A role for adenosine in metabolic depression in the marine invertebrate *Sipunculus nudus*

ANKE REIPSCHLÄGER, GÖRAN E. NILSSON, AND HANS O. PÖRTNER
Alfred-Wegener-Institut für Polar- und Meeresforschung, Biologie I/Ökophysiologie, D-27515 Bremerhaven, Germany; Vertebrate Physiology and Behavior Unit, Department of Limnology, Uppsala University, S-75236 Uppsala, Sweden; and Division of General Physiology, University of Oslo, N-0316 Oslo, Norway

Reipschläger, Anke, Göran E. Nilsson, and Hans O. Pörtner. A role for adenosine in metabolic depression in the marine invertebrate *Sipunculus nudus*. *Am. J. Physiol.* 272 (*Regulatory Integrative Comp. Physiol.* 41): R350–R356, 1997.—Involvement of neurotransmitters in metabolic depression under hypoxia and hypercapnia was examined in *Sipunculus nudus*. Concentration changes of several putative neurotransmitters in nervous tissue during anoxic or hypercapnic exposure or during combined anoxia and hypercapnia were determined. Among amino acids (γ-aminobutyric acid, glutamate, glycine, taurine, serine, and aspartate) and monoamines (serotonin, dopamine, and norepinephrine), some changes were significant, but none were consistent with metabolic depression under all experimental conditions applied. Only the neuromodulator adenosine displayed concentration changes in accordance with metabolic depression under all experimental conditions. Levels increased during anoxia, during hypercapnia, and to an even greater extent during anoxic hypercapnia. Adenosine infusions into coelomic fluid via an indwelling catheter induced a significant depression of the normocapnic rate of O_2 consumption from 0.36 ± 0.04 to a minimum of 0.24 ± 0.02 (SE) $\mu mol \cdot g^{-1} \cdot h^{-1}$ after 90 min ($n = 6$). Application of the adenosine antagonist theophylline caused a transient rise in O_2 consumption 30 min after infusion during hypercapnia but not during normocapnia. Effects of adenosine and theophylline were observed in intact individuals but not in isolated body wall musculature. The results provide evidence for a role of adenosine in inducing metabolic depression in *S. nudus*, probably through the established effects of decreasing neuronal excitability and neurotransmitter release. In consideration of our previous finding that metabolic depression in isolated body wall musculature was elicited by extracellular acidosis, it is concluded that central and cellular mechanisms combine to contribute to the overall reduction in metabolic rate in *S. nudus*.

monoamine transmitters; amino acid transmitters; anoxia; hypercapnia

ANOXIA TOLERANCE, present in a number of invertebrate and lower vertebrate species, is closely linked to the ability to reduce metabolic rate (4, 8, 31). ATP-consuming processes like protein synthesis, ion pumping, and locomotor activity have been shown to be depressed during anoxia (2, 10, 19). However, little is known about the mechanisms that mediate a downregulation of energy consumption, especially in invertebrates.

Brain neurotransmitter levels exhibit changes during anoxia in several lower vertebrates and are considered to play a key role in anoxic survival. The concentrations of glycine and γ-aminobutyric acid (GABA), both being inhibitory, increased, whereas the content of glutamate (an excitatory transmitter) decreased in the brain of anoxia-tolerant species like the crucian carp (*Carassius carassius*), the freshwater turtle (*Pseudemys scripta elegans*), and the loggerhead sea turtle (*Caretta caretta*) (13, 16, 18). Another substance of interest is adenosine, a product of the breakdown of high-energy purines like ATP. Extracellular adenosine levels increased in the brain striatum of the freshwater turtle during the early phase of anoxia (17), and for crucian carp pharmacological evidence has been given for a role of adenosine in metabolic depression (14). Adenosine is also known to exhibit neuroprotective effects during ischemia in the anoxia-intolerant mammalian brain (28). By decreasing neuronal excitability (postsynaptic inhibition) as well as neurotransmitter release (presynaptic inhibition) (25, 30), adenosine tends to reduce the energy demand of the mammalian brain and raises the threshold for the initiation of pathophysiological processes caused by ischemia.

Although invertebrates comprise the major number of anoxia-tolerant species, they have, with one exception (20), never been examined for neurotransmitter changes during anoxia. The aim of the present study was to investigate the role of neurotransmitters in the regulation of metabolic rate in *Sipunculus nudus*. *S. nudus* depresses its metabolic rate by ~70% during anoxia (7) and by 20–35% during hypercapnia (H. O. Pörtner, A. Reipschläger, and N. Heisler, unpublished observations). We examined the concentrations of various putative neurotransmitters in the nervous tissue after exposure of the animal to anoxia, hypercapnia, or anoxic hypercapnia. Adenosine levels are shown to increase under all three conditions. A possible role of adenosine in mediating metabolic depression was then tested by agonist and antagonist infusion experiments.

MATERIALS AND METHODS

Animals. Specimens of *S. nudus* were dug up from sandy sediments of the intertidal zone in Locquémeau and Morgat, Brittany, France, and were kept in aquaria with aerated artificial seawater and a bottom layer of sand (15–25 cm) at 11–15°C for up to several weeks. For the incubation experiments with subsequent nervous tissue sampling, animals collected in February 1994 were used. Determinations of oxygen consumption rates were carried out with animals collected in April 1995.

