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Changes in metabolic rate and N excretion in the marine invertebrate *Sipunculus nudus* under conditions of environmental hypercapnia: identifying effective acid–base variables

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Summary

Increased CO_2 partial pressures (hypercapnia) as well as hypoxia are natural features of marine environments like the intertidal zone. Nevertheless little is known about the specific effects of CO_2 on metabolism, except for the well-described effects on acid–base variables and regulation. Accordingly, the sediment-dwelling worm *Sipunculus nudus* was used as an experimental model to investigate the correlation of acid–base-induced metabolic depression and protein/amino acid catabolism, by determining the rates of oxygen consumption, ammonia excretion and O/N ratios in non-perfused preparations of body wall musculature at various levels of extra- and intracellular pH, P_{CO_2} and $[HCO_3^-]$. A decrease in extracellular pH from control level (7.9) to 6.7 caused a reduction in aerobic metabolic rate of both normocapnic and hypercapnic tissues by 40–45 %. O/N ratios of 4.0–4.5 under control conditions indicate that amino acid catabolism meets the largest fraction of aerobic energy demand. A significant 10–15 % drop in ammonia excretion, a simultaneous reduction of O/N ratios and a transient accumulation of intracellular bicarbonate during transition to extreme acidosis suggest a reduction in net amino acid catabolism and a shift in the selection of amino acids used, favouring monoamino dicarboxylic acids and their amines (asparagine, glutamine, aspartic and glutamic acids). A drop in intracellular pH was identified as mediating this effect. In conclusion, the present data provide evidence for a regulatory role of intracellular pH in the selection of amino acids used by catabolism.

Key words: *Sipunculus nudus*, hypercapnia, acid–base variable, intracellular pH, amino acid catabolism.

Introduction

During low tide, animals living in marine estuaries or the intertidal zone have to cope with reduced gas exchange as a result of the restricted access to surface water. Accordingly, regular periods of environmental hypoxia combined with increasing CO_2 partial pressures (P_{CO_2}; hypercapnia) characterize life in these habitats (Diaz and Rosenburg, 1995). Much work has focused on organismal adaptations to hypoxia or anoxia (for a review, see Grieshaber et al., 1994); however, little is known about the specific effects of hypercapnia on marine animals and ecosystems. The development of strategies and techniques for an ocean disposal of CO_2 by several industrial countries also urges for a better understanding of the long-term effects of hypercapnia, especially with respect to changes in metabolism, growth and reproduction.

One of the most important adaptations during extended periods of combined hypoxia and hypercapnia seems to be the reduction of the animals' aerobic energy demand. A number of studies conducted on brine shrimp embryos (*Artemia franciscana*) and on land snails (*Otala lactea*) have shown that hypercapnia alone is suitable to elicit a depression of metabolic rate (Barnhart and McMahon, 1988; Barnhart, 1989; Rees and Hand, 1990). For brine shrimp embryos the effect of increased P_{CO_2} is interpreted as mainly mediated by an acidotic shift in intracellular pH (pHi). Pörtner et al. (1998) reported that exposure to environmental hypercapnia (1 % CO_2) causes respiratory acidosis in both intra- and extracellular compartments of the intertidal worm *Sipunculus nudus*. While pHi was completely restored within 48 h, extracellular pH (pHe) remained only partially compensated. *In vitro* experiments demonstrated that in *Sipunculus nudus* isolated body wall musculature a reduction in pHe rather than a moderate fall in pHi causes a depression of aerobic energy turnover (Reipschläger and Pörtner, 1996).

Pörtner et al. (2000) demonstrated that a decrease in the rate of acid–base regulation during extracellular acidosis contributed to metabolic depression. In addition, further mechanisms are probably involved in the reduction of energy turnover. At the whole-organism level, metabolic depression is supported by the downregulation of neuronal and motoric activity through hypercapnia-induced accumulation of the

neurotransmitter adenosine (Nilsson and Lutz, 1992; Reipschläger et al., 1997). An extreme reduction of energy demand, as in the brine shrimp embryos, also includes a depression of cellular protein synthesis (Hofmann and Hand, 1994; Van Breukelen et al., 2000).

The question arises whether protein or amino acid metabolism are also affected during moderate metabolic depression, as observed in *Sipunculus nudus*, and which variables might be relevant for triggering such a change. Earlier work showed a decrease in O/N ratios and an increase in net acid excretion, indicating enhanced protein or amino acid catabolism during the period of hypercapnia (Pörtner et al., 1998), which was possibly related to a shift in the use of substrate or a decrease in protein synthesis rate. The role of intra- *versus* extracellular acid–base status in this context is not clear.

