Intracellular pH regulation in mantle epithelial cells of the Pacific oyster, *Crassostrea gigas*

Kirti Ramesh · Marian Y. Hu · Frank Melzner · Markus Bleich · Nina Himmerkus

Received: 18 November 2019 / Revised: 31 July 2020 / Accepted: 9 August 2020 / Published online: 20 August 2020
© The Author(s) 2020

Abstract
Shell formation and repair occurs under the control of mantle epithelial cells in bivalve molluscs. However, limited information is available on the precise acid–base regulatory machinery present within these cells, which are fundamental to calcification. Here, we isolate mantle epithelial cells from the Pacific oyster, *Crassostrea gigas* and utilise live cell imaging in combination with the fluorescent dye, BCECF-AM to study intracellular pH (pHi) regulation. To elucidate the involvement of various ion transport mechanisms, modified seawater solutions (low sodium, low bicarbonate) and specific inhibitors for acid–base proteins were used. Diminished pH recovery in the absence of Na⁺ and under inhibition of sodium/hydrogen exchangers (NHEs) implicate the involvement of a sodium dependent cellular proton extrusion mechanism. In addition, pH recovery was reduced under inhibition of carbonic anhydrases. These data provide the foundation for a better understanding of acid–base regulation underlying the physiology of calcification in bivalves.

Keywords Mollusc · Calcification · Acid–base · Ion transport proteins

Introduction
Shells of the Pacific oyster, *Crassostrea gigas*, a mollusc species with enormous economic and ecological value (Zhang et al. 2012) are characterized by the presence of two calcareous valves. In adult *C. gigas* molluscs, shell formation occurs under the control of the mantle tissue, where mantle epithelial cells take part in the transport of calcification substrates (Gong et al. 2008) (Ca²⁺ and HCO₃⁻). Recently, Sillanpaa et al. (2018) reported that up to 60% of the Ca²⁺ transport in *C. gigas* mantle epithelia occurs via the transcellular pathway. However, it is unknown at present by which pathways bivalve molluscs control the precipitation of the calcareous shell. Since proton production is a by-product of mineral formation from the bicarbonate (HCO₃⁻) substrate (Zeebe and Wolf-Gladrow 2001), specific mechanisms have to be present in calcifying molluscs to minimize fluctuations in intracellular pH (pHi). For calcification to take place, mantle epithelial cells would be responsible for generating and maintaining a highly regulated microenvironment. This makes intracellular pH homeostasis intricately linked to biomineralization as HCO₃⁻ and Ca²⁺ import will require the export or buffering of protons to maintain pHᵢ.

Regulation of pHi is a key aspect of cell physiology and cells make use of evolutionarily conserved membrane-bound transport proteins or intracellular buffering to accomplish stable pHᵢ (Laurent et al. 2014). Membrane-bound transport proteins can achieve proton extrusion (sodium/hydrogen exchangers (NHEs), V-type proton ATPases), bicarbonate uptake (sodium driven bicarbonate transporters) and most importantly generate the electrochemical gradient driving pHᵢ regulation (sodium–potassium ATPases). In addition to pHi regulation, these membrane-transporters are also crucial for calcification by providing the substrates necessary through cellular ion transport. This occurs via primary (calcium-ATPases)
and secondary (sodium/calcium exchangers and sodium driven chloride/bicarbonate exchangers) pathways. Additionally, membrane transporters also aid in the removal of proton by-products from the calcification substrate HCO$_3^-$ (Ramesh et al. 2019). Molecular evidence for the presence of such transporters in molluscs comes from cloning of calcium transporting proteins within the mantle tissue in the pearl oyster, *Pinna nobilis* (Dwyer and Burnett 1996). Therefore, acid–base regulators and can only partially compensate for molluscs, oysters are considered to be weak extracellular and Heuer 2019; Melzner et al. 2020). Similar to many quently alters extracellular acid–base homeostasis (Zlatkin nied with elevated dissolved carbon dioxide which conse- quently alters extracellular acid–base homeostasis (Zlatkin and Heuer 2019; Melzner et al. 2020). Regulation of pH$_i$ can also be achieved via intracellular modulation of bicarbonate concentrations via carbonic anhydrases and a range of other proton equivalent exchange processes. Carbonic anhydrases can produce HCO$_3^-$ from intracellular CO$_2$ and their role in biomineralisation has been studied in a range of molluscs (Medakovic 2000; Marie et al. 2008). However, there is limited information regarding the role of carbonic anhyd- rases in pH$_i$ regulation, particularly in calcifying tissue. Characterization of the ability of bivalve mantle cell acid–base regulatory mechanisms during induced stress, is rudimentary, although knowledge of the pH$_i$ control mechanisms in bivalve models such *C. gigas* is particularly important in the context of anthropogenic seawater acidifi- cation. Anthropogenic seawater acidification is accompa- nied with elevated dissolved carbon dioxide which conse- quently alters extracellular acid–base homeostasis (Zlatkin and Heuer 2019; Melzner et al. 2020). Similar to many molluscs, oysters are considered to be weak extracellular acid–base regulators and can only partially compensate for induced acidosis (Dwyer and Burnett 1996). Therefore, the relationship between seawater acidification, and pH$_i$ regulation is intricate and our ability to accurately predict the response of calcifying bivalves to seawater acidifica- tion is severely hindered by our limited understanding of the cellular mechanisms driving pH homeostasis in these organisms.

