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MODULATION OF THE COST OF pHi REGULATION DURING METABOLIC DEPRESSION: A ^{31}P-NMR STUDY IN INVERTEBRATE (*SIPUNCULUS NUDUS*) ISOLATED MUSCLE

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Summary

Extracellular acidosis has been demonstrated to play a key role in the process of metabolic depression under long-term environmental stress, exemplified in the marine invertebrate *Sipunculus nudus*. These findings led to the hypothesis that acid–base regulation is associated with a visible cost depending on the rate and mode of H^+-equivalent ion exchange. To test this hypothesis, the effects of different ion-transport inhibitors on the rate of pH recovery during hypercapnia, on energy turnover and on steady-state acid–base variables were studied in isolated body wall musculature of the marine worm *Sipunculus nudus* under control conditions (pHe 7.90) and during steady-state extracellular acidosis (pHe 7.50 or 7.20) by *in vivo* ^{31}P-NMR and oxygen consumption analyses. During acute hypercapnia (2 % CO_2), recovery of pHi was delayed at pHe 7.5 compared with pHe 7.9. Inhibition of the Na^+/H^+-exchanger by 5-(*N*,*N*-dimethyl)-amiloride (DMA) at pHe 7.5 delayed recovery even further. This effect was much smaller at pHe 7.9. Inhibition of anion exchange by the addition of the transport inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) prevented pH recovery at pHe 7.5 and delayed recovery at pHe 7.9, in accordance with an effect on Na^+-dependent Cl^-/HCO_3^- exchange. The effects of ouabain, DIDS and DMA on metabolic rate were reduced at low pHe, thereby supporting the conclusion that acidosis caused the ATP demand of Na^+/K^+-ATPase to fall. This reduction occurred *via* an inhibiting effect on both Na^+/H^+- and Na^+-dependent Cl^-/HCO_3^- (i.e. $Na^+/H^+/Cl^-/HCO_3^-$) exchange in accordance with a reduction in the ATP demand for acid–base regulation during metabolic depression. Considering the ATP stoichiometries of the two exchangers, metabolic depression may be supported by the predominant use of $Na^+/H^+/Cl^-/HCO_3^-$ exchange under conditions of extracellular acidosis.

Key words: acidosis, ATP turnover, pHe, H^+-ATPase, pHi, metabolic rate, Na^+/H^+ exchange, Na^+/K^+-ATPase, Na^+-dependent Cl^-/HCO_3^- exchange, ^{31}P-NMR, transport energetics, *Sipunculus nudus*.

Introduction

The maintenance of ion gradients over cell membranes constitutes an important fraction of cellular energy consumption in all tissues. Depending on the function of the tissue and the allocation of energy to other processes, the contribution of ion transport to total ATP turnover varies. However, during periods of limited energy supply, ion transport can be expected to be one target for a reduction of energy expenditure. The concept of ‘channel arrest’ during anoxia (Hochachka, 1985) has gained wide attention in this context, and several studies have provided direct or indirect evidence for an anoxia-induced down-regulation of ion channels, especially Na^+ and K^+ channels, in anoxia-tolerant animals (Chih et al., 1989; Pérez-Pinzón et al., 1992; Buck and Hochachka, 1993; Nilsson et al., 1993; Krumschnabel et al., 1996). To our knowledge, these relationships have not been investigated for acid–base regulation since it has not commonly been considered that acid–base regulation bears a visible cost that might be modulated by environmental factors.

We recently demonstrated that a reduction in extracellular pH (pHe), independent of changes in intracellular pH (pHi) or extra- and intracellular bicarbonate levels, induces a depression of aerobic energy turnover rate in *Sipunculus nudus* (Reipschläger and Pörtner, 1996; Pörtner et al., 1998). Full recovery of pHi during hypercapnic (extracellular) acidosis (Pörtner et al., 1998) suggested that the full capacity of intracellular acid–base regulation is maintained at reduced extracellular pH. It was proposed that metabolic rate can be lowered by reducing the energy demand of ionic pH regulation as a result of a shift from less ATP-efficient to more ATP-efficient ion transporters. The transport systems of intracellular pH regulation in animals differ in the stoichiometry of ATP molecules hydrolysed per acid–base equivalent transported.

The primary active H^+-ATPase has a stoichiometry of 0.5 ATP per proton, the secondary active Na^+/H^+ exchanger leads (indirectly *via* Na^+/K^+-ATPase activity) to the hydrolysis of 0.33 ATP per proton extruded from the cell, and electroneutral Na^+-dependent Cl^-/HCO_3^- exchanger uses (indirectly) only 0.17 ATP per acid–base equivalent. Thus, the energetic efficiencies of these transporters differ by a factor of up to 3, making room for potential energy savings by shifting from a less efficient to a more efficient transporter.

The present study was designed to test this hypothesis and to examine whether a shift to more ATP-efficient transport mechanisms truly occurs during acidosis. Moreover, we investigated the extent to which a reduction in the rate of H^+-equivalent ion exchange may contribute to metabolic depression. We used the isolated body wall musculature of *S. nudus* as an experimental model (Reipschläger and Pörtner, 1996) and investigated the sensitivity of different ion transporters involved in pHi regulation to changes in extracellular pH. Transporter activities, estimated from changes in the time course of pH recovery or tissue oxygen consumption after application of specific transport inhibitors, were compared at different values of extracellular pH. The present data suggest a modification of the original hypothesis in that they demonstrate a reduction in the overall rate of intracellular acid–base regulation during extracellular acidosis. In addition, the ATP efficiency of acid–base regulation is likely to increase during acidosis as a result of the predominant use of Na^+-dependent Cl^-/HCO_3^- exchange. At high extracellular bicarbonate levels, and despite reduced rates of pHi regulation, this supports full recovery of intracellular pH during hypercapnia (see Pörtner et al., 1998).

Materials and methods

Animals

Large specimens (39.3±1.7 g; mean ± S.E.M., N=30) of *Sipunculus nudus* L. were dug up from sandy sediments of the intertidal zone in Locquémeau, Brittany, France, and transported to the Alfred-Wegener-Institute, Bremerhaven, Germany. The animals were kept in aquaria with aerated artificial sea water and a bottom layer of sand (15–25 cm deep) at 11–15 °C for up to several weeks.