Experimental procedure. Individuals [40.5 ± 11.4 (SD) g body wt] were transferred to darkened 20-liter containers (8 individuals at a time) filled with seawater that was preequilibrated and continuously bubbled with different gas mixtures supplied by a gas-mixing pump (model 2M303/a-F, Wösthoff) at 15°C. The gas mixtures were 40% air-60% nitrogen (con-

trol), 100% nitrogen (anoxia), 40% air-59% nitrogen-1% CO_2 (hypercapnia), and 99% nitrogen-1% CO_2 (anoxic hypercapnia). Gas mixtures containing oxygen below air saturation were used, because P_{O_2} levels as high as those in air proved to be damaging to this sediment-dwelling animal. After 24 h the animals were opened dorsally, and the brain ganglion and ventral nerve cord were removed within <1 min and immediately frozen and stored in liquid nitrogen. The frozen nervous tissue was weighed and sonicated in 4% (wt/vol) ice-cold perchloric acid (PCA) containing 0.2% EDTA and 0.05% sodium bisulfite with an MSE 100-W ultrasonic disintegrator. Brain ganglia and ventral nerve cords weighed 32.4 ± 9.8 (SD) mg (n = 32). After sonication the homogenates [~10% (wt/vol) tissue] were centrifuged at 15,000 g for 5 min at 4°C. Supernatants were stored for ≤8 days at −80°C and, for GABA analysis, in liquid nitrogen for several weeks.

Analyses. Amino acids (for GABA see below) were quantified by reverse-phase high-performance liquid chromatography (HPLC) with fluorescence detection after derivation with *o*-phthaldialdehyde (OPA). A C_{18} Nucleosil 120 column, 4.6 × 125 mm and 3 μm diameter (Macherey-Nagel, Düren, Germany), was used. Excitation was set at 330 nm, and emission was measured at 450 nm. The solvent system and gradient program were described by Tossman (32). *Buffer A* contained 90 mmol/l sodium acetate, 10% methanol, and 2.5% tetrahydrofuran, pH 6.95. *Buffer B* consisted of 90% methanol. The following gradient was run (in percentage of *buffer A*): 100% between 0 and 2.4 min, 90% at 9.6 min, 75% at 15.6 min, 60% at 17.6 min, 40% at 21.6 min, 0% between 24 and 26.4 min, and finally back to 100% at 31.2 min. The flow rate was 1 ml/min. Ten microliters of the supernatant obtained after PCA extraction and centrifugation were mixed with 190 μl of the complete OPA reagent (P0532, Sigma Chemical). After exactly 1 min, 20 μl of the mixture were injected into the sample loop, and the run was started at the same time. Concentrations were calculated by comparison with standards prepared in the same PCA solution as the extracts.

GABA could not be measured in the tissue extracts by this method, because taurine and GABA eluted together (which did not interfere with taurine quantification, because peak height was measured and the crest of the taurine peak had passed before the retention time of GABA was reached). An isocratic method (60 mmol/l K_2HPO_4, 30 mmol/l sodium citrate, 0.6 mmol/l EDTA, 35% methanol, pH 6.3) run on the same column, also in combination with OPA derivation, did not result in a better separation. For this reason, GABA was determined with a different HPLC method. Samples were neutralized with 5 M KOH and derivated with dabsyl chloride [4′-(dimethylamino)~azobenzene-4′-sulfonylchloride] by use of a dabsylation kit (Beckman, San Ramon, CA). Absorbance of the derivatives was measured with a spectrophotometric detector at 436 nm. A C_{18} reverse-phase column (Ultrasphere ODS-DABS, 4.6 × 250 mm, 5 μm diameter, Beckman), thermostated to 30°C, was used. *Buffer A* consisted of 14 mmol/l sodium citrate and 4% dimethylformamide, pH 6.5, and *buffer B* contained 30% *buffer A* and 70% acetonitrile with 4% dimethylformamide. The gradient was as follows (in percentage of *buffer A*): 75% between 0 and 0.5 min, 63% at 3 min and between 3 and 8 min, 58% at 24 min, 0% at 26 min and between 26 and 31 min, and finally back to 75% at 31.5 min. The flow rate was 1.4 ml/min. GABA was eluted before taurine, and the peaks were almost baseline separated.

The monoamines dopamine, serotonin, and norepinephrine were quantified by HPLC with electrochemical detection. An LC-3 electrochemical detector with a glassy carbon working electrode set at +750 mV vs. an Ag-AgCl reference electrode (all from Bioanalytical Systems, West Lafayette, IN) and a reverse-phase column (C_{18} Nucleosil 120, 4.6 × 75 mm, 3 μm diameter, Macherey-Nagel, Düren, Germany), thermostated to 40°C, were used. The mobile phase consisted of 100 mmol/l NaH_2PO_4, 0.5 mmol/l EDTA, 30 mg/l sodium octylsulfate, and 6% methanol, pH 3.7, and the flow rate was 1 ml/min. Standards were dissolved in H_2O and diluted 1:1,000 with 4% (wt/vol) PCA containing 0.2% EDTA and 0.05% sodium bisulfite.