Therefore, the aim of the present study was to determine the specific influence of intra- and extracellular pH, P_{CO_2} and [HCO_3^-] on ammonia excretion of isolated body wall musculature of *Sipunculus nudus* compared to changes in tissue metabolic rate. The use of isolated muscle tissue as a non-perfused viable preparation allowed us to control extracellular acid–base status and thereby clamp the steady-state intracellular acid–base variables. Tissue oxygen consumption, ammonia excretion rates and O/N ratios were analysed under both hypercapnic (P_{CO_2}=1.01 kPa) and normocapnic (P_{CO_2}=0.03 kPa) conditions and different pHe levels. As outlined previously, correlated changes in metabolic variables and acid–base status and the comparison of changes under hypercapnic and normocapnic conditions enable the identification of the effective acid–base variable that elicits the metabolic shift (Reipschläger and Pörtner, 1996). Focusing on the specific effects of gaseous CO_2, analyses were always performed under aerobic conditions.

Materials and methods

Animals

Large specimens (20–50 g) of *Sipunculus nudus* L. were dug up from sandy sediments of the intertidal zone in Locquémeau, Brittany, France, in October 1999. The animals were kept in aquaria with natural sea water and a bottom layer of sand (10–20 cm) from the original habitat for several weeks before the beginning of the experiments. The aquaria were supplied with aerated and recirculated sea water at 10–15 °C.

Experimental procedure

The experimental approach basically followed the rationale of Reipschläger and Pörtner (1996) and Pörtner et al. (2000). For the preparation of isolated body wall musculature, individuals were killed by ‘decapitating’ them behind the base of the introvert retractor muscles. The animals were opened dorsally and all inner organs including the ventral nerve cord were removed. The body wall musculature was cut transversally to obtain three more or less equal-sized pieces. Each piece was used for one experiment. The tissue was fixed with a fine needle and thread onto a plastic frame to ensure full equilibration with the ambient medium.

Each tissue preparation was first subjected to 15 h of normocapnic control conditions at pH 7.90. For each incubation the three tissue pieces of one animal were placed together in a closed recirculating system containing a volume of 8 l of 34‰ artificial sea water (455 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 24 mmol l^{-1} $MgCl_2.6H_2O$, 10 mmol l^{-1} $CaCl_2.6H_2O$, 28 mmol l^{-1} $MgSO_4.7H_2O$) with 0.1 g l^{-1} streptomycin, 10^5 i.u. l^{-1} penicillin and 20 mmol l^{-1} Hepes at a temperature of 15±0.5 °C. The medium was equilibrated and bubbled continuously with a hypoxic gas mixture of 40 % air, 60 % nitrogen supplied by a gas mixing pump (2M303/a-F, Wösthoff, Germany). Moderate hypoxia (P_{O_2}=8.47 kPa) was chosen since normoxic P_{O_2} levels can be damaging to this sediment-dwelling animal.

After 15 h of incubation, the control rate of oxygen consumption was determined for two of the three tissue segments in artificial sea water medium at pHe 7.90. Water samples were taken before and after the measurement for determination of the control rate of ammonia excretion. The third piece of tissue was freeze-clamped and stored in liquid nitrogen for further analyses of control levels of intracellular acid–base variables.

Subsequently, both remaining tissues were subjected to a second incubation period of 45 h under normocapnic (40 % air, 60 % nitrogen; P_{CO_2} 0.03 kPa) or hypercapnic (40 % air, 59 % nitrogen, 1 % CO_2; P_{CO_2} 1.01 kPa) conditions in media at one of various pH values. Normocapnic incubations were carried out at pH 7.90, 7.20 or 6.70 and hypercapnic incubations at pH 7.90, 7.55, 7.20 or 6.70. Values of 7.2 and 7.55 were chosen because they represent the plasma pHe of acute, uncompensated hypercapnia (1 % CO_2) and the steady-state pHe reached after compensation of hypercapnic acidosis, respectively (Pörtner et al., 1998). The lowest medium pH of 6.7 mimics the higher environmental P_{CO_2} conditions that prevent full compensation of tissue pHi *in vivo*. 1 % CO_2 was used as a high P_{CO_2} level experienced by the animals in their natural environment. Normocapnic as well as hypercapnic solutions were equilibrated with the corresponding gas mixture for several hours and the pH was then adjusted by the addition of solid $NaHCO_3$. Media bicarbonate levels used to set the respective pHe covered a broad range of concentrations reaching from 0.7 mmol l^{-1} to 27.0 mmol l^{-1}. They are referred to as extracellular bicarbonate concentrations in the following text. The appropriate amount was calculated from the Henderson–Hasselbach equation using a value of pK‴ determined according to Heisler (1986). Medium pH was checked at the beginning and at the end of the experiment to make sure that variations remained within ±0.03 pH units of the initial value.

After 45 h of incubation in the different media, rates of oxygen consumption and ammonia excretion were determined again. Water samples were removed before and after the period of P_{O_2} recording and finally tissues were freeze-clamped. Water and tissue samples were stored under liquid nitrogen for further analyses.