Experimental procedure

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 the inner organs including the ventral nerve cord were removed. The anterior part of the body wall musculature was cut to obtain six tissue pieces of equal size (1.0–1.5 g) for oxygen consumption analyses. Larger pieces of body wall musculature (6 cm^2) were used for ^{31}P-NMR (nuclear magnetic resonance) analysis. Each piece of tissue was punctured at the edges with a fine needle and fixed with thread to a plastic frame. The tissue preparations were then incubated for 24 h at pH 7.90, 7.50 or 7.20 in 1.5 l of saline (concentrations in mol l^{-1}: NaCl, 0.455; KCl, 0.010; $MgCl_2$, 0.024; $MgSO_4$, 0.028; $CaCl_2$, 0.010; variable [$NaHCO_3$] as calculated from the Henderson–Hasselbalch equation according to pH and P_{CO_2}) with 0.1 g l^{-1} streptomycin, 10^5 units l^{-1} penicillin and 0.01 mol l^{-1} tetraethylammonium (TEA^+)/HCl at 15 °C (final pH adjustment was achieved by the addition of HCl under continuous and long-term equilibration with the required gas mixture). pH values were chosen according to extracellular pH values and associated changes in metabolic rate observed *in vivo* during 1 % hypercapnia (Pörtner et al., 1998). The saline had been equilibrated and was bubbled continuously with 50 % air in nitrogen (normocapnia) or with 2 % CO_2 and 50 % air in nitrogen (hypercapnia). Gas mixtures were supplied by a gas-mixing pump (2M303/a-F, Wösthoff, Germany). A hypoxic gas mixture was used because long-term exposure to normoxic P_{O_2} levels can be harmful to this sediment-dwelling animal (H. O. Pörtner, personal observation).

The 180 ml chamber used for ^{31}P-NMR studies was reperfused continuously at a minimum rate of 75 ml min^{-1} from a total volume of 400 ml. The tissue was first allowed to equilibrate for approximately 12 h inside the magnet in saline set to pHe 7.9 or 7.5 under 50 % air in nitrogen. During this time, ^{31}P-NMR spectra were recorded to make sure that steady-state levels of phosphorus metabolites and pH were reached. Although absolute values of pHi are rather imprecise when analysed by NMR, continuous recordings yield reliable estimates of the pH change in individual preparations (Ellington and Wiseman, 1989). For an analysis of the rate of pH recovery, the medium was replaced with saline with or without transport inhibitors (see below), pre-equilibrated with a humidified mixture of 2 % CO_2 and 50 % air in nitrogen at the required temperature. 2 % CO_2 was chosen for its large effect on intracellular pH, as required for optimized recordings of pH changes and pH recovery. Under this high level of P_{CO_2}, a low pHe of 7.5 allowed full pHi recovery under hypercapnia (Pörtner et al., 1998). Moreover, earlier studies had shown that a pHe of 7.5 and below proved to be effective in causing metabolic depression, irrespective of P_{CO_2} or intra- and extracellular bicarbonate concentration (Reipschläger and Pörtner, 1996; Pörtner et al., 1998).

Transport inhibitors (obtained from Sigma, Deisenhofen, Germany) were dissolved in water or ethanol and were mixed with hypercapnic saline (^{31}P-NMR) or injected into the tissue chamber (oxygen consumption analyses). In control experiments, ethanol was shown to exert no effect on tissue respiration in the applied concentration range (maximal 1 %). Final inhibitor concentrations were as follows: ouabain, 10^{-3} mol l^{-1}; 5-(*N*,*N*-dimethyl)-amiloride (DMA), 5×10^{-4} mol l^{-1}; bafilomycin A_1, 10^{-7} mol l^{-1}; 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), 10^{-4} mol l^{-1}. Maintenance of inhibition by DIDS and DMA over the time course of exposure (see Figs 3, 4) and their maximum effect in the longest experiment (see Fig. 4) confirmed the long-term stability of the inhibitory effect.

^{31}P spectra were recorded under 2 % CO_2 for between 48

and 72 h depending on pHe and the addition of inhibitors. Signals were confirmed to be stable under control conditions for a minimum period of 72 h. At pHe 7.9, both the return to control pHi during hypercapnic exposure and pH recovery in the presence of DMA or DIDS were investigated in consecutive experiments in the same tissue samples. This procedure proved to be rather lengthy at pHe 7.5, so separate tissues were used for investigating pH recovery under control conditions and pH recovery in the presence of DMA or DIDS.

Control rates of oxygen consumption were recorded for 90 min at pHe 7.9 or 7.2 prior to the application of transport inhibitors at the same pHe. P_{CO_2} was not varied for oxygen consumption analyses since, among acid–base variables, only pHe was responsible for eliciting changes in metabolic rate in this tissue preparation (Reipschläger and Pörtner, 1996). A low pHe of 7.2 was chosen to give a large effect. The rate of oxygen consumption after application of the inhibitor was recorded for 120 min. At the end of the experiment, the tissue was freeze-clamped and stored under liquid nitrogen for later analyses of intracellular acid–base variables. In general, water samples were checked for constant pH at the end of the experiments.

Analyses

In vivo ^{31}P-NMR studies were performed at 81 MHz using a 47/40 Bruker Biospec DBX system with actively shielded gradient coils. Radio frequency (RF) pulses were transmitted by using a 5.0 cm diameter ^{1}H/^{31}P/^{13}C surface coil placed directly under the perfusion chamber. ^{31}P-NMR spectra were recorded continuously with a sweep width of 4000 Hz, 60 ° hard pulses of 50 µs and a repetition time of 1.2 s for 1000 scans, resulting in an acquisition period of 20 min (mean signal-to-noise ratio was between 20 and 60 depending on the size of the tissue, accuracy was ±0.05 pH units). For a reliable determination of the chemical shift from the inorganic phosphorus signal, data were averaged for up to 1 h depending on the signal-to-noise ratio. This was possible because of the very slow rate of pHi recovery in this tissue compared with vertebrate muscle preparations. The quantification of pH was carried out by setting the chemical shift of phospho-L-arginine to zero and using the equations given by Doumen and Ellington (1992) and the dissociation constant for inorganic phosphate determined at 15 °C by Pörtner (1990), which is in close agreement with that given by Kost (1990).