Here, we use primary mantle cell cultures to investiga- te the previously uncharacterized pH$_i$ regulatory capac- ity of mantle epithelial cells in *C. gigas* using live cell imaging and the pH-sensitive fluorescent probe, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM). Furthermore, we utilize pharma- ceutical blockers and modifications in ion composition to investigate the role of key ion transport pathways to provide crucial information on the mechanisms of pH$_i$ regulation in molluscan calcifying tissue.

**Materials and methods**

**Oyster collection and maintenance**

Adult aquaculture raised *C. gigas* were purchased from Ditt- meyer’s Austern Compagnie GmbH (‘Sylter Royal’, List, Germany) and delivered over night to GEOMAR Helmholtz Centre for Ocean Research Kiel. Animals were utilized for experiments within 30 days following arrival. Animals were maintained at a temperature of 11 °C and pH$_{NBS}$ of 8.0 ± 0.1 in an aerated, re-circulating seawater system without feeding in seawater prepared using Instant Ocean to a salinity of 31 ± 2 psu. Water in the culture system was exchanged on a weekly basis. Ammonium concentrations were tested using a JBL NH$_4^+$ aquaria kit and maintained below a concentra- tion of 0.05 mg/L.

**Mantle cell culture**

Primary mantle cells were cultured according to Gong et al. (2008) with slight modifications. Briefly, oysters were dis- sectet, and pallial mantle tissue as described in Gong et al. (2008) was excised for cell culture. The mantle tissue was sanitized for 20 min in an artificial seawater solution (ASW, Supplementary Table 1) containing 0.5 mg/ml streptomycin, 500 IU/ml penicillin,100 IU/ml gentamicin, and 2 μg/ ml nystatin. After rinsing 3 times in a calcium and magne- sium free ASW (Supplementary Table 1), the pallial man- tle tissue was minced into small fragments (approx. 2 mm diameter) and these fragments were planted onto the center of lysine coated glass coverslips (Eydam, Germany), placed into sterile petridishes. Cell cultures were maintained in a culture medium that is detailed in Supplementary Table 2. Cells of interest were allowed to migrate out of explants for 24 h, explants were removed and sedentary culture cells remaining on the coverslips were used for microfluorimetry. All reagents were purchased from Sigma-Aldrich unless oth- erwise specified.

**Preparation of solutions**

Artificial seawater (ASW) solutions were prepared accord- ing to Zeebe and Wolf-Gladrow (2001) (Supplementary Table 1). Osmolality (1104 ± 5 mOsm kg$^{-1}$) and salinity (31 ± 2 psu) were selected to match the seawater values in the culture system (1113 ± 8 mOsm kg$^{-1}$). Inhibitors were dissolved in DMSO and added at final concentrations of 20 μM (ethylisopropyl amiloride, EIPA) and 1 mM
(acetazolamide, ACZM) to ASW. DMSO concentrations did not exceed 0.1%.

**BCECF dye loading**

To measure mantle epithelial cell pHᵢ, cover slips containing cell cultures were affixed to glass perfusion chambers (Supplementary Fig. 1) using a hydrophobic, silicone gel and bathed in ASW with a final BCECF-AM concentration of 10 μM at 19 °C for 30 min in the dark. Following dye loading, cells that were firmly attached to the coverslip were used for measurements. The flow rate of the perfusion system was 1–2 mL min⁻¹ and experiments were performed at 19 °C.

**Microfluorimetry**

Microfluorimetric measurements were performed on an inverted microscope (Zeiss Axio Observer. D1) equipped with a 40 × objective (Zeiss) and a CoolSNAP HQ² CCD camera (Photometrics, USA). The dye was excited alternately at two wavelengths, 486 nm and 439 nm (± 10 nm bandwidth) for 24 and 60 ms, respectively. Emission was recorded at 525 nm and fluorescence was monitored with the imaging system Visitron. The ratio of the emission intensities at the two excitation wavelengths over mantle cell was calculated, following background subtraction of camera offset using the software Metafluor 7.6.1. From each coverslip, the recordings of one to six mantle epithelial cells were collected and averaged. For each treatment, between 4 and 7 individual oysters were used as biological replicates. The ionophore nigericin was used to calibrate pHᵢ of mantle cells as previously described by Stumpp et al. (2012). Mantle cells were exposed to 10 μM nigericin in the presence of 160 mM potassium [K⁺] at pH 6.5, 7.0, 7.5 and 8.0. This K⁺ concentration was chosen to be in the range of intracellular [K⁺] reported for marine molluscs (Potts 1958; Ellington 1993). The calibration curve allowed calculation of the relationship between recorded emission ratio of BCECF and the corresponding pHᵢ. For pHᵢ recovery experiments, mantle cells were exposed to ASW for 10 min followed by a 20 mM NH₃/NH₄⁺ pulse. Alkalosis compensation rates were calculated as the slope during this ammonia prepulse phase and are indicative of the rate of active pHᵢ acidification during the NH₃/NH₄⁺ prepulse (Table 1). Acidosis was consecutively induced by the washout of NH₃/NH₄⁺ using the following solutions: ASW as control condition and 5 mM Na⁺, low HCO₃⁻ or ASW plus inhibitors (see above) to assess the involvement of different ion transport systems. Consecutive experiments (control followed by treatment experiments) were not performed due to the required length of such experiments and therefore, the current experimental design is associated with natural differences in cell-to-cell variability. Recovery rates were estimated from the compensatory slope after induced acidosis for the linear phase of recovery marked by the red lines in Fig. 2.