Analyses

Oxygen consumption rates were determined by closed-system respirometry in 40 ml respiration chambers equipped with polarographic oxygen sensors (Eschweiler, Germany). The chambers were filled with media identical to those that had been used for tissue equilibration. Oxygen consumption was recorded for 2–3 h. Tissue oxygen consumption rates were calculated after correction for the electrode drift and for the minor oxygen consumption of the medium caused by bacterial growth (approximately 10 % of experimental value). Measurements in chambers without tissue demonstrated that bacterial growth was effectively inhibited by the added antibiotics.

For the quantification of ammonia concentrations, water samples were taken from the respiration chambers before and after oxygen measurements and analysed enzymatically according to Bergmeyer (1984). Tissue excretion rates were calculated from the difference in ammonia concentrations between water samples. To determine tissue ammonia concentrations body wall musculature was ground under liquid nitrogen and extracted in ice-cold perchloric acid, as described by Beis and Newsholme (1975). Extracts were pH neutralized with 5 mol l^{-1} KOH and solid K_2CO_3/$KHCO_3$ (1:6 w/w).

O/N ratios were calculated from the amount of atomic oxygen consumed by the tissue and the amount of ammonia-N excreted during the same period, reflecting the contribution of protein or amino acid metabolism, respectively, to overall metabolic rate.

Intracellular acid–base variables were analysed using the homogenate technique (Pörtner et al., 1990). Tissue samples were ground under liquid nitrogen and the frozen powder (80–120 mg) was added to 0.2 ml of a solution containing KF (160 mmol l^{-1}) and nitrilotriacetic acid (1 mmol l^{-1}) in a 0.6 ml Eppendorf tube. The tube was filled to the top with the same solution, closed and mixed on a vortex mixer. After brief centrifugation (15 s, 20 000 ***g***, 4 °C), the supernatant was used for pHi measurement. Total CO_2 (C_{CO_2}) was analysed using a gas chromatograph (Hach Carle, USA) and apparent HCO_3^- concentrations were calculated as $[HCO_3^-]=C_{CO_2}-\alpha_{CO_2}\times P_{CO_2}$ using values of pK‴ and solubility coefficient α_{CO_2} determined according to Heisler (1986).

Statistics

For each treatment (hypercapnia or normocapnia), oxygen consumption rates, ammonia excretion rates and the resulting O/N ratios under control and experimental conditions were compared using two-factorial analysis of variance (ANOVA) or analysis of covariance (ANCOVA). Oxygen consumption and ammonia excretion rates are expressed as a percentage of the respective control values at pHe 7.90 and P_{CO_2} 0.03 kPa in order to faciliate comparisons between different experimental conditions. When a significant influence of a single variable was indicated by ANOVA/ANCOVA, the different treatments were compared using the Student–Newman–Keuls *post-hoc* test. In all cases, $P<0.05$ was accepted to indicate a significant difference. All values are calculated as means ± S.D., N=5–6.

Results

Steady-state intracellular acid–base variables and oxygen consumption rates

By controlling pHe and P_{CO_2} of the medium it was possible to clamp steady-state intracellular variables such as pHi, P_{CO_2} and $[HCO_3^-]$. Changes in tissue acid–base variables are summarized in Fig. 1A,B. As shown in Fig. 1A, pHi decreased with medium pH in a significant and linear way (ANCOVA; $F_{1/38}$=1090.931, $P<0.0001$). Interestingly, pHi values were always significantly higher in hypercapnic than in normocapnic samples. At the control pHe of 7.9, pHi was 7.42±0.03 during hypercapnia and 7.29±0.02 during normocapnia. In accordance with our previous study (Reipschläger and Pörtner, 1996) this difference in pHi values became smaller at lower pHe. Medium HCO_3^- concentrations, used to set the respective pHe at different P_{CO_2}, covered a broad range of concentrations (see Fig. 1B) with up to 100-fold higher concentrations of HCO_3^- in hypercapnic than in normocapnic solutions. The corresponding intracellular HCO_3^- concentrations changed in a similar way to pHi, with identical values in the lower range of pHe but with a considerable difference between normocapnia and hypercapnia at control pHe 7.90.

As shown previously, oxygen consumption rates of *Sipunculus nudus* isolated body wall musculature (Fig. 2A,B) were significantly influenced by decreasing pHe (ANCOVA; $F_{1/37}$=284.938, $P<0.0001$) and correlated with the subsequent lowering of pHi (ANCOVA; $F_{1/36}$=344.170, $P<0.0001$). Under normocapnia and at a low pHe of 7.20 (pHi=7.06±0.04), oxygen consumption was depressed to approximately 75 % of the control rate and fell further to 60 % at pHe 6.70 (pHi=6.91±0.03). At pHe 7.20 (pHi=7.18±0.01), hypercapnic tissues exhibited a similar reduction of 25 % below the control oxygen consumption rate, but an even greater depression to approximately 54 % of control metabolism was observed at the lowest experimental pHe of 6.70 (pHi=7.00±0.03). Comparison of normocapnic and hypercapnic treatments clearly demonstrated that hypercapnic samples consumed the same amount of oxygen as normocapnic ones at different pHi values but at the same pHe values (ANCOVA; $F_{1/37}$=1.001, P=0.323). For identical pHi values a significant difference in oxygen consumption between the two P_{CO_2} treatments results (ANCOVA; $F_{1/36}$=26.679, $P<0.0001$).