Rates of oxygen consumption were determined by closed-system respirometry in 100 ml respiration chambers equipped with a polarographic oxygen sensor (Eschweiler, Kiel, Germany). The chambers were filled with solutions identical to those in which the incubations had been carried out. The determination of oxygen consumption rates in chambers without tissue showed that bacterial growth was completely inhibited by the added antibiotics. The inhibitors used did not have an effect on oxygen recordings.

Intracellular acid–base variables were determined according to Pörtner et al. (1990) in tissues subjected to oxygen consumption analyses. Total CO_2 concentrations (C_{CO_2}) of tissue and water samples were measured using a gas chromatograph (Hach Carle, USA) and apparent HCO_3^- concentrations were calculated using values of pK‴ and of the solubility coefficient α_{CO_2} determined according to Heisler (1986).

Calculations and statistical analyses

pH recordings by ^{31}P-NMR over time were examined for significance by analysis of variance (ANOVA) (Superanova, Abacus Concepts) performing contrasts for paired sample comparisons. The oxygen consumption rates before and after application of an inhibitor were compared using a Student's *t*-test for paired values. Oxygen consumption rates are expressed both as µmole of O_2 consumed per hour per gram tissue wet mass and as percentage changes from the respective control values to facilitate comparison among different treatments. Pairwise comparisons of the percentage changes after different treatments were carried out using a Student's *t*-test for unpaired values. This test was also applied for pairwise comparisons of intracellular pH values with the control value. In all cases, $P<0.05$ was accepted as a significant difference. Maximum rates of net proton release were calculated by multiplying the maximum rates of pH change over time by the non-bicarbonate buffer value of 20 mmol pH unit^{-1} kg^{-1} adopted from Pörtner et al. (1984). Proton turnover during degradation of phospho-L-arginine (PLA) in the presence of DMA was considered as outlined by Pörtner (1987a,b). Because there was less variability in the signal (see Fig. 5), the accumulation of P_i more reliably reflected this influence than the depletion of PLA. Values are presented as means ± S.E.M. throughout.

Results

Fig. 1 depicts ^{31}P-NMR spectra collected under control conditions (pH 7.9), under the acute effect of 2 % CO_2 (20 min after changing the medium and P_{CO_2}) and after 10 h of recovery. As a result of metabolic depression at pHe 7.5 (see Reipschläger and Pörtner, 1996; Pörtner et al., 1998), some of the tissue preparations showed rather low levels of inorganic phosphate. No significant changes in signal intensity of the high-energy phosphates (phospho-L-arginine, ATP) were observed. This reflects the excellent stability of the preparation and the resting state of the tissue as well as the equilibrium of arginine kinase (Ellington, 1989); however, it led to relatively poor signal-to-noise ratios with a reduced accuracy in the determination of absolute pH values and, in consequence, a rather large scatter of individual pH values in Figs 2–4. The use of deoxyglucosephosphate as a pH indicator (Wiseman and Ellington, 1989) proved impossible since it was metabolized by this muscle tissue. However, continuous recordings allowed pH changes to be monitored over time.

The recovery of intracellular pH under acute hypercapnic acidosis (2 % CO_2) took approximately four times longer (35 h compared with 8 h) when extracellular pH was reduced from 7.9 to 7.5 (Fig. 2). Recovery at pHe 7.2 took longer than at pHe 7.5 (data not shown); therefore, further

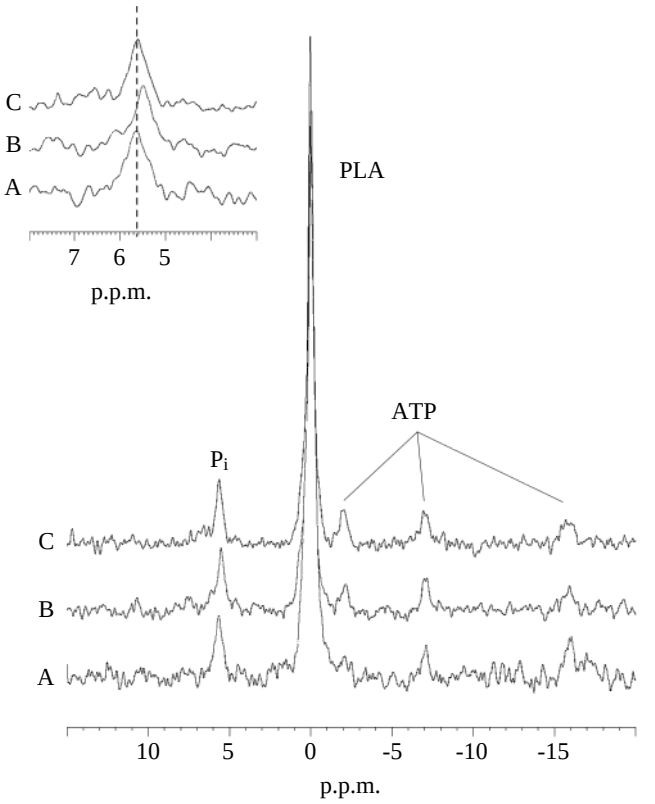

Fig. 1. *In vivo* ^{31}P-NMR spectra (acquisition time 20 min) for isolated body wall muscle tissue from *Sipunculus nudus* at pHe 7.9 acquired under control conditions (A), 20 min after the addition of 2 % CO_2 (P_{CO_2}=1.97 kPa) (B) and 10 h later when the intracellular acidification was reversed (C). P_i, inorganic phosphate; PLA, phospho-L-arginine; ATP, adenosine triphosphate. The inset shows the chemical shift of inorganic phosphate for A, B and C.