**Buffer capacity**

Buffer capacity (β) was estimated using the NH₃/NH₄⁺ pulse as described by Boron (1977) and is expressed as Slykes (mM/pH unit). Concentrations of NH₄⁺ in ASW were assumed to be negligible and nominally set to zero (Boron 1977). Following NH₃/NH₄⁺ pulse, β was calculated with the following formula:

\[ \beta = \frac{\Delta[\text{NH}_4^+]}{\Delta[\text{pH}]} \]

**Data analysis**

All data were analysed using R (Version 3.3.2, R Development Core Team, R: http://www.R.org/. 2011). Data were tested for normality and homogeneity using Shapiro-Wilks test and Bartlett test, respectively. If assumption for normality was not met, data were transformed by applying Box-Cox transformations. To determine the ability of mantle epithelial cells to recover from an NH₃/NH₄⁺ induced acidosis, the alkalosis compensation rates, pHᵢ recovery rates and final pHᵢ (after 60 min) were tested for fixed effects of washout solution and the random effects of animals as replicate. A mixed effects model using the lmer function in the lmerTest package

| Baseline pHᵢ | Alkalosis Compensation rate | Treatment after NH₃/NH₄⁺ pulse | Acidosis pHᵢ | Recovery pHᵢ | β       | N (cells) | N (animals) |
|--------------|-----------------------------|---------------------------------|--------------|--------------|---------|-----------|-------------|
| 6.84 ± 0.04  | −0.0019 ± 0.0006            | ASW                             | 6.54 ± 0.04  | 6.96 ± 0.07  | 22.53 ± 1.26 | 11        | 6           |
| 6.72 ± 0.02  | −0.0033 ± 0.0005            | Low Na⁺                         | 6.36 ± 0.02  | 6.47 ± 0.03  | 19.6 ± 0.86  | 12        | 7           |
| 6.89 ± 0.06  | −0.0025 ± 0.0006            | Low HCO₃⁻                       | 6.46 ± 0.03  | 6.73 ± 0.04  | 21.19 ± 1.35 | 10        | 5           |
| 6.81 ± 0.03  | −0.0022 ± 0.0003            | 20 μM EIPA                      | 6.39 ± 0.05  | 6.61 ± 0.07  | 22.12 ± 2.3  | 17        | 5           |
| 6.89 ± 0.04  | −0.0021 ± 0.0002            | 1 mM ACZM                       | 6.49 ± 0.04  | 6.83 ± 0.05  | 21.57 ± 1.04 | 29        | 7           |
was applied and significant effects were determined using the ANOVA function. Post hoc analyses were performed via Tukey HSD tests. Data on Δ[H+] were analysed using a Kruskal–Wallis test followed by a Dunn’s posthoc test.

**Results**

**Mantle epithelial cell culture**

Following 24 h of cultivation, three typical cell populations were commonly observed around mantle explants, namely mantle epithelial cells, granular hemocytes and hyalinocytes (agranular hemocytes) (Fig. 1a–d). In addition, certain

![Image](image-url)

**Fig. 1** A Phase contrast image of mantle tissue cell culture in *Crassostrea gigas* depicting an epithelial cell (e), granular hemocytes (g) and hyalinocytes (h). Higher magnification images of all the three cell types labelled in (A): (B) epithelial cell (C) granular hemocytes (D) hyalinocyte. Scale bars: 20 μm in (A), 40 μm in (B), 30 μm in (C) and 50 μm in (D). E Fluorometric pH measurement in a mantle epithelial cell. F Calibration curve of BCECF-AM in mantle epithelial cells of *Crassostrea gigas* allowing the conversion of ratios to pH, values. Dots represent individual cells.
cultures contained the presence of spindle-like muscle cells (not shown). Cells were identified based on size, morphology and characteristic movement of the two hemocyte cell types as described previously (Awaji 1991; Gong et al. 2008). Although hemocytes have been linked to calcification in oysters (Mount et al. 2004; Ivaina et al. 2017), microfluorimetric measurements were not performed for these cell types due to a vesicular concentration of BCECF in addition to the cytosolic signal. Regions of the cell cultures containing higher abundance of the roundish, stationary epithelial cells were selected for microfluorimetric measurements.