Adenosine was determined by a method described by Hagberg et al. (5) using HPLC (isocratic) with spectrophotometric detection. The supernatants obtained after PCA extraction were neutralized with 25% (vol/vol) 0.85 M K_2CO_3. A reverse-phase column (C_{18} Nucleosil 120, 4.6 × 75 mm, 3 μm diameter, Macherey-Nagel) was used. The buffer consisted of 10 mmol/l NaH_2PO_4, 0.25 mmol/l EDTA, and 6% methanol, pH 6.5. The flow rate was 1 ml/min, and concentrations were calculated by comparison with standards prepared in the same neutralized PCA solution as the extracts.

Determination of oxygen consumption rates. Animals weighing 5.47 ± 0.70 (SD) g were catheterized by introduction of a 1-cm length of polyethylene tubing (0.86 mm ID, 1.27 mm OD, 35 cm long) filled with seawater into the body cavity after the posterior end of the body was punctured. The tubing was secured with cyanoacrylate glue. The animals were allowed to acclimate for 24 h in a darkened container filled with seawater that was bubbled continuously with a gas mixture containing 50% air-50% nitrogen (supplied by a gas-mixing pump, model 2M303/a-F, Wösthoff) at 15°C. Then one animal at a time was gently placed in a 100-ml respiration chamber filled with seawater that had been equilibrated with the same gas mixture. The end of the catheter was fed through a small hole (isolated by Tygon tubing filled with seawater) in the respiration chamber and was connected to a three-way valve. The respiration chamber was located in a darkened box filled with tap water thermostated to 15°C. Oxygen consumption rates were recorded by closed-system respirometry with a polarogaphic oxygen sensor (Eschweiler, Kiel, Germany) inserted into the lid of the chamber. The water was continuously mixed by a small stirrer separated from the animal by a 1-cm plastic wall. The first 30–40 min of the measurement period were neglected. The changes in P_{O_2} during the following 30–40 min were recorded as reflecting the control rate of oxygen consumption before infusion. Subsequently, 300 μl (the dead space volume) were withdrawn slowly from the indwelling catheter by means of a 1-ml syringe. With a second syringe, 300 μl of coelomic fluid were withdrawn. Then animals were infused with 10 μl/g body wt of saline containing 1.5 mmol/l adenosine or with pure saline; 300 μl of coelomic fluid were reinfused, and the catheter was flushed with 300 μl of seawater. The oxygen consumption rate was determined for another 120 min. The animal was returned to the acclimation chamber, and oxygen consumption was measured again 16 h after infusion. The rate of P_{O_2} decrease 20 min before and 30 min, 90 min, and 16 h after infusion was used to calculate oxygen consumption rates. Values were corrected for a small fraction of bacterial respiration (<5%). During the measurement period up to 90 min after infusion, P_{O_2} in the respiration chamber decreased from 10.59 to 6.49 ± 0.45 (SD) kPa. The critical P_{O_2} of small specimens of *S. nudus* is 4.0 kPa (23). It has previously been shown (23) that, in the applied range of P_{O_2}, oxygen consumption remains close to constant with decreasing P_{O_2}, a finding that is confirmed by our measurements.

The effect of theophylline infusion on oxygen consumption rates was examined in a similar experimental setup. One group of animals was acclimated for 24 h to seawater that was bubbled continuously with 50% air-50% nitrogen; the other

group was acclimated to seawater preequilibrated and bubbled with 50% air-49% nitrogen-1% CO_2. Oxygen consumption was measured in seawater equilibrated with the same gas mixtures. Animals weighed 4.94 ± 1.21 (SD) g. Theophylline (10 mg/ml) was dissolved in saline to which 5 µl/ml of 3 mol/l NaOH was added. The final pH was 8.2. Theophylline was infused using a volume of 10 µl/g body wt, resulting in a dose of 100 mg/kg. During the oxygen consumption measurements, P_{O_2} decreased from 10.59 to 5.38 ± 0.71 kPa.

For the determination of oxygen consumption rates of isolated body wall musculature before and after adenosine or theophylline application, individuals were killed by "decapitation" behind the base of the introvert retractor muscles. During this step the brain ganglion was removed. The animals were opened dorsally, and all inner organs including the ventral nerve cord were removed using tissue paper. The remaining body wall musculature weighed 1.98 ± 0.23 (SD) g. Each piece of body wall musculature was punctured at the edges with a fine needle and fixed with thread to a plastic frame. The tissue preparations were incubated for 24 h in saline (concentrations in mol/l: 0.455 NaCl, 0.010 KCl, 0.024 $MgCl_2$, 0.028 $MgSO_4$, 0.010 $CaCl_2$, 0.002 $NaHCO_3$) with 0.1 g/l streptomycin, 10^5 U/l penicillin, and 0.01 mol/l tetraethylammonium·HCl, pH 7.9, at 15°C. The saline was preequilibrated and bubbled continuously with 50% air-50% nitrogen (normocapnic group) or 50% air-49% nitrogen-1% CO_2 (hypercapnic group). After 24 h, each piece of tissue was transferred to a 100-ml respiration chamber filled with the same saline, and oxygen consumption was determined as described above for ~60 min. Then 1.5 ml of 2 mmol/l adenosine (dissolved in saline) were added to the saline of the normocapnic group (final concentration 30 µmol/l), and 2 ml of 10 mg/ml theophylline were added to the saline of the hypercapnic group (final concentration 200 mg/l). The oxygen consumption of the tissue was recorded for another 120 min. In previous experiments we demonstrated that isolated body wall musculature of *S. nudus* remained viable for ≥36 h on the basis of constant oxygen consumption rates and ATP levels.