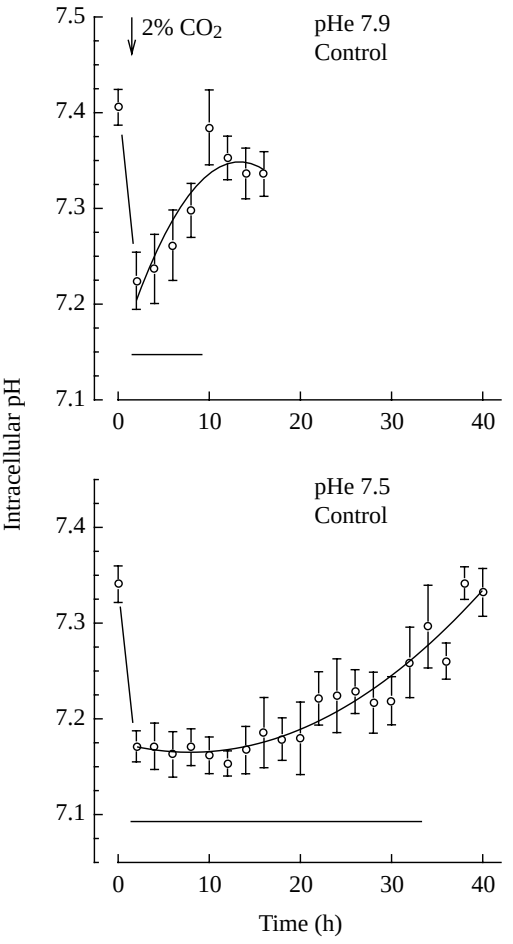

Fig. 2. Time course of pHi recovery during acute hypercapnia under 2 % CO_2 at pHe 7.9 (to maintain pHe between control and hypercapnic conditions, $[HCO_3^-]_e$ was increased from 0.1 to 52 mmol l^{-1}) and at pHe 7.5 ($[HCO_3^-]_e$ was increased from 0.04 to 19 mmol l^{-1}). Note the delay in the onset of pH recovery and the fourfold longer time course at pHe 7.5. Horizontal lines indicate the onset and maintenance of a significant effect of the treatment. Values are means ± S.E.M., N=4.

experimentation was carried out at pHe 7.9 and 7.5 to ensure full recovery of pHi in both cases. Under the effect of 2 % CO_2, a similar drop in pHi of, on average, 0.16–0.18 pH units occurred at both pHe values. pHi recovery set in immediately at pHe 7.9 but only after a 15–20 h delay during extracellular acidosis (pHe 7.5). Moreover, the maximum rate of pHi increase observed was approximately 2.3 times faster at pHe 7.9 than at pHe 7.5. This factor also quantifies the reduction in the net rate of proton release from the intra- to the extracellular space during extracellular acidosis from 0.47±0.11 μmol g^{-1} h^{-1} (between 4 and 10 h of analysis at pHe 7.9) to 0.20±0.09 μmol g^{-1} h^{-1} (between 30 and 40 h of experimentation at pHe 7.5).

When DMA and DIDS were applied at pH 7.9, the rate of recovery was lower in the presence of DIDS than in the presence of DMA (Fig. 3). The inhibitory effect of DMA on the rate of pH recovery became more prominent at low pHe (7.5) but was not evident at pHe 7.9 (Figs 3, 4). As part of the effect at pHe 7.5, both DMA and DIDS caused an extension of the delay period observed prior to the onset of pH recovery during acidosis. During the delay in the presence of DMA, pH continued to decrease by another 0.05–0.1 units before it finally started to rise after 30 h of hypercapnia. The addition of DIDS caused full inhibition of pHi recovery at pHe 7.5 followed by a trend for pHi to decrease further after 30 h (Fig. 4).

Exposure to hypercapnia in the presence of DMA caused phospho-L-arginine levels to decrease in association with an increase in inorganic phosphate levels and the development of muscular contracture during the period of intracellular acidosis and recovery. The accumulation of inorganic phosphate (P_i) mirrored the depletion of phospho-L-arginine (Fig. 5).

The effects of transport inhibitors on rates of oxygen consumption, pHi and intracellular bicarbonate concentrations under control conditions (pHe 7.90) and during acidosis (pHe 7.20) are summarized in Table 1 and Figs 6 and 7. The effects of pHe on pHi in the present study (pHe 7.90 resulted in pHi 7.28, and pHe 7.20 resulted in pHi 7.15) were the same as reported previously (Reipschläger and Pörtner, 1996). At pHe 7.90, all inhibitors except for the combination of DIDS,

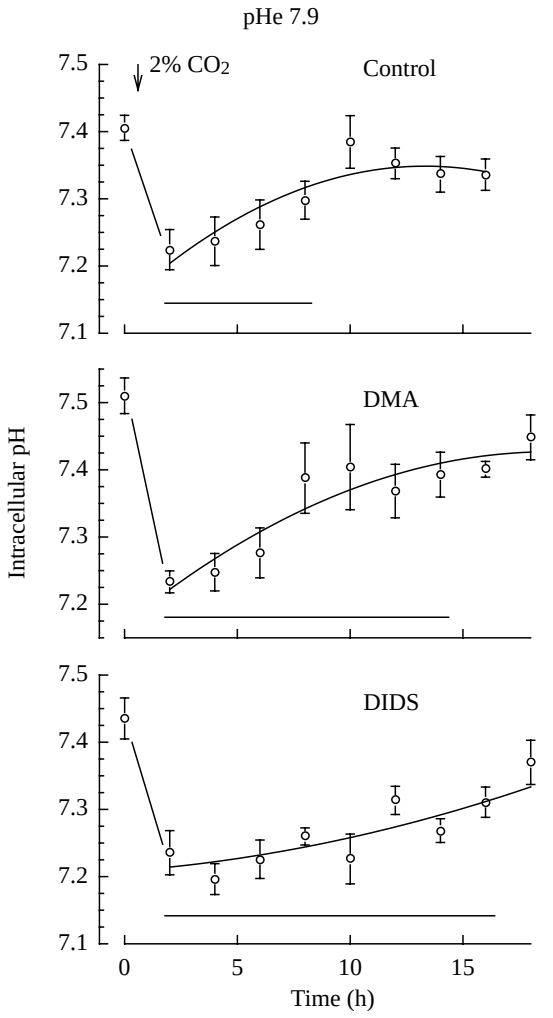

Fig. 3. Effects of DMA and DIDS on the time course of pHi recovery under 2 % CO_2 at pHe 7.9. The effect of DMA was small, with no evident change in the rate of pH recovery. Addition of DIDS caused a delay in recovery. The horizontal lines indicate the onset and maintenance of a significant effect of the treatment. Values are means ± S.E.M., *N*=4.