Changes in other acid–base variables, P_{CO_2} or extracellular $[HCO_3^-]$, were not consistently related to oxygen consumption and ammonia excretion (not shown) under all the experimental conditions analysed. As seen in Fig. 3, medium HCO_3^- concentrations differed considerably between both P_{CO_2} treatments at the same pHe, but nevertheless oxygen consumption rates were identical.

Dependence of ammonia excretion rates on experimental pH values

Ammonia excretion rates are expressed as a percentage fraction of the respective control values determined at pHe 7.90 and P_{CO_2} 0.03 kPa. Even under control conditions, values at the end of the experimental period never reached 100 % of

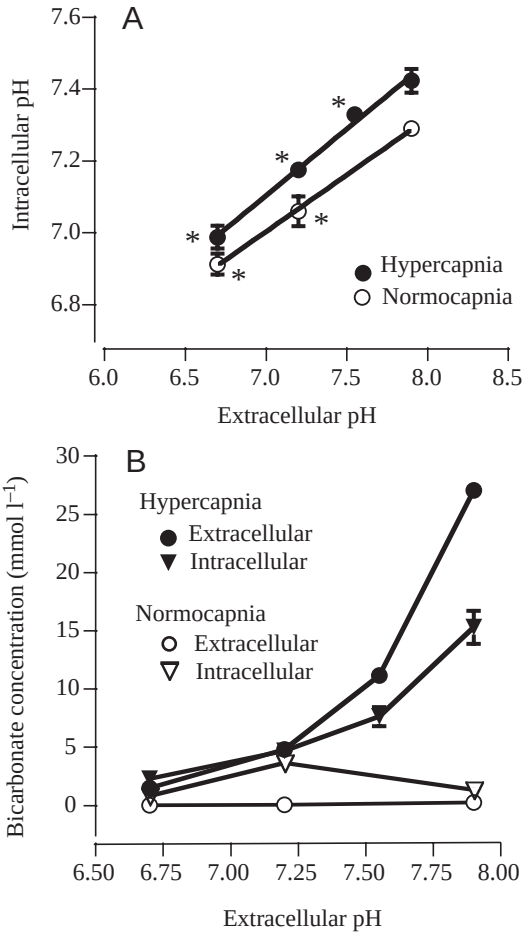

Fig. 1. (A) Steady-state intracellular pH values of *Sipunculus nudus* isolated body wall tissue under normo- and hypercapnia, measured after long-term exposure to media of different pH. *pHi significantly different from the control value at pHe 7.9. (B) Intracellular bicarbonate concentrations of isolated tissue exposed to media of different pH and bicarbonate concentrations. Values are means ± S.D., *N*=5–6, except for extracellular bicarbonate levels, which are media concentrations, used to set the specific pHe, and therefore given without S.D.

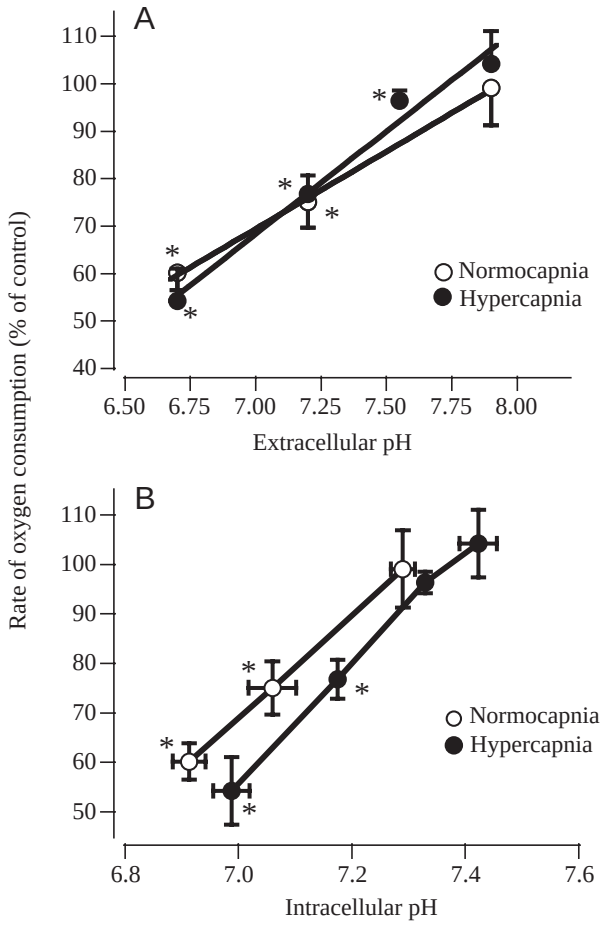

Fig. 2. Oxygen consumption rates of *Sipunculus nudus* isolated body wall musculature measured at different values of extracellular (A) and intracellular (B) pH during normocapnic or hypercapnic incubation. *Oxygen consumption rate significantly different from the respective control value at pHe 7.9. Values are means ± S.D., *N*=5–6.

the initial level, indicating that tissue samples showed a continuous decrease in metabolic rate possibly owing to progressive relaxation from long-term tonic contractions and the associated energetic costs (Fig. 4).