**pH regulatory proteins in oyster mantle epithelia**

Figure 1e shows a typical fluorometric measurement of one single mantle epithelial cell under resting conditions and after challenging the pH by an ammonium pulse experiment. Cells where perfused with ASW (resting, control situation). Superfusion with 20 mM of the mild base NH$_3$/NH$_4^+$ leads to alkalization (alkalosis) with slight counter-reaction of the cells (blue line), representing the alkalosis compensation rate, which was not significantly different between experiments ($p > 0.05$). This prepulse was followed by an acidosis phase upon wash-out of NH$_3$/NH$_4^+$ leaving protons behind which have been produced as a result of alkalosis compensation. The following recovery phase (recovery) can be used to estimate proton extrusion and buffer capacity in cells (red lines in Fig. 2). Nigericin calibration was used to translate ratio into pH$_i$ (Fig. 1f). N = 4–7 experiments were summarized for each of the measuring conditions in the following figures. An experiment was defined as cells isolated from $N = 1$ oyster. Figure 2a shows the control experiment where the wash-out was done by ASW (control). Cells maintained a resting pH$_i$ of 6.84 ± 0.04. This pH$_i$ was found to be stable for at least 180 min (Supplementary Fig. 2a, shown for one cell) and was used to establish cell culture status for further experiments. When cells were perfused with 20 mM NH$_3$/NH$_4^+$, pH$_i$ increased to a value of 7.45 ± 0.04. Removal of 20 mM NH$_3$/NH$_4^+$ and perfusion with ASW induced an intracellular acidosis (pH$_i$, 6.54 ± 0.04) followed by gradual recovery in pH$_i$ to 6.96 ± 0.07. A recovery rate (red line) of approx. 0.01 pH units/min was estimated (Fig. 2) for control conditions. Baseline pH$_i$ for the different cell preparations varied under resting conditions and this variation may be biological or depend on seasonality. For this reason, we investigated rates of pH$_i$ recovery following acidosis and Δ[H$^+$] between resting and recovery phases. Figure 2b, c summarize the results for experiments where the washout of NH$_3$/NH$_4^+$ was performed under low Na$^+$ (B) and low bicarbonate (C) conditions where pH$_i$ recovery in oyster mantle epithelial cells are significantly inhibited by modification of ASW in comparison to control experiments (ANOVA, $F = 18.17$, $p < 0.05$). Specifically, ASW treatments containing reduced Na$^+$ or pharmacological inhibitors of the NHE (EIPA) resulted in significantly lower rates of pH$_i$ recovery (Fig. 3, Tukey HSD, $p < 0.05$ respectively). To consider potential effects of DMSO, resting pH (prior to administration of inhibitors) between control and the two inhibitor experiments were examined and no significant differences were observed ($p > 0.05$, one-way ANOVA). Cell vitality following perfusion with these modified ASW solutions was confirmed by observing recovery to resting pH$_i$ upon addition of control ASW containing comparable concentrations of DMSO (Supplementary Fig. 2b). In addition, rates of pH$_i$ recovery were decreased when mantle epithelial cells were perfused with the carbonic anhydrase inhibitor, ACZM (Tukey HSD, $p < 0.05$). No significant effect on pH$_i$ recovery rates was observed when mantle epithelial cells were perfused in ASW containing low HCO$_3^-$ (Tukey HSD, $p > 0.05$). However, final pH$_i$ values following recovery period from the ammonium prepulse were significantly different in experiments where mantle epithelial cells were perfused with modified ASW solutions (ANOVA, $F = 6.46$, $p < 0.05$) where, experiments in the presence of low Na$^+$ (Tukey HSD, $p < 0.01$) and EIPA (Tukey HSD, $p < 0.01$) revealed significantly lower pH$_i$ at 60 min. Similarly, Δ[H$^+$] were found to be significantly different (Kruskal–Wallis, $X^2 = 24.01$, $p < 0.05$), where significant differences were found for the low HCO$_3^-$, low Na$^+$ and EIPA washouts (Dunn’s Test, $p < 0.05$). Recovery rates ([H$^+$]/minute) were $-3.67E-08 ± 7.82E-09$, $-4.1E-08 ± 4.33E-09$, $-3.98E-09 ± 1.75E-09$, $-1.22E-08 ± 2.53E-09$ and $-5.49E-09 ± 9.65E-10$ for ASW, low bicarbonate, low Na$^+$, EIPA and acetazolamide washouts respectively (Supplementary Fig. 4).

**Determination of buffer capacity ($\beta$)**

We estimated $\beta$ by perfusing mantle epithelial cells with 20 mM NH$_3$/NH$_4^+$ followed by control ASW and observed a mean $\beta$ value of 22.53 ± 1.26 mM/pH unit ($n = 11$ cells). Between experimental groups, within this short experimental period, calculated $\beta$ was not different (Table 1).