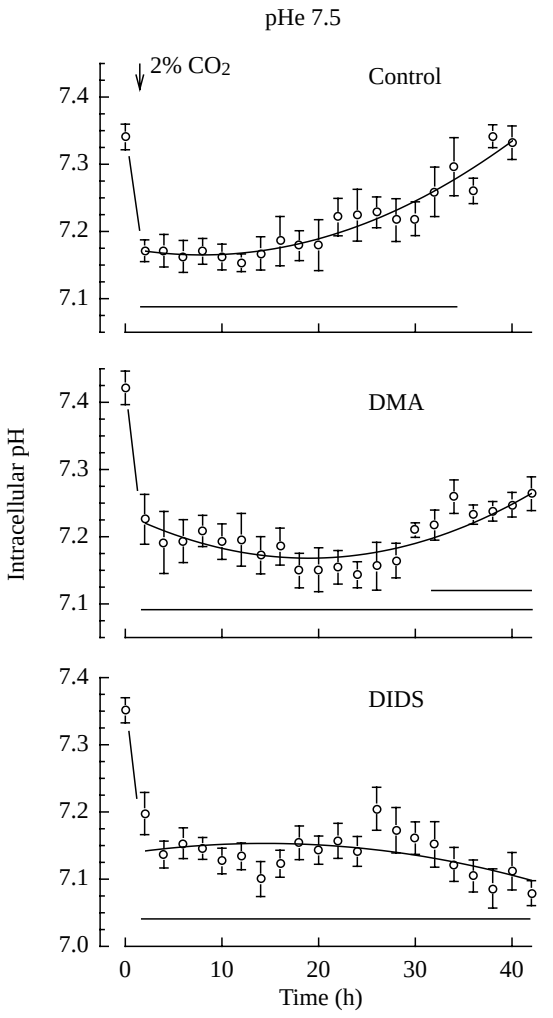

Fig. 4. Effects of DMA and DIDS on the time course of pHi recovery under 2 % CO_2 at pHe 7.5. Addition of DMA caused a delay in recovery, whereas the addition of DIDS completely inhibited pH recovery. The horizontal lines indicate the onset and maintenance of a significant effect of hypercapnic exposure (lower lines) and the onset of a significant recovery during long-term hypercapnia in the presence of DMA (upper line). Values are means ± S.E.M., *N*=4.

DMA and bafilomycin A_1 induced significant changes in the rate of tissue oxygen consumption. DIDS caused a rise in the rate of oxygen consumption rather than the decrease seen in the presence of the other inhibitors. At pHe 7.2, the effects of ouabain, DMA and DIDS on rates of oxygen consumption were smaller than at pHe 7.9, starting from a somewhat lower mean rate of metabolism (Table 1). Monitoring of ^{31}P-NMR spectra at unchanged extracellular pH confirmed that DMA did not cause a decrease in phospho-L-arginine levels during the period of oxygen consumption analyses (results not shown).

Ouabain depressed the rate of tissue oxygen consumption by 25.2 % at pHe 7.90 (Fig. 6B) and by only 17.4 % during acidosis (Fig. 7B). When the percentage change in ATP turnover was normalized with respect to the ATP turnover seen at pHe 7.9, the reduction was only 14.6 % (Table 1). DMA induced a depression of the rate of oxygen consumption by 13.7 % under control conditions (Fig. 6B) and only by a small non-significant amount during acidosis (Fig. 7B; Table 1). The reduction in the rate of tissue oxygen consumption caused by bafilomycin A_1 was similar under control conditions (13.9 %) and during acidosis (12.3 % with respect to the control rate at pHe 7.2, Fig. 7B, or 11.2 % with respect to the control rate at pHe 7.9, see Table 1).

DIDS led to an increase in oxygen demand of 63.0 % at pHe 7.90 (Fig. 6B) but of only 11.9 % (not significant) during acidosis (Fig. 7B). Simultaneous application of DMA and DIDS reduced the increment at both pH values. At pH 7.9, the increase was completely abolished when DIDS, DMA and bafilomycin A_1 were combined. A combination of DIDS and DMA or of DIDS, DMA and bafilomycin A_1 also eliminated the small rise in the rate of oxygen consumption seen at pHe 7.2.

Table 1. *Effects of various inhibitors and inhibitor combinations on rates of oxygen consumption, intracellular bicarbonate concentrations and the percentage change in ATP turnover under control conditions (pH_e 7.90) or after 24 h of pre-exposure to acidosis (pH_e 7.20)*

	pH_e 7.90, $[HCO_3^-]_e$=1.0 mmol l^{-1}				pH_e 7.20, $[HCO_3^-]_e$=0.2 mmol l^{-1}			
Treatment	$\dot{M}_{O_2a}$ (μmol O_2 g^{-1} h^{-1})	$\dot{M}_{O_2b}$ (μmol O_2 g^{-1} h^{-1})	$[HCO_3^-]_i$ (mmol l^{-1})	$\Delta\dot{M}_{ATP}$ (%)	$\dot{M}_{O_2a}$ (μmol O_2 g^{-1} h^{-1})	$\dot{M}_{O_2b}$ (μmol O_2 g^{-1} h^{-1})	$[HCO_3^-]_i$ (mmol l^{-1})	$\Delta\dot{M}_{ATP}$‡ (%)
Control	0.725±0.070	0.706±0.075	1.08±0.10	–2.6±1.1	0.617±0.085	0.603±0.072	0.65±0.04	–1.9±0.6
Ouabain	0.779±0.062	0.570±0.054*	0.87±0.18	–25.2±2.7*	0.652±0.112	0.535±0.086*	0.47±0.05*	–14.6±1.9*
DMA	0.715±0.047	0.622±0.061*	1.24±0.30	–13.7±3.4*	0.755±0.139	0.690±0.109	0.63±0.11	–7.7±2.3
Bafilomycin A_1	0.761±0.043	0.654±0.038*	1.07±0.20	–13.9±2.3*	0.691±0.115	0.601±0.098*	0.69±0.11	–11.2±2.9*
DIDS	0.631±0.014	1.028±0.077*	0.77±0.25	+63.0±10.3*	0.556±0.097	0.597±0.073	0.50±0.04*	+10.5±7.3
DIDS + DMA	0.553±0.038	0.722±0.069*	0.99±0.09	+30.6±6.9*	0.707±0.077	0.667±0.052	0.55±0.15	–3.3±9.1
DIDS + bafilomycin A_1	0.731±0.106	1.015±0.101*	0.66±0.18	+42.9±10.9*	0.816±0.135	0.839±0.120	0.64±0.11	+6.6±8.3
DIDS + DMA + bafilomycin A_1	0.743±0.106	0.678±0.083	0.85±0.21	–5.7±8.7	0.746±0.047	0.720±0.053	0.61±0.10	–3.1±5.1