Fig. 4A depicts the relationship between ammonia excretion rate and pHe. In the high pHe range the rate of ammonia excretion of both normocapnic and hypercapnic tissues remained constant at approximately 90 % of the respective control values. Only below a threshold value of pHe 7.20, were rates significantly reduced by approximately 10–15 % (ANOVA; $F_{2/26}$=27.16, P<0.0001) independent of ambient P_{CO_2} (ANOVA; $F_{1/26}$=0.40, P=0.535). Note that the rate of ammonia excretion in both treatments was still at control levels at a low pHe of 7.20, when a depression of aerobic metabolic rate by 25 % had already occurred (see Fig. 2).

The correlation of ammonia excretion with pHi is similar (see Fig. 4B), except that the threshold value for the significant reduction in ammonia excretion (ANCOVA; $F_{1/34}$=19.37, P<0.0001) was found at a higher pHi in hypercapnic (reduction below pHi=7.15) compared to normocapnic tissue samples (reduction below pHi=7.05). A significant difference between normo- and hypercapnic treatments was evident only for the relationship of ammonia excretion rate and pHi (ANCOVA; $F_{1/34}$=4.33, P=0.0045), whereas the change of ammonia excretion rate with pHe was the same in normocapnic and hypercapnic treatments.

To make sure that the depression of the rates of ammonia excretion under conditions of low pHe was not caused by an increased accumulation of the protonated form NH_4^+ in acidotic cells, samples of body wall musculature were analysed for ammonium content. The results showed that tissue ammonia concentration even decreased significantly from 1.10±0.15 μmol NH_4^+ g^{-1} fresh mass at pHe 7.90 to 0.63±0.08 μmol NH_4^+ g^{-1} fresh mass at pHe 6.70, indicating that ammonia was not trapped intracellularly at low values of pHi.

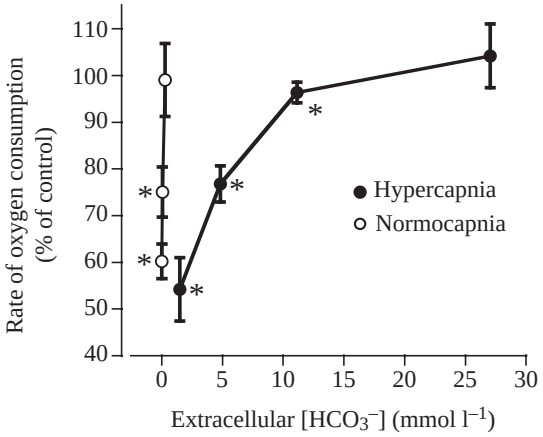

Fig. 3. Relationship between oxygen consumption rates of *Sipunculus nudus* isolated body wall musculature and extracellular bicarbonate concentrations ([HCO_3^-]). Bicarbonate levels in hypercapnic media cover a broad range of concentrations while normocapnic levels are all very low (0.27–0.015 mmol l^{-1} HCO_3^-). *Oxygen consumption rate significantly different from the respective control value at pHe 7.9. Values are means ± S.D., *N*=5–6.