Statistics. Changes in oxygen consumption rates over time were tested for significance using one-way analysis of variance (ANOVA) for paired samples. When significant differences were indicated by the ANOVA ($P \leq 0.05$), each point in time was compared with the control value using Student-Newman-Keuls post hoc test. Neurotransmitter concentrations in the nervous tissue were analyzed using the nonparametric Kruskal-Wallis ANOVA followed by the nonparametric Mann-Whitney test (InStat GraphPAD Software). Unless otherwise stated, values are means ± SE.

RESULTS

Concentrations of putative neurotransmitters in the nervous tissue. Figure 1 shows the concentrations of the amino acids taurine, glycine, aspartate, glutamate, serine, and GABA determined in the brain ganglion and ventral nerve cord after exposure of the animals to anoxia, hypercapnia, or anoxic hypercapnia for 24 h. Significant differences between treatments were observed for aspartate, glutamate, and serine levels. Aspartate decreased from 26.6 ± 1.2 (control) to 15.1 ± 1.5 µmol/g nervous tissue during anoxia and to 16.0 ± 1.0 µmol/g nervous tissue during anoxic hypercapnia. Glutamate concentrations increased during anoxia and anoxic hypercapnia from 8.6 ± 0.3 (control) to 10.3 ± 0.4 and 10.6 ± 0.4 µmol/g nervous tissue, respectively. Serine concentrations increased from 1.2 ± 0.1 µmol/g nervous tissue under control conditions to 2.0 ± 0.2 µmol/g nervous tissue during hypercapnia.

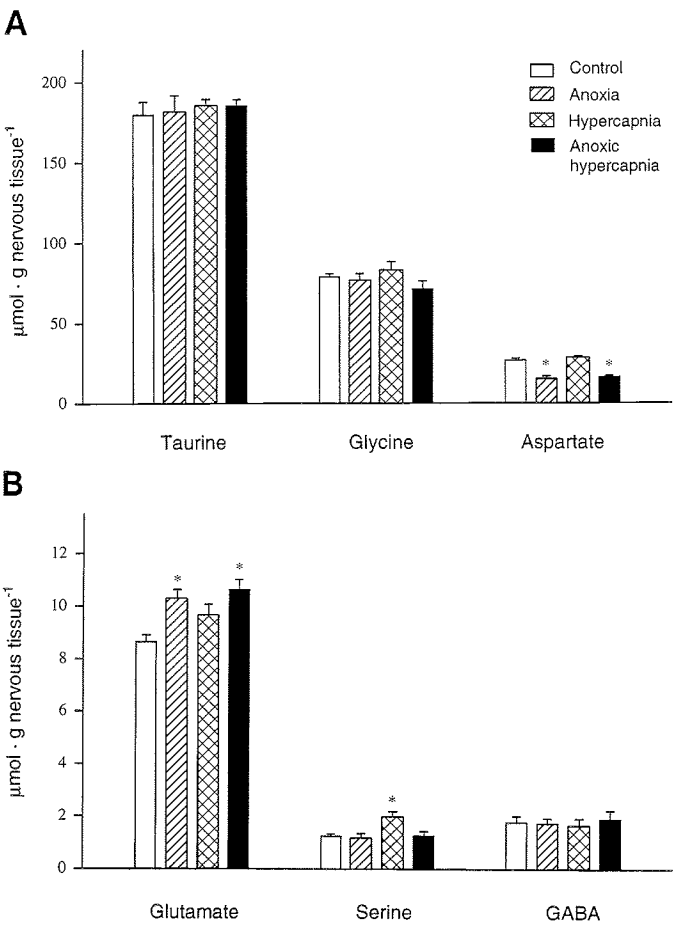

Fig. 1. Taurine, glycine, and aspartate concentrations (*A*) and glutamate, serine, and GABA levels (*B*) in nervous tissue after exposure of *Sipunculus nudus* to anoxia, hypercapnia, or anoxic hypercapnia for 24 h. Values are means ± SE (n = 8; for GABA, n = 5). * Significantly different from respective control value.

The concentrations of the monoamines dopamine, serotonin, and norepinephrine are shown in Fig. 2*A*. Dopamine and serotonin levels in the nervous tissue were not affected by exposure of the animals to anoxia, hypercapnia, or anoxic hypercapnia. In contrast, the norepinephrine content decreased significantly during anoxia and hypercapnia, but not during anoxic hypercapnia.

Adenosine concentrations (Fig. 2*B*) in the nervous tissue were affected by all three treatments. The control level of adenosine was 1.9 ± 1.1 nmol/g nervous tissue. During anoxia, adenosine increased to 5.7 ± 0.9 and during hypercapnia to 5.6 ± 2.0 nmol/g nervous tissue. An even higher level of 8.4 ± 1.9 nmol/g nervous tissue was reached during anoxic hypercapnia.