Rates of oxygen consumption ($\dot{M}_{O_2}$) before ($\dot{M}_{O_2a}$) and after ($\dot{M}_{O_2b}$) inhibitor application are given in μmol O_2 g^{-1} h^{-1}.
* indicates a significant difference from the control value.
Values are means ± S.E.M.
N values range between 4 and 6.
‡The percentage change in ATP turnover ($\Delta\dot{M}_{ATP}$) at pH 7.2 was normalized by setting the ATP turnover rate at pH 7.9 to 100 %.
Note that intracellular bicarbonate was analysed at the end of approximately 120 min of $\dot{M}_{O_2}$ analyses (see Figs 6, 7).

At pHe 7.9, only the combined effect of the three inhibitors caused pHi to decrease significantly from 7.28 (control) to 7.23 (Fig. 6A). Intracellular bicarbonate concentrations were not significantly affected by inhibitors applied individually or in various combinations for 2 h at pHe 7.90 (Table 1). All inhibitor combinations, as well as ouabain and DIDS alone, induced significant decreases in pHi by nearly 0.10 units at pHe 7.2 (Fig. 7A). In addition, ouabain and DIDS induced a reduction of intracellular bicarbonate concentration, emphasizing the non-respiratory nature of the acidosis elicited by the effects on ion exchange (Table 1). The effect of DMA on pHi was smaller than the effect of DIDS at pHe 7.2.

Discussion

The study of pH recovery by ^{31}P-NMR, of the changes in rates of oxygen consumption and of the deviations from steady-state pHi in the presence of ion-transport inhibitors should reveal which mechanisms of acid–base regulation in the muscle tissue are affected by low extracellular pH and how this

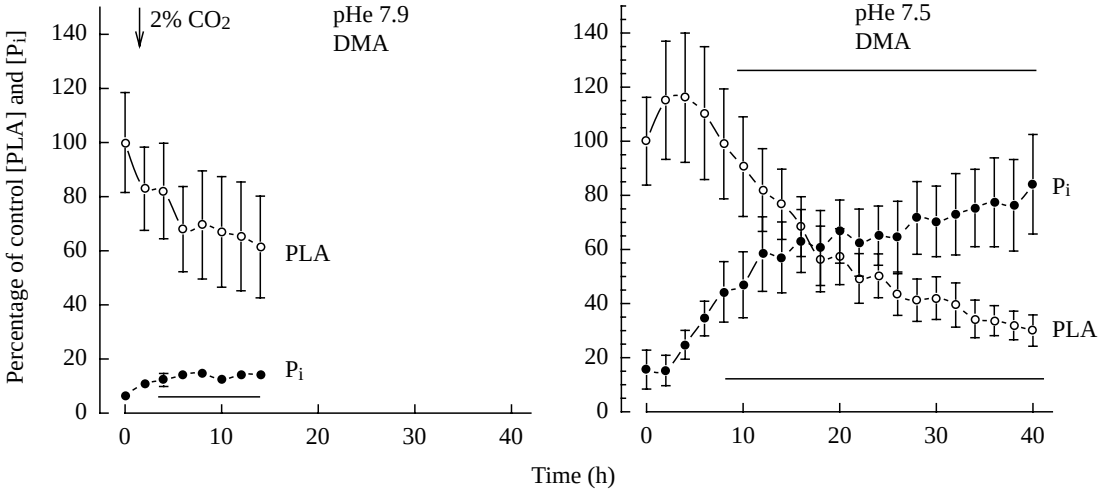

Fig. 5. Relative changes in phospho-L-arginine (PLA) (open circles) and inorganic phosphate (P_i) levels (filled circles) during exposure to DMA under 2 % CO_2 at pHe 7.9 and at pHe 7.5. Note the delayed onset of PLA degradation at pHe 7.5. The accumulation of P_i mirrored the reduction in PLA levels. The horizontal lines indicate the onset and maintenance of a significant effect of the treatment. Values are means ± S.E.M., *N*=4.

