non-specific inhibitor of K^+ channels Cs^+, applied basolaterally**,** inhibited the Isc within the same order of magnitude as hypoxia. Combined effects of hypoxia and NPPB blocks more than 90% of Isc. Upon subsequent reoxygenation, NPPB induced inhibition of Isc was not recovered. During hypoxia and reoxygenation, the antioxidant enzyme catalase sustained the same high level as that observed in the control DSW acclimated crabs. An initial slight decrease in Isc impaired by 2 mM H_2O_2 was followed by a rise and full recovery of Isc after reoxygenation. Results showed that Trolox C was unable to change hypoxia-induced Isc transport generated by Na^+,K^+-ATPase. The lack of Trolox C effect suggest that the ROS signaling does not mediate changes in hypoxia inhibition and reoxygenation of the isolated gill epithelium.

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