**Discussion**

Mollusc mantle epithelia have been long been studied to elucidate the biological control of calcification (Neff 1972; Joubert et al. 2010; Herlitze et al. 2018). It is crucial to understand the fundamental cellular acid–base regulatory pathways utilized by these cells to secrete calcified shells. The stable average pH$_i$ of approx. 6.8 recorded by in vivo fluorescence imaging is comparable to those reported in other molluscan cells, including C. gigas hemocytes (Beckmann 2011; Michaelidis et al. 2005). Ammonium prepulse
Fig. 2 Acid-base regulatory capacities of mantle epithelial cells in *Crassostrea gigas* under the presence of various ASW solutions prepared according to Supplementary Table 1 and pharmacological inhibitors of specific cellular ion transport proteins. pH\textsubscript{i} recordings in the presence of a ASW (control recovery) b low HCO\textsubscript{3}\textsuperscript{-} c low Na\textsuperscript{+} d 1 mM acetazolamide and e 20 µM EIPA. Values are presented as mean ± SEM for various replicates as described in Table 1. Blue and red lines indicate slope of alkalosis and acidosis compensation respectively (color figure online)
experiments demonstrate that oyster mantle epithelial cells have the capacity to recover from cellular acid load by a sodium dependent, EIPA-sensitive pathway. Additionally, we describe the buffer capacity (β) of calcifying mantle epithelial cells which may shed light on the ability of these cells to regulate pH when exposed to environmental hypercapnia and ongoing ocean acidification.

Using the ammonium prepulse technique, we observe a consistent cellular acidosis below control pH in *C. gigas* mantle epithelial cells, down to pH 6.54. This acidosis was followed by a recovery phase back to stable pH values around 6.96 (Table 1). Similar observations have been observed in other mollusc cell types including cardiac myocytes, oyster hemocytes and limpet oocytes (Ellington 1993; Vilain et al. 1993; Beckmann 2011). Our results demonstrate that the rates of pH recovery are significantly lower when cells are perfused with modified ASW solutions containing low Na⁺ or a pharmacological inhibitor, EIPA (Fig. 2, 3). The involvement of NHE proteins in pH regulation in mussel hemocytes and isolated mantle/gonad cells has been observed utilising pharmacological techniques (Kaloyianni et al. 2005; Banakou and Dailianis 2010). This group of ion-regulatory proteins has also previously been observed to exhibit upregulated mRNA expression in the mantle of the pearl oyster, *Pinctada fucata* during seawater acidification (Li et al. 2016). Within the genus *Crassostrea*, there are multiple genes encoding NHE transport proteins with similarity to cell membrane and intracellular (mitochondrial) isoforms in *Homo sapiens* and *Mus musculus*. However, an elementary molecular description for this group of antiporter proteins has been conducted in only one mollusc species, the giant clam, *Tridacna squamosa*, where NHE-3 isoforms have a predicted role in calcification (Hiong et al. 2017). This group of proteins has also been demonstrated to be crucial in the tolerance to seawater acidification in another mollusk, the squid, *Sepioteuthis lessoniana* (Hu et al. 2013, 2014). Taken together with the results from the present study, these observations indicate that NHE’s which are sensitive to EIPA are crucial to pH regulation in *C. gigas* mantle epithelial cells. Rates of pH recovery in *C. gigas* mantle epithelial cells are ca. 10 times slower than recovery rates observed in hemocytes within the same species (Beckmann 2011) but are comparable to recovery rates observed in cnidarian cells (Laurent et al. 2014), where NHE pathways have also been established to be important for pH regulation in *C. gigas* mantle epithelial cells. The compensation of induced alkalosis was very weak in oyster mantle epithelial cells in comparison to mammalian cells types (Bourgeois et al. 2018). This may be because these cells rarely experience such an extracellular alkalosis in the environment (seawater) or hemolymph and similar weak compensations to an ammonia induced alkalosis have also been observed in sea urchin larvae (Stumpp et al. 2012; Hu et al. 2018).
In addition to proton extrusion pathways mediated by NHEs and other proteins, cellular mechanisms of bicarbonate (HCO$_3^-$) transport can also play an important role in pH$_i$ homeostasis. Our experiments indicate that modified ASW containing low HCO$_3^-$ has no significant effect on the rates of pH$_i$ recovery and final pH$_i$ values following induced cellular acidosis. However, it has to be mentioned here that little is known regarding the bicarbonate affinity of bicarbonate transporters in molluscs. Typically, bicarbonate affinities of acid/base proteins in marine invertebrates are matched to their environment and/or extracellular fluids (Tresguerres 2014). In the present study, the low HCO$_3^-$ solution was measured to contain 158 μmol kg$^{-1}$ seawater HCO$_3^-$ and therefore, we cannot exclude the possibility of residual transport enabled by the minor fractions of HCO$_3^-$.

In contrast to the results obtained on the role of extracellular HCO$_3^-$ in C. gigas pH$_i$ regulation in mantle cells, significantly lower pH$_i$ recovery rates in the presence of the carbonic anhydrase inhibitor, acetazolamide were observed. The enzyme carbonic anhydrase facilitates the reversible hydration of CO$_2$ to HCO$_3^-$ and has long been suggested to be an important enzyme in mollusc shell forming tissue such as mantle epithelia (Medakovic 2000; Miyamoto et al. 2005; Yu et al. 2006; Aguilera et al. 2017). Seventeen genes encoding both extracellular and cytosolic isoforms of carbonic anhydrase proteins have been identified in the C. gigas genome (Zhang et al. 2012). Recent molecular cloning and characterisation of carbonic anhydrase II in C. gigas has revealed that this protein has highly conserved catalytic domains, is expressed in all tissues and its inhibition affects pH$_i$ homeostasis (Wang et al. 2017). Further, in oysters, this carbonic anhydrase isoform is localised to the outer epithelium of mantle tissue and is observed to exhibit significant mRNA upregulation in response to CO$_2$ exposure (Li et al. 2016; Wang et al. 2017).