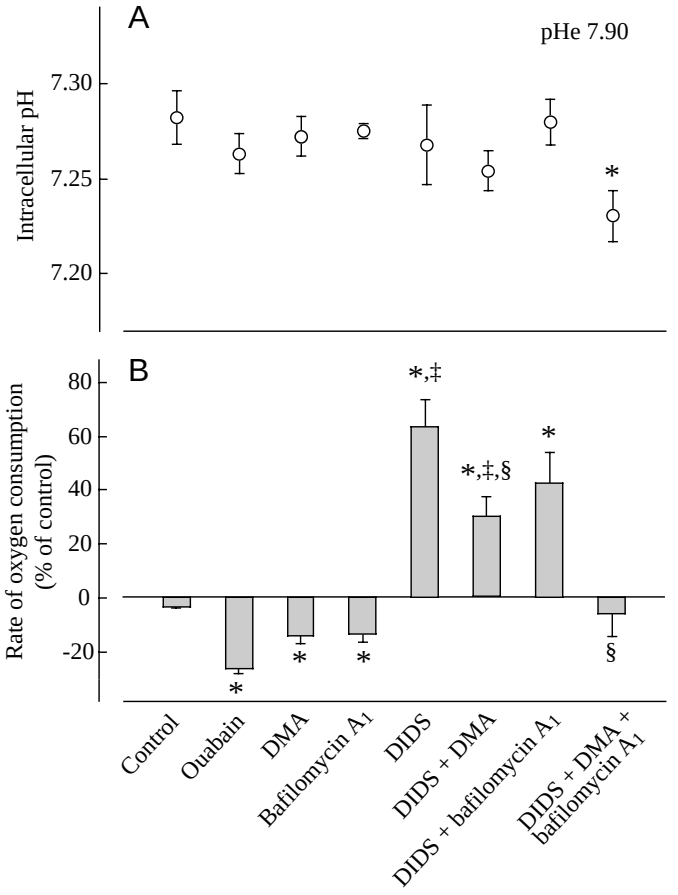

Fig. 6. Changes in intracellular pH (A) and the rate of oxygen consumption (B) after application of transport inhibitors under control conditions (pHe 7.90, P_{CO_2}=0.04 kPa). The following statistical comparisons were carried out. (i) Rates of oxygen consumption and pHi values were compared with the respective control value; significant differences are indicated by *. (ii) Percentage changes in rates of oxygen consumption were compared with those after the same treatment during acidosis (see Fig. 7); significant differences are indicated by ‡. (iii) The percentage changes in the rate of oxygen consumption after treatments with DIDS combined with other inhibitor(s) were compared with the change induced by DIDS alone; § indicates a significant difference. Note that pHi was analysed at the end of approximately 120 min of oxygen consumption analyses. Values are means ± S.E.M., *N*=4–6.

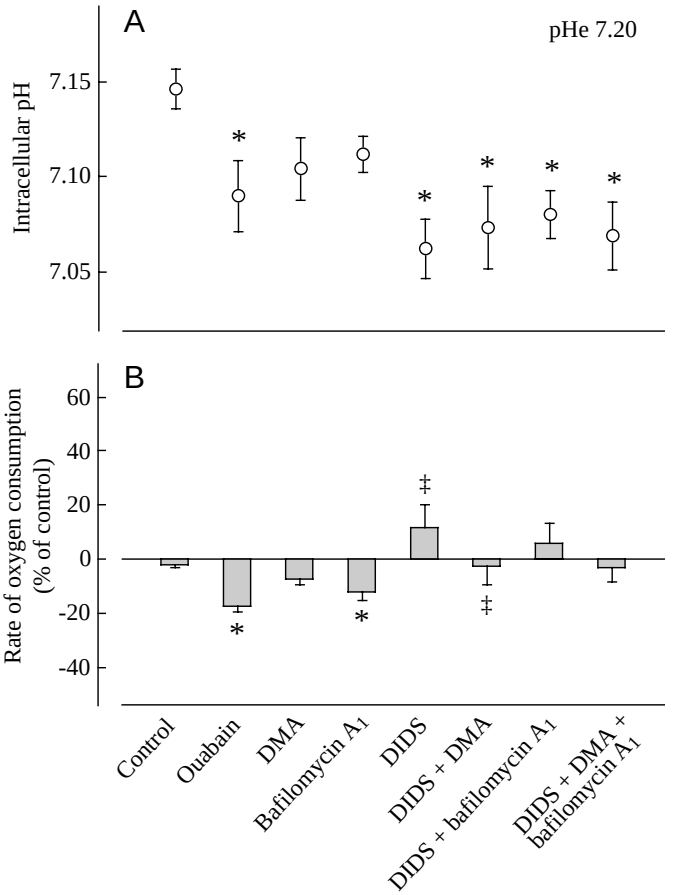

Fig. 7. Changes in intracellular pH (A) and the rate of oxygen consumption (B) after application of transport inhibitors during acidosis (pHe 7.20, P_{CO_2}=0.04 kPa). * indicates a significant difference from the respective control value and ‡ denotes that the percentage changes in the rate of oxygen consumption were significantly different from those after the same treatment under control conditions (pHe 7.90). See also Fig. 6 and Table 1. Values are means ± S.E.M., *N*=4–6.

effect relates to changes in energy turnover. In this context, it must be considered whether the applied inhibitor is specific for the respective acid–base exchange mechanism, whether it does or does not enter the cell and whether they might affect respiration by other means.

Among the transport inhibitors applied, both DIDS and amiloride (and its derivatives such as DMA) may block not only carrier-mediated transport but also channel-mediated transport of Cl^- and Na^+ (see Kleyman and Cragoe, 1988; Jensen and Skott, 1996). The distribution of Cl^- across the cell membrane is generally at or near its electrochemical equilibrium (see Madshus, 1988). An inhibitory effect of DIDS on Cl^- channels is therefore unlikely to cause short-term changes in ion-transport rates or other energy-dependent cellular processes. DIDS also inhibits Na^+-independent Cl^-/HCO_3^- exchange, but this exchanger passively follows the gradients for Cl^- and HCO_3^-, only contributing to pHi regulation after an alkali load (Madshus, 1988). In our case, a contribution of the Na^+-dependent Cl^-/HCO_3^- exchanger is much more likely since this carrier contributes to pHi regulation following an acid load, often in concert with the Na^+/H^+ exchanger (e.g. Kahn et al., 1990; Neylon et al., 1990; Little et al., 1995). Accordingly, DIDS caused an inhibition of recovery from acidosis (Figs 3, 4). However, it elicited a stimulation of respiration in isolated body wall tissue (Figs 6, 7). The latter effect needs explanation (see below), but argues against an effect of DIDS on mitochondrial respiration considering that an inhibitory effect was found in isolated rat liver mitochondria (Bernardes et al., 1997). The stimulatory effect seen at pHe 7.9 is larger than the ATP turnover associated with all Na^+ transport estimated in the presence of

ouabain without the addition of DIDS. We will discuss below how DIDS may stimulate the futile cycling of ATP-dependent ion transport.