Effects of adenosine, theophylline, and saline infusion on oxygen consumption rates. The oxygen consumption rate of *S. nudus* under control conditions was significantly affected by adenosine infusion into the coelomic fluid to a final concentration of 15 nmol/g body wt (Fig. 3*A*). Oxygen consumption declined from 0.36 ± 0.04 µmol·

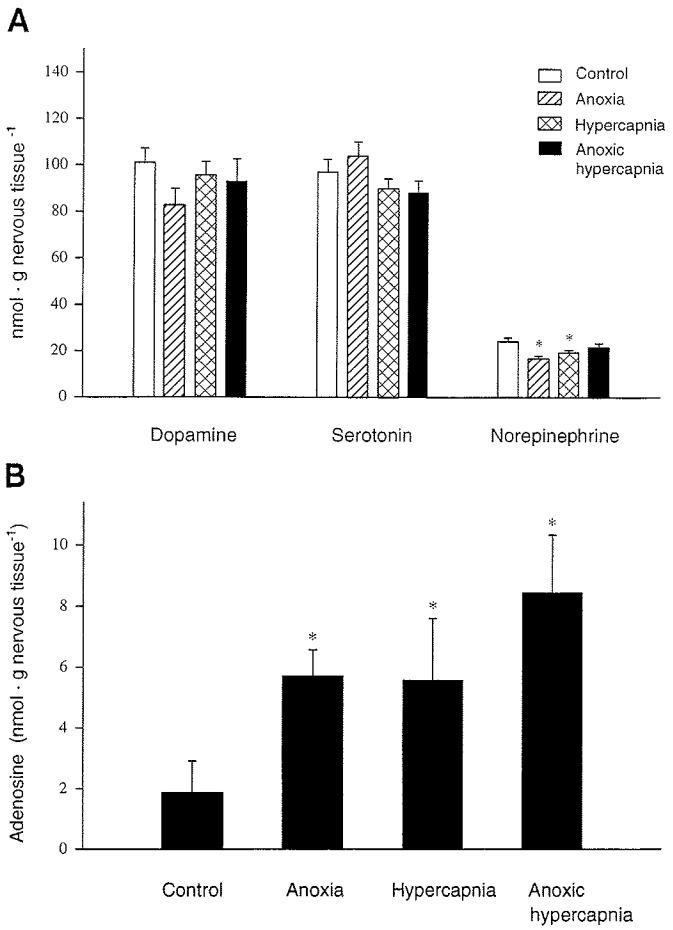

Fig. 2. Monoamine concentrations (*A*) and adenosine levels (*B*) in nervous tissue after exposure of *S. nudus* to 24 h of anoxia, hypercapnia, or anoxic hypercapnia. Values are means ± SE (n = 8). *Significantly different from respective control value.

$g^{-1} \cdot h^{-1}$ before infusion to 0.27 ± 0.02 and 0.24 ± 0.02 $\mu mol \cdot g^{-1} \cdot h^{-1}$ 30 and 90 min after infusion, respectively. This is equivalent to a 25% and a 33% decrease in aerobic metabolic rate 30 and 90 min after infusion, respectively. After 16 h the oxygen consumption rate was no longer significantly different from the value before adenosine infusion. Infusion of the same volumes of saline did not lead to significant changes in oxygen consumption.

Theophylline infusion at 100 mg/kg body wt during hypercapnia elicited a transient increase in oxygen consumption rate from 0.34 ± 0.03 to 0.43 ± 0.03 $\mu mol \cdot g^{-1} \cdot h^{-1}$ 30 min after infusion (Fig. 4*B*). Ninety minutes after infusion the oxygen consumption rate returned to control levels, and 16 h after theophylline infusion the control rate of oxygen consumption was maintained. Theophylline infusion under normocapnia did not lead to significant changes in oxygen consumption (Fig. 4*A*). Several days after the experiment all animals were alive and in good condition.

Isolated body wall musculature (Fig. 3*B*) did not show a significant change in oxygen consumption during normocapnia or hypercapnia when adenosine (30 µmol/l) or theophylline (200 mg/l) was present in the same concentrations as in the coelomic fluid of intact animals after infusion.

DISCUSSION

In the present study we intended to identify systemic factors contributing to the depression of metabolic rate in *S. nudus* during anoxia and hypercapnia. To this aim, we first examined the concentrations of a large number of putative neurotransmitters in the nervous tissue under control conditions and during anoxia, hypercapnia, and anoxic hypercapnia. Among all substances analyzed, adenosine levels changed consistently in a way that is in accordance with a role in metabolic depression under all conditions investigated. By agonist/antagonist infusion experiments, we substantiated the role of adenosine in the regulation of metabolic rate.