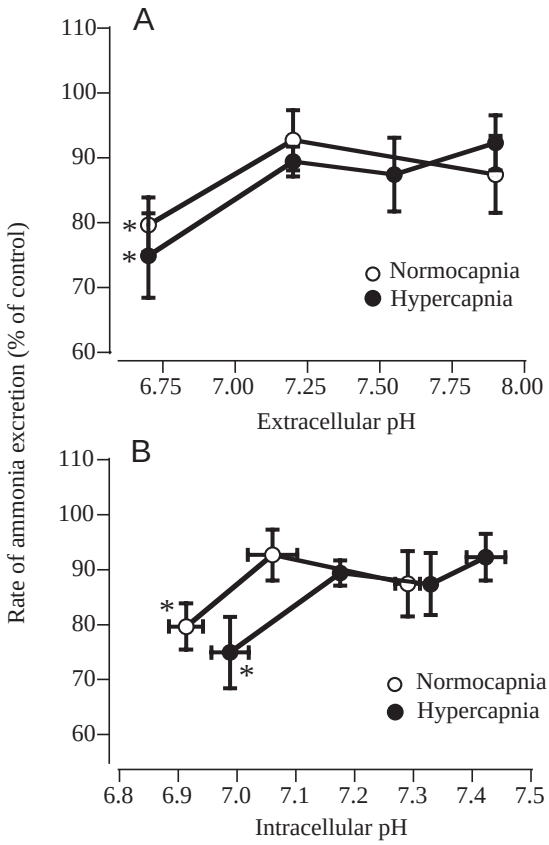

Fig. 4. The effect of changing extracellular (A) and intracellular (B) pH on ammonia excretion rates of *Sipunculus nudus* isolated body wall musculature. Tissue samples were analysed after long-term exposure to normocapnic or hypercapnic media of different pH. *Ammonia excretion rate significantly different from the respective control value at pHe 7.9. Values are means ± S.D., *N*=5–6.

O/N ratios

Fig. 5 shows the dependence of O/N ratios on pHe (Fig. 5A) and pHi (Fig. 5B). During hypercapnia, the ratios initially remained constant with decreasing pHe, whereas during normocapnia the O/N ratios fell progressively, starting at high pHe. A significant depression in both normocapnic and hypercapnic tissues (ANOVA; $F_{2/25}$=21.63, P<0.0001) to approximately 70–75 % of the respective control value was, however, observed at a low pHe value of 6.70. For the dependence of O/N ratios on pHi a threshold value occurred somewhere between pHi 7.20 and 7.30, below which O/N ratios of both CO_2 treatments started to decrease with falling pHi (ANCOVA; $F_{1/33}$=36.19, P<0.001).

The influence of intracellular as well as extracellular pH was found to be independent of ambient P_{CO_2} because no significant difference in O/N ratios was found between normocapnic and hypercapnic tissues at the same pHe (ANOVA; $F_{1/25}$=0.55, P=0.465) or pHi (ANCOVA; $F_{1/33}$=1.45, P=0.237), respectively. Both pHe and pHi seemed to have a significant influence on the pattern of change in O/N ratios, although pHe will influence cellular processes by modifying pHi (see Fig. 1A). For that reason we checked the correlation of pHe and O/N ratios after taking into account the dependence of pHi on pHe. O/N ratios determined at different pHe and the respective pHi values were normalized for the mean of all pHi data (pHi_{mean}=6.97) as if O/N ratios had been measured at constant pHi, and thus were solely dependent on pHe. In a subsequent analysis of the relationship between pHe and standardized O/N data, we were no longer able to find a significant influence of pHe (ANOVA; $F_{2/24}$=0.792, P=0.465).

Another aspect of interest is the relationship between intracellular [HCO_3^-] and O/N ratios (Fig. 6). Bicarbonate is involved since amino acid degradation results in the net production of bicarbonate and ammonium ions. Fig. 6 shows clearly that there is no overall correlation of intracellular [HCO_3^-] with O/N ratios, especially during normocapnia. Starting from high pHi values and a low value of 1.33±0.14 mmol l^{-1} intracellular [HCO_3^-], normocapnic intracellular [HCO_3^-] increased significantly with a reduction in O/N ratio. At O/N values below 3.5 a parallel decrease in intracellular [HCO_3^-] occurred under normo- and hypercapnic conditions.

Discussion

The present study examined the correlation of acid–base-induced metabolic depression and protein/amino acid catabolism in isolated body wall tissue of *Sipunculus nudus* by measuring rates of oxygen consumption, ammonia excretion and O/N ratios in relation to intra- and extracellular acid–base variables. Oxygen consumption rates clearly reflect a drastic reduction of aerobic metabolic rate, dependent on pHe, but without any influence of intra- or extracellular levels of P_{CO_2} and [HCO_3^-]. Tissue pHi values were clamped by extracellular acid–base conditions and samples of both CO_2 treatments showed significantly different oxygen consumption rates at

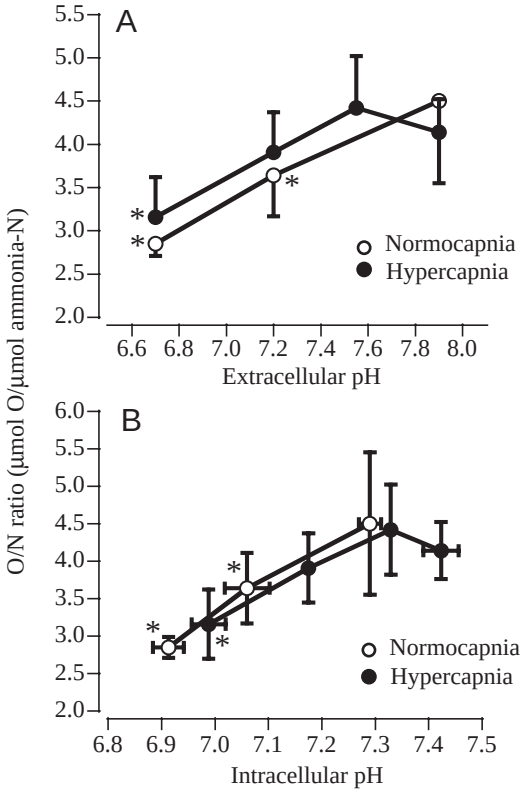

Fig. 5. O/N ratios of *Sipunculus nudus* body wall musculature, calculated from oxygen consumption and ammonia excretion rates under normo- and hypercapnia, *versus* different values of extracellular (A) and intracellular (B) pH. *O/N ratio significantly different from the respective control value at pHe 7.9. Values are means ± S.D., *N*=5–6.