Our results demonstrate that the activity of specific ion regulatory proteins such as NHEs and carbonic anhydrase are crucial for acid–base regulation. Interestingly, these proteins have also been associated with biomineralization (Medakovic et al. 2000; Zhang et al. 2012). Specifically, a suite of novel molecular studies lend support to the role of these carbonic anhydrases in acquisition of inorganic carbon during calcification (Wang et al. 2017; Koh et al. 2018; Chew et al. 2019). Additionally, it has been suggested that NHE proteins promote calcification by aiding in the removal of proton byproducts (Hiong et al. 2017; Cao-Pham et al. 2019). In bivalves, NHEs exhibit peaks in gene expression at the onset of larval calcification (Ramesh et al. 2019) and are also implicated in adult shell formation based on shell Na:Ca ratios (Zhao et al. 2017a, b). The concurrent activity of NHE and sodium/calcium exchange (NCX) proteins (Na$^+$-dependent elevation of calcium) in mollusc calcification has not been studied. However, apical NCX proteins are suggested to be involved in calcium transfer across the oyster mantle epithelia (Sillanpaa et al. 2018) and whether Na$^+$ exchange for calcium occurs following NHE mediated Na$^+$ entry requires validation.

Although the role of ion transport is pivotal in pH$_i$ homeostasis, cells may also minimize the effects of extracellular pH change through their cellular buffering capacity (β). The β of a specific cell type is related to the osmotic pressure, [HCO$_3^-$], glycolysis, sensitivity to pH and cation disturbances, the degree to which cells have the role of buffering the extracellular fluid and concentration of compounds containing histidine residues (Burton 1978; Abe 2000). In the present study, we used the NH$_3$/NH$_4^+$ prepulse technique to determine β and observed a mean value of 22.53 Slykes, which is in the range reported for other molluscan cell types such as snail neurons and whelk radula muscle (25 Slykes, Thomas 1974 and 30 Slykes, Wiseman and Ellington 1989). However, oyster hemocytes within the same species have been observed to exhibit distinctly lower β (8 Slykes, Beckmann 2011). The relatively high β in C. gigas mantle epithelial cells may be consistent with the necessity of these cells to protect themselves from acid load during calcification, where protons are generated as byproducts.

In the context of global environmental change, there is little information on plasticity of pH$_i$ regulation in these calcifying cells. One study that indirectly estimates pH$_i$ in hemocytes suggests that C. gigas elevates pH$_i$ upon CO$_2$ exposure (Wang et al. 2016). Further, in response to seawater acidification, oysters have demonstrated an increased metabolic demand for NHEs which has been associated with increased proton extrusion (Stapp et al. 2018). Simultaneously, seawater acidification has been linked to an increased elimination of metabolic CO$_2$ in oysters, a potential resilience mechanism (Stapp et al. 2018). However, without direct measurements, it is difficult to estimate the degree to which these organisms can respond to seawater pH reductions, particularly in the long term.

**Conclusion**

We describe pH$_i$ measurements in the mantle epithelial cells of C. gigas using an established microfluorimetric cell-imaging technique. The detected differences in pH$_i$ regulatory capacities are a first step in identifying the functional cellular pathways for acid–base homeostasis of these cells. The decreased capacities of mantle epithelial cells to recover from an induced cellular acidosis as a result of exposure to low Na$^+$ and all three pharmacological inhibitors indicate that Na$^+$-driven ion transport pathways and carbonic anhydrases are an important component of the pH$_i$ regulatory machinery in these cells. These findings are summarized in a first preliminary model of the pH$_i$ homeostasis machinery.
in mantle cells, highlighting the necessity for maintaining a Na\(^+\) gradient as driving force (NKA and NHE) and of CA in facilitating proton and bicarbonate generation (Supplementary Fig. 3). At present, several open questions remain regarding the physiology of molluscan calcification including the identification of bicarbonate transporters involved and the role of septate junctions in extracellular calcium transport.

Acknowledgements Open access funding provided by University of Gothenburg. This study is funded by the European Union’s Seventh Framework Programme [FP7] ITN project ‘CACHE’ under REA Grant agreement #605051. M. Hu is funded by the Emmy-Noether Program (403529967) of the German Research Foundation (DFG).

Author contributions KR and FM designed the study. FM and MB contributed to reagents and analytical tools. KR, MYH, NH conducted experiments. KR analysed the data and wrote the manuscript. All authors contributed to manuscript revisions.

Data availability Data can be accessed through PANGAEA database (https://doi.org/10.1594/PANGAEA.920870).