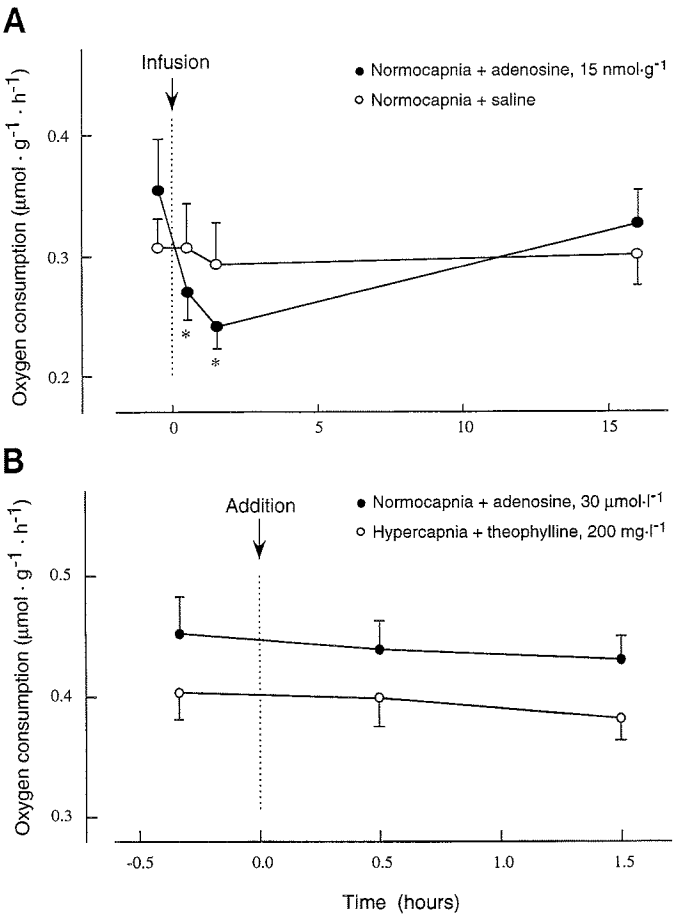

Fig. 3. *A*: effect of adenosine infusion on oxygen consumption rate of *S. nudus*. Adenosine dissolved in saline or pure saline was infused into coelomic fluid of whole animals via an indwelling catheter. Final concentration of adenosine in coelomic fluid was chosen to be equal to concentration measured in nervous tissue under anoxia and hypercapnia (Fig. 2*B*), with assumption that all adenosine measured in tissue was present in extracellular space. Values are means ± SE (n = 6). *Significantly different from value before infusion. *B*: oxygen consumption rates of isolated body wall musculature of *S. nudus* before and after addition of adenosine and theophylline to ambient medium during normocapnia and hypercapnia, respectively. Final concentrations in seawater were equal to those in coelomic fluid of intact animals after infusion. Values are means ± SE (n = 5).

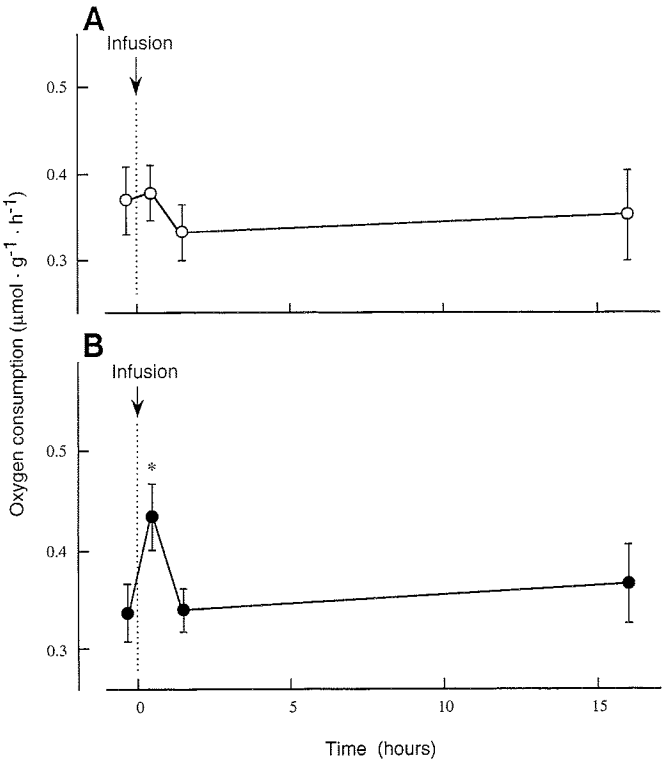

Fig. 4. Oxygen consumption rates of *S. nudus* before and after infusion of a theophylline solution (100 mg/kg body wt) during normocapnia (*A*) and hypercapnia (*B*). Values are means ± SE (n = 6). * Significantly different from value before infusion.