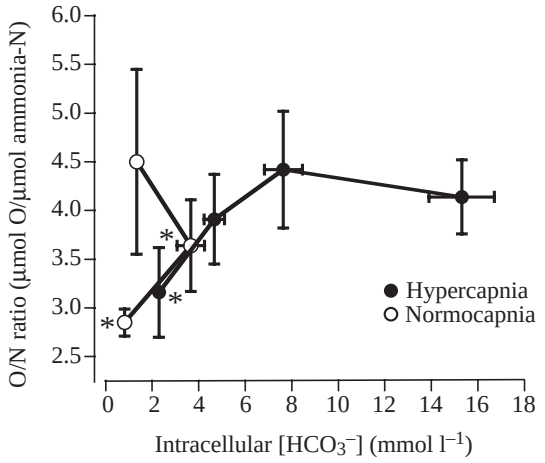

Fig. 6. Correlated changes in O/N ratios and intracellular bicarbonate concentrations determined in *Sipunculus nudus* isolated body wall tissue. Data were measured after long-term exposure to normocapnic and hypercapnic media of different pH and bicarbonate content. *O/N ratio significantly different from the respective control value at pHe 7.9. Values are means ± S.D., *N*=5–6.

identical pHi levels. At pHe 6.70, oxygen consumption decreased by up to 45 %, far beyond the moderate 20 % reduction of metabolic rate recently observed at pHe 7.20 (Reipschläger and Pörtner, 1996). Whereas the moderate depression was largely attributed to a decreased energy cost of cellular acid–base regulation (Pörtner et al., 2000), additional cellular mechanisms may become more prominent during extreme acidosis. One possible target could be the process of protein biosynthesis and turnover because up to 26 % of basal energy metabolism is used just to preserve the cellular protein pool (Hawkings, 1991). Under conditions of anoxia or starvation many organisms show a reduced incorporation rate of labeled amino acids into cellular protein (Hand and Hardewig, 1996). A reduction of protein synthesis, combined with an extension of protein half life under conditions of reduced energy turnover, has been observed in anoxic *Artemia franciscana* embryos (Anchordoguy et al., 1993). The present study did not examine the pH-dependence of protein synthesis, but analysed associated changes in ammonia excretion and O/N ratios. In this way, the effect of subtle changes in individual acid–base variables on overall N metabolism should become visible.

One has to keep in mind that ammonia excretion rates cannot be seen independently of the respective rates of oxygen consumption and energy turnover, which are reduced during metabolic depression and, subsequently, less protein or amino acids should be catabolized. Only at the lowest pHe tested (6.70), however, were ammonia excretion rates significantly depressed by approximately 10–15 % in both normocapnic and hypercapnic tissue samples. At the same time, oxygen consumption had fallen by 40–45 %. O/N ratios reflect the proportion of amino acids being used to fuel aerobic energy metabolism and were found to be approximately 4.0–4.5 in *Sipunculus nudus* isolated body wall tissue under control conditions. These values show that amino acid catabolism covers by far the largest fraction, if not all, of the energy demand in this invertebrate tissue (Cowey and Corner, 1963; Snow and Williams, 1971). O/N ratios reached even lower values during acidosis. At pHe 6.70 the reduction in ammonia excretion did not prevent a further decrease in O/N ratios to values of approximately 3.0, since oxygen consumption rates fell to a greater extent compared to ammonia excretion.

The lowest O/N ratios expected from catabolism of a mix of amino acids in proportion to their presence in the proteins are around 7.0, and indicate the sole use of protein (Cowey and Corner, 1963). An O/N ratio lower than this value is expected for the predominant oxidation of glycine (approximately 1.0). Oxidative metabolism of alanine or glutamic acid leads to O/N values of 7.0 and 3.0, respectively (Lehninger, 1975; Mayzaud and Conover, 1988). Therefore, a decreasing O/N ratio during acidosis suggests a shift in metabolic substrate. The values found do not suggest elevated overall amino acid catabolism but rather a shift in the selection of the different amino acids. A larger participation of asparagine (O/N=3.3), glutamic acid (O/N=3.0), glutamine (O/N=1.5), glycine (O/N=1.0) or histidine (O/N=3.0) in metabolic amino acid degradation would result in lower than control O/N ratios. All of these are non-essential amino acids that could easily be channelled into

the Krebs cycle *via* α-ketoglutarate, pyruvate or oxaloacetate. At the same time an enhanced oxidative decarboxylation of dicarboxylic acids (aspartic and glutamic acid) would yield a higher net production of HCO_3^-, supporting the compensation of pHi under acidotic conditions. However, the question of whether protein synthesis and turnover rates were lowered under these conditions remains open.