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Abe H (2000) Role of histidine-related compounds as intracellular proton buffering constituents in vertebrate muscle. Biochem Soc Trans 28:757–765

AgUILera F, McDougall C, Degnan BM (2017) Co-option and de novo gene evolution underlie molluscan shell diversity. Mol Biol Evol 34:779–792

Awaji M (1991) Separation of outer epithelial cells from pearl oyster mantle by dispase digestion. In: Fransen, M.J. Jr. (ed.) Proceedings of the 8th International Conference on Invertebrate and Fish Tissue Culture, June 16–20, 1991, Anaheim. Tissue Culture Association, Columbia, MD, pp 43–46

Banakou E, Diallamis S, (2010) Involvement of Na\(^+\)/H\(^+\) exchanger and respiratory burst enzymes NAPDH oxidase and NO synthase, in Cd-induced lipid peroxidation and DNA damage in haemocytes of mussels. Comp Biochem Physiol C: Toxicol Pharmacol 152:346–352

Beckmann MJ (2011) Modulation of intracellular pH of hemocytes from the pacific oyster Crassostrea gigas to changes in extracellular pH. Master thesis, Alfred Wegener Institute, Bremerhaven, Germany

Boron WF (1977) Intracellular pH transients in giant barnacle muscle fibers. Am J Physiol 233:C61–C73

Bourgeois S, Bounoure L, Mouro-Chanteloup I, Colin Y, Brown D, Wagner CA (2018) The ammonia transporter RhCG modulates urinary acidification by interacting with the vacuolar proton-ATPases in renal intercalated cells. Kidney Int 93:390–402

Burton RF (1978) Intracellular buffering. Resp. Physiol 33:51–58

Cao-Pham AH, Hiong KC, Boo MV, Choo CYL, Pang CZ, Wong WP, Neo ML, Chiew SF, Ip YK (2019) Molecular characterization of cellular localization and light-enhanced expression of Beta-Na\(^+\)/H\(^+\) Exchanger-like in the whitish inner mantle of the giant clam Tridacna squamosa denote its role in light-enhanced shell formation. Gene 695:101–112

Chiew SF, Koh CZY, Hiong KC, Choo CYL, Wong WP, Neo ML, Ip YK (2019) Light-enhanced expression of carbonic anhydrase 4-like supports shell formation in the fluted giant clam Tridacna squamosa. Gene 683:101–112

De Wit P, Durland E, Ventura A, Langdon C (2018) Gene expression correlated with delay in shell formation in larval Pacific oysters (Crassostrea gigas) exposed to experimental ocean acidification provides insights into shell formation mechanisms. Mol Ecol 19:160

Dwyer JJ, Burnett LE (1996) Acid-Base Status of the Oyster Crassostrea virginica in Response to Air Exposure and to Infections by Perkinsus marinus. Biol Bull 190:139–147

Ellington WR (1983) The extent of intracellular acidification during anoxia in catch muscle of two bivalve molluscs. J Exp Zool 227:313–317

Ellington WR (1993) Studies of intracellular pH regulation in cardiac myocytes from the marine bivalve mollusk, Mercenaria Campechiensis. Biol Bull 184:209–215

Gong N, Li Q, Huang J, Fang Z, Zhang G, Xie L, Zhang R (2008) Culture of outer epithelial cells from mantle tissue to study shell matrix protein secretion for biomineralization. Cell Tiss Res 333:493–501

Herlitze L, Marie B, Marin F, Jackson DJ (2018) Molecular modularity and asymmetry of the molluscan mantle revealed by a gene expression atlas. GigaScience 7:1–15

Hiong KC, Cao-Pham AH, Choo CYL, Boo MV, Wong WP, Chiew SF, Ip YK (2017) Light-dependent expression of a Na\(^+\)/H\(^+\) exchanger 3-like transporter in the ctenidium of the giant clam, Tridacna squamosa, can be related to increased H\(^+\) excretion during light-enhanced calcification. Physiol Rep 5:e13209

Hu MY, Lee JR, Lin LY, Shih TH, Stumpp M, Lee MF, Hwang PP, Tseng YC (2013) Development in a naturally acidified environment: Na\(^+\)/H\(^+\) -exchanger 3-based proton secretion leads to CO2 tolerance in cephalopod embryos. Front Zool 10:51

Hu MY, Guh YJ, Stumpp M, Lee JR, Chen RD, Sung PH, Chen YC, Hwang PP, Tseng YC (2014) Branchial NH\(^+\)4-dependent acid-base transport mechanisms and energy metabolism of squid (Sepioteuthis lessoniana) affected by seawater acidification. Front Zool 11:55

Hu MY, Yan JJ, Peterson I, Himmerkus N, Bleich M, Stumpp M (2018) SLC4 family bicarbonate transporter is critical for intracellular pH regulation and biomineralization in sea urchin embryos. eLife. https://doi.org/10.7554/eLife.36600

Ivanina AV, Falushynska HI, Beniash E, Piontkivska H, Sokolova IM (2017) Biomineralization-related specialization of hemocytes and mantle tissues of the Pacific oyster Crassostrea gigas. J Exp Biol 220:3209–3221

Joubert C, Piquemal D, Marie B, Manchon L, Pierrat F, Zanella-Cléon I, Cochennec-Laureau N, Gueguen Y, Montagnani C
(2010) Transcriptome and proteome analysis of *Pinctada margaritifera* califying mantle and shell: focus on biomineralization. BMC Genomics 11:613