Amino acids. The concentration changes of amino acids in the nervous tissue of *S. nudus* after 24 h of anoxia differ considerably from those of other anoxia-tolerant animals that have been examined previously (mainly lower vertebrates). GABA levels in the nervous tissue of *S. nudus* did not change after 24 h of anoxia (Fig. 1*B*). GABA is the major interneuronal inhibitory transmitter in vertebrates and also has an important role as an inhibitory motor transmitter in arthropods, annelids, and nematodes (33). Brain GABA levels have been shown to be elevated during anoxia in the crucian carp *C. carassius* (after 4.5 h as well as after 17 days of anoxia) (13, 15), the freshwater turtle *P. scripta elegans* (up to ≥13 h of anoxia) (16), and also in the shore crab *Carcinus maenas* (up to ≥24 h of anoxia) (20). Pharmacological evidence for a role of GABA in mediating metabolic depression has been given for crucian carp (15), and increased GABA levels have generally been considered to be of crucial importance for anoxic survival. For *S. nudus* this does not hold true: GABA levels remained unaffected by 24 h of anoxia. Glutamate was significantly elevated during anoxia and also during anoxic hypercapnia in the nervous tissue of *S. nudus* (Fig. 1*B*). In contrast, all facultative anaerobes mentioned above showed an increase of GABA and a corresponding reduction of glutamate levels in the brain (same references as above). Glutamate is the direct precursor of GABA, and the reciprocal changes in these species have been interpreted to be of potential functional significance, since glutamate acts as an excitatory neurotransmitter in vertebrates, arthropods, and molluscs (33). However, nothing is known about the role of glutamate in the nervous system of *S. nudus*. Furthermore, as for every other substance measured in nervous tissue homogenates, it remains unclear whether the concentration changes of glutamate also reflect extracellular fluctuations. The increase of glutamate and decrease of aspartate (Fig. 1*A*) in the nervous tissue of *S. nudus* during anoxia and anoxic hypercapnia are likely due to intracellular anaerobic metabolism. Aspartate is a substrate of anaerobic mitochondrial energy metabolism, and it enters the succinate-propionate pathway after transamination, leaving glutamate behind. In the body wall musculature of *S. nudus*, further transamination leads to the formation of alanine from glutamate and pyruvate. In this tissue, aspartate levels decreased, glutamate remained unaffected, and alanine levels increased after 24 h of anoxia (22).

The levels of the amino acids glycine, serine, and taurine in the nervous tissue of *S. nudus* did not change after 24 h of anoxia (Fig. 1). Conversely, all three amino acids increased during anoxia in the freshwater turtle brain and are possibly relevant for the anoxic survival of the turtle brain (16). In summary, amino acid changes in the nervous tissue of *S. nudus* during anoxia have, except for the decrease in aspartate levels that is also observed in *C. carassius* and *C. maenas* (13, 20), nothing in common with the other anoxia-tolerant species that have been examined. Unless the density of the respective receptors is down- or upregulated during anoxia, the general picture of the importance of GABA, glutamate, and glycine for the anoxic survival of the vertebrate brain is not valid for the invertebrate *S. nudus*, which readily survives 24 h of anoxia. However, resistance to anoxia is less in *S. nudus* than in some other marine invertebrates (e.g., *Arenicola marina*), and further comparative research is required to evaluate the importance of amino acid neurotransmitters in other species.

Monoamines. The monoamines serotonin, dopamine, and norepinephrine require oxygen for their synthesis from tryptophan and tyrosine in vertebrates, and vertebrate-like enzymatic pathways for monoamine synthesis are already present in cnidarians (21). This suggests that anoxia would affect monoamine levels in the nervous tissue of *S. nudus*. Nevertheless, serotonin and dopamine levels remained unaffected by 24 h of anoxia, whereas norepinephrine decreased significantly (Fig. 2*A*). Because not only the synthesis but also the breakdown of serotonin is oxygen dependent, the constant serotonin level after 24 h of anoxia might be explained by a total halt in serotonin metabolism. In contrast, dopamine and norepinephrine can probably be degraded anaerobically (12). The halt of monoamine synthesis during anoxia could be compensated for by effective uptake mechanisms and low metabolic turnover rates and/or by simply keeping sufficient amounts stockpiled. In fact, the normoxic levels of serotonin, dopamine, and norepinephrine in the nervous tissue of *S. nudus* (97.0, 101.0, and 24.2 nmol/g, equivalent to

17.1, 15.5, and 4.1 μg/g) are several times higher than those in mammalian brain (~0.3, 1.0, and 0.4 μg/g) (11) and also higher than those in anoxia-tolerant freshwater turtle brain (16). The maintenance of inhibitory monoamine neurotransmitter levels during anoxia may be of special importance for anoxic survival. However, the actions of serotonin, dopamine, and norepinephrine in invertebrate nervous systems are not uniform. Already within molluscs, serotonin and dopamine can depolarize (excitatory action) as well as hyperpolarize (inhibitory action) (9). Norepinephrine levels in the nervous tissue of *S. nudus* were maintained neither during anoxia nor during hypercapnia. However, the combination of both conditions did not lead to a decrease in norepinephrine levels (Fig. 2*A*), an observation that remains unexplained.