Besides the identification of potential changes in N metabolism, another aim of the study was to analyse the regulatory role of individual acid–base variables for the different physiological processes. With respect to oxygen consumption and ammonia excretion rates, the statistical evaluation clearly showed that both processes are influenced by pHe. Neither P_{CO_2} nor extracellular [HCO_3^-] exert a regulatory influence. In line with previous studies (Reipschläger and Pörtner, 1996; Pörtner et al., 2000), the pHe-dependent regulation of oxygen consumption rates seems to be partly mediated by an inhibition of net proton transport across the cell membrane during acidosis. The resulting decrease in the overall rate of acid–base regulation and a shift to more energy-efficient transport (see below) lowers the cost of this cellular process and contributes to an energy-saving strategy during metabolic depression.

With respect to the effect of acid–base status on O/N ratios, no clear picture emerged from the statistical evaluation of the present data, for both pHe and pHi showed a significant influence. However, when the correlation of intra- and extracellular pH was considered from the calculation of pHi-normalized O/N data, no specific effect of extracellular pH on O/N ratios could be observed. Therefore, changes in N metabolism seem to be determined predominantly by intracellular pH, which in itself is influenced by pHe.

A decrease in pHi may also not directly affect N metabolism, which led us to investigate any potential effect of intracellular bicarbonate. If we exclude a further influence of pHe-inhibited ion exchange on metabolic rate in the low range of pHe and pHi, changes in intracellular [HCO_3^-], ammonia excretion and O/N ratios are consistently correlated. The transient but significant accumulation of intracellular HCO_3^- with a decrease in pHe from 7.90 to 7.20 (see Fig. 6, normocapnic data) cannot be explained by a shift of pHi regulation from the Na^+/H^+ antiporter to Na^+-dependent Cl^-/HCO_3^- exchange. Although investigations by Pörtner et al. (2000) indicate a predominant use of this more ATP-efficient transport system under conditions of acidosis, the exchange rates of all membrane proteins participating in pHi homeostasis were downregulated below control rates in acidotic tissue, leaving no room for any accumulation of bicarbonate. It seems very likely that the changes in intracellular [HCO_3^-], clearly visible in the normocapnic data of Fig. 6, have to be viewed in close connection with N metabolism. In hypercapnic tissue samples, such findings are hidden by high ambient HCO_3^- concentrations at high pHe, which lead to a strong passive influx of HCO_3^- and elevated pHi levels (see Fig. 1). On the one hand, bicarbonate, resulting from the decarboxylation of the amino acid α-carboxylic group, represents an end product of protein degradation; on the other hand, it plays an important role in the buffering of intracellular pH. An augmented breakdown of dicarboxylic amino acids (e.g. glutamic acid and, after deamination, asparagine or glutamine), thought to be favoured under conditions of acidosis, may explain the higher intracellular [HCO_3^-] in normocapnic tissue at pHe 7.20. As the medium pH was further reduced to pHe 6.70, overall amino acid catabolism fell owing to decreased energy demand, causing the normocapnic intracellular [HCO_3^-] to fall below the respective control value (see Fig. 6). A full compensation of pHi, as reported by Pörtner et al. (1998) for the whole animal after 48 h of incubation at 1 % CO_2, would have been supported by metabolic bicarbonate production. The mechanism that triggers the disproportional use of dicarboxylic amino acids and their amines remains unclear; however, it probably involves the decrease in pHi. This leaves the accumulation of intracellular bicarbonate as a dependent process which does not exert a regulatory role.

In summary, the present data provide evidence for an influence of intracellular acid–base variables on N metabolism, specifically on the selection of amino acids used by catabolism. This effect is correlated with a decrease in pHi. The shift in N metabolism, leading to a decrease in O/N ratios despite lowered ammonia excretion rates, is evident under conditions of extreme acidosis. This indicates that under pronounced metabolic depression catabolism prefers low O/N amino acids like asparagine, glutamine or their dicarboxylic acids. These changes cause a release of bicarbonate and, thus, support the regulation of pHi.

It remains to be investigated whether, during long-term exposure to high levels of P_{CO_2}, this metabolic shift may damage the cellular protein pool and also influence growth and reproduction. As a working hypothesis to be examined in further studies we propose a downregulation of protein biosynthesis at low levels of pHi. Free amino acids originating from ongoing protein degradation would not be incorporated in the cellular protein pool, but rather would be diverted into catabolic energy metabolism.

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