Kaloyianni M, Stamiou R, Dailianis S (2005) Zinc and 17 β-estradiol induce modifications in Na+/H+ exchanger and pyruvate kinase, activity, through protein kinase C in isolated mantle/gonad cells of *Mytilus galloprovincialis*. Comp Biochem Physiol C-Tox Pharm 141:257–266

Koh CZY, Hiong KC, Choo CYL, Boo MV, Wong WP, Chew SF, Sillanpaa K, Sundh H, Sundell KS (2018) Calcium transfer across the Potts WTW (1958) The inorganic and amino acid composition of some *Miyamoto H, Miyoshi F, Kohno J* (2005) The carbonic anhydrase Michaelidis B, Haas D, Grieshaber MK (2005) Extracellular and intra-Stumpp M, Hu MY, Melzner F, Gutowska MA, Dorey N, Him- Stapp LS, Parker LM, O’Connor WA, Bock C, Ross PM, Pörtner HO, Zlatkin RL, Heuer RM (2019) Ocean acidification affects acid–base sensing. Biochim Biophys Acta 1842:2629–2635 Vilain JP, Rodeu JL, Gaillard S (1993) Fluorescent probe measurement of intracellular pH during meiosis reinitiation by ammonia in oocytes of the Mollusc, *Patella Vulgata*. Comp Biochem Physiol 104A:479–482 Wang X, Fan W, Xie L, Zhang R (2008) Molecular cloning and distribution of a plasma membrane calcium ATPase homolog from the pearl oyster *Pinctada fucata*. Tsinghua. Sci Technol 13:439–446 Wang X, Wang M, Jia Z, Wang H, Jiang S, Chen H, Wang L, Song L (2016) Ocean acidification stimulates alkali signal pathway: a bicarbonate sensing soluble adenylyl cyclase from oyster *Cras- sostrea gigas* mediates physiological changes induced by CO₂ exposure. Aquat Toxicol 181:124–135 Wang X, Wang M, Jia Z, Qiu L, Wang L, Zhang A, Song L (2017) A carbonic anhydrase serves as an important acid-base regulator in Pacific Oyster *Crasostrea gigas* Exposed to Elevated CO₂: implication for physiological responses of mollusk to ocean acidification. Mar Biotechnol 19:22–35 Wiseman RW, Ellington WR (1989) Intracellular buffering in molluscan muscle: superfused muscle versus homogenates. Physiol Zool 62:541–558 Yu Z, Xie L, Lee S, Zhang R (2006) A novel carbonic anhydrase from the mantle of the pearl oyster (*Pinctada fucata*). Comp. Biochem Physiol B 143:190–194 Zange J, Grieshaber MK, Jans AWH (1990) The regulation of intracel- lular pH estimated by P-NMR spectroscopy in the anterior byssus retractor muscle of *Mytilus edulis* L. J Exp Biol 150:95–109 Zeebe RE, Wolf-Gladrow DA (2001) CO₂ in seawater: equilibrium, kinetics, isotopes. In: Halpern D (ed) Elsevier oceanography series, vol 65. Elsevier, Amsterdam, p 346

Zhang G, Fang X, Guo X, Li L, Luo R, Xu F, Yang P, Zhang L, Wang X, QI H, Xiong Z, Que H, Xie Y, Holland PWH, Paps J, Zhu Y, Wu F, Chen Y, Wang J, Peng C, Meng J, Yang L, Liu J, Wen B, Zhang N, Huang Z, Zhu Q, Feng Y, Mount A, Hedgecock D, Xu Z, Liu Y, Domazet-Lošo T, Du Y, Sun X, Zhang S, Liu L, Cheng P, Jiang X, Li J, Fan D, Wang W, Fu W, Wang T, Wang B, Zhang J, Peng Z, Li Y, Li N, Wang J, Chen M, He Y, Tan F, Song X, Zheng Q, Huang R, Yang H, Du X, Chen L, Chen Y, M, Gaffney PM, Wang S, Luo L, Shi Z, Ming Y, Huang W, Zhang S, Huang B, Zhang X, Qu T, Ni P, Miao G, Wang J, Wang Q, Stehren CEW, Wang H, Li N, Qian L, Zhang G, Li Y, Yang H, Liu X, Wang J, Yin Y, Wang J (2012) The oyster genome reveals stress adaptation and complexity of shell formation. Nature 490:49–54 Zhao L, Schöne BR, Mertz-Kraus R, Yang F (2017a) Insights from sodium into the impacts of elevated pCO₂ and temperature on bivalve shell formation. J Exp Mar Biol Ecol 486:148–154 Zhao L, Schöne BR, Mertz-Kraus R, Yang F (2017b) Sodium provides unique insights into transgenerational effects of ocean acidifica- tion on bivalve shell formation. Sci Total Environ 577:360–366 Zlatkin RL, Heuer RM (2019) Ocean acidification affects acid–base physiology and behaviour in a model invertebrate, the Californ- nia sea hare (*Aplysia californica*). R Soc Open Sci. https://doi. org/10.1098/rsos.191041

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.