Adenosine. Adenosine levels in the nervous tissue of *S. nudus* increased during anoxia and hypercapnia and to an even greater extent during anoxic hypercapnia (Fig. 2*B*). Therefore, adenosine is a promising candidate for being a metabolic depressant not only during anoxia, but also during hypercapnia. Hypercapnia has been shown to induce a depression of aerobic metabolic rate in *S. nudus* as well as in many other animals (1, 6, 26; H. O. Pörtner, A. Reipschläger, and N. Heisler, unpublished observations), and the mechanisms responsible for hypercapnic metabolic depression are even less understood than those responsible for anoxic metabolic depression. To test whether the observed elevation of adenosine levels in the nervous tissue during anoxia and hypercapnia is involved in the depression of metabolic rate under both conditions, we infused adenosine or theophylline solutions into the coelomic fluid of *S. nudus* while determining oxygen consumption. The application of adenosine via the coelomic fluid is suitable to mimic increased extracellular adenosine concentrations in the nervous tissue, since the brain ganglion and the ventral nerve cord of *S. nudus* are directly exposed to the coelomic fluid. However, infusion also results in increased extracellular adenosine levels in the body wall musculature (and other tissues). To distinguish between the effects on nervous tissue and muscle (the most dominant fraction of tissue), parallel experiments with isolated body wall musculature were carried out where agonist or antagonist was added to the medium. Our results clearly indicate that adenosine and its antagonist theophylline significantly altered the aerobic metabolic rate of intact individuals (Figs. 3*A* and 4), but not that of isolated body wall musculature when infused or added to the same final concentrations (Fig. 3*B*), thus demonstrating the action via the nervous tissue. Adenosine applied under control conditions, when the adenosine level in the nervous tissue is low (Fig. 2*B*), induced a depression of oxygen consumption for ≥90 min. During hypercapnia, when adenosine levels are elevated (Fig. 2*B*) and the aerobic metabolic rate is depressed (H. O. Pörtner, A. Reipschläger, and N. Heisler, unpublished observations), blockage of adenosine receptors with theophylline led to a rise in oxygen consumption rate 30 min after infusion (Fig. 4*B*), most probably by reversing the depressive effect of adenosine. In accordance, theophylline was not effective under normocapnia (Fig. 4*A*). It should be pointed out that, apart from being a potent inhibitor of adenosine receptors, theophylline has been reported to inhibit phosphodiesterases (29). However, the stimulatory action of theophylline in the central nervous system of mammals has been shown to correlate better with its ability to block adenosine receptors than with the inhibitory action on phosphodiesterases (29). The fact that, in *S. nudus*, theophylline was effective only in hypercapnic intact animals, and not in the normocapnic control group or in isolated body wall musculature, also does not support stimulatory effects other than blockage of adenosine receptors.

In the mammalian brain, adenosine decreases neuronal excitability as well as neurotransmitter release (25, 30) and it probably acts similarly in the nervous tissue of invertebrates. Derby et al. (3) reported that adenosine depressed the spontaneous activity and the responsiveness of interneurons to electrical or chemical stimuli in the brain of the spiny lobster. A similar effect could lead to the depression of energy turnover that we observed in *S. nudus*. As a product of the breakdown of ATP and other high-energy purines, adenosine is thought to be formed under conditions of limited energy supply. Possibly it is also formed as a consequence of acidosis during hypercapnia, when metabolism remains aerobic. Intracerebral microdialysis in the anoxic mammalian brain and in the anoxic freshwater turtle brain showed that adenosine was not only formed but also released to the extracellular space (5, 17). In anoxic crucian carp, blockage of adenosine receptors by aminophylline infusion resulted in a threefold increase in the rate of ethanol (the main anaerobic end product in crucian carp) excretion (14). We also monitored the main anaerobic end products (succinate, acetate, and propionate) in the coelomic fluid and the incubation water in *S. nudus* during anoxia after theophylline infusion. An influence of theophylline on the rate of end product release could, however, not be detected, probably because of the short-term duration of the effect of theophylline (Fig. 4).

Conclusions. The mechanisms mediating metabolic depression in marine invertebrates have long remained obscure. Taken together, the results of this study identify adenosine as the most likely mediator of neurally induced depression of metabolic rate during hypoxia and hypercapnia in *S. nudus*. The animal frequently encounters both conditions during low tide, and a concomitant depression of metabolic rate has been shown previously (7; H. O. Pörtner, A. Reipschläger, and N. Heisler, unpublished observations). Among the putative neurotransmitters present in the nervous tissue of *S. nudus*, adenosine was the only substance displaying concentration changes in accordance with metabolic depression under all experimental conditions. The depressive effect of adenosine infusion on oxygen consumption rate together with the transient increase of oxygen consumption during hypercapnia after theophylline application support our conclusion that adenosine plays a central role in the

regulation of whole organism metabolic rate. Recently, we could demonstrate that a decrease in extracellular (coelomic fluid) pH causes metabolic depression in isolated denervated body wall musculature of *S. nudus* (27). We therefore conclude that both central nervous and cellular mechanisms combine to elicit metabolic depression during environmental stress in this species.

Perspectives

Our study is the first to provide evidence for a role of adenosine in metabolic depression in an invertebrate. Accordingly, future comparative investigations might elucidate whether the importance of adenosine in metabolic downregulation and protection during periods of unfavorable environmental conditions is as high in invertebrates as in several vertebrates (14, 17, 28). In the future, identification and characterization of adenosine receptors and related second messenger systems in invertebrates should contribute to a better understanding of the role of adenosine in invertebrate systems.

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Address for reprint requests: H. O. Pörtner, Alfred-Wegener-Institut für Polar- und Meeresforschung, Biologie I/Ökophysiologie, Postfach 120161, D-27515 Bremerhaven, Germany (E-mail: hpoertner@AWI-Bremerhaven.DE).

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