Non-specific effects of DMA may include an inhibition of Na^+ channels, resulting in reduced Na^+/K^+-ATPase activity. In consequence, the contribution of Na^+/H^+ exchange to the rate of oxygen consumption may have been overestimated. However, amiloride analogues such as DMA, which bear constituents on the 5-amino nitrogen atom, exhibit a substantial loss in activity against Na^+ channels. Also, DMA has a very low potency in inhibiting the Na^+/Ca^{2+} exchanger and the Na^+/K^+-ATPase, with I_{50} values in the millimolar range (Kleyman and Cragoe, 1988). This lies above the applied concentration of 0.5 mmol l^{-1} DMA, which will therefore affect mainly the Na^+/H^+ exchanger. In addition, we are not aware of any direct effects of DMA on mitochondrial respiration. A limitation of mitochondrial respiration may finally occur *via* inhibition of Na^+/H^+ exchange and the subsequent inhibition of Ca^{2+} entry (Hotta et al., 1998; Alvarez et al., 1999). Accordingly, an early effect on baseline mitochondrial respiration during the period of oxygen consumption analyses appears unlikely. The release of Ca^{2+} from internal stores elicited by low pHi in the presence of DMA may even counteract this effect and may explain the contracture that developed in the isolated muscle preparation during hypercapnic acidosis in the presence of DMA. This probably caused the continuous decrease in phosphagen (phospho-L-arginine) levels, reflecting a rise in ATP demand as a result of contracture. Since phosphagen depletion causes proton consumption, this effect may support a somewhat faster pH recovery in the presence of DMA. However, phospho-L-arginine degradation did not occur during the short period of oxygen consumption analysis, when the results actually suggest a reduction in energy requirements in the presence of DMA.

In summary, our data do not indicate that our conclusions have been influenced significantly by unknown side effects, especially when it is considered that our first priority was to evaluate the changing contribution of the transporters examined to pHi regulation at various values of extracellular pH. This also eliminates uncertainties about the secondary effects of these inhibitors on tissue respiration in the sense that they remain negligible as long as they are not dependent on extracellular pH.

The findings that pH recovery is delayed in the presence of DMA and DIDS together with significant effects of ouabain, DMA, bafilomycin A_1 and DIDS on aerobic energy turnover strongly suggest that a Na^+/K^+-ATPase, a Na^+/H^+ exchanger, an H^+-ATPase and an anion exchanger operate in this tissue. Since application of DIDS delayed pH recovery from acidosis and induced an acidosis rather than an alkalosis at pHe 7.2 (Fig. 7B), we conclude that a Na^+-dependent Cl^-/HCO_3^- exchanger contributes to pHi regulation. In consequence, the following discussion will focus on the relative contributions of these transporters to acid–base regulation and energy turnover.

^{31}P-NMR analyses

Acute exposure to hypercapnia at pHe 7.9 and pHe 7.5 revealed a slow rate of pH recovery compared with other, especially vertebrate, systems (e.g. Vandenberg et al., 1994; Shi et al., 1997) and confirmed the suitability of ^{31}P-NMR for investigating the long-term pattern of acid–base regulation in the tissue model chosen. Other continuous methods of pH analysis (e.g. microelectrodes or fluorescent dyes) would have been less suitable because of their limited stability over time. The slow rate of pH recovery may reflect the simplicity of the system and its early evolutionary position.

Recovery of intracellular pH took approximately four times longer at pHe 7.5 than at pHe 7.9, starting from somewhat lower pHi values, in agreement with previous findings (Reipschläger and Pörtner, 1996). Moreover, the onset of pH compensation was delayed and was then completed at a lower rate. Not only proton-equivalent ion exchange but also the flexibility to respond to acid–base disturbances appear to be reduced at low extracellular pH. The detailed mechanism of this delay remains obscure; however, such a delay might become evident when, similar to the stimulation of a metabolic pathway, ion exchange has to be stimulated starting from a low rate of futile cycling of the carrier. The extended delay in the presence of DMA or DIDS confirms this idea and demonstrates that both Na^+/H^+ exchange and Na^+-dependent Cl^-/HCO_3^- exchange contribute to acid–base regulation during acidosis; however, at a lower rate than at pHe 7.9. This is reminiscent of the situation in mammalian heart, in which net proton extrusion from the intracellular space was reduced depending on the degree of extracellular acidosis (Vandenberg et al., 1994). A reduction in the rate of net proton extrusion at low pHe has also been reported in barnacle muscle fibres (Boron et al., 1981).

A change in transporter activities between control conditions and acidosis could be elicited either by changes in transporter densities or by changes in the activity of a constant number of transporters. In suspensions of rabbit kidney proximal tubules, the Na^+/H^+ exchanger is predominantly regulated by pH, with a constant number of transporter sites, whereas the H^+-ATPase is regulated by HCO_3^- *via* changes in transporter abundance (Soleimani et al., 1995). Our findings are consistent with an inhibitory effect of extracellular protons on the Na^+/H^+ exchanger during acidosis, similar to the effect demonstrated in isolated neurons of the crayfish *Astacus leptodactylus* (Gaillard and Rodeau, 1987). Competition with extracellular Na^+ for the transport site (Aronson et al., 1983; Krapf and Alpern, 1993) appears unlikely considering the high extracellular Na^+ levels, but mixed-type and even non-competitive inhibition by extracellular protons has been reported (Grinstein et al., 1984; Grinstein and Rothstein, 1986). In barnacle muscle fibres, the reduction in net proton extrusion was attributed to a rise in the Michaelis–Menten constant (K_m) for bicarbonate of Na^+-dependent Cl^-/HCO_3^- exchange (Boron et al., 1981).

An inhibitory effect of DMA on pH recovery was not evident at pHe 7.9, although DMA caused a reduction in the

rate of oxygen consumption (Fig. 6). Proton consumption during phospho-L-arginine degradation probably slightly reduced the maximum degree of acidosis developing during early hypercapnia and may have prevented some delay in the onset of pH recovery. However, phosphagen degradation no longer contributed when the maximum rate of pH recovery was observed up to 10 h after the onset of hypercapnia (Figs 3, 5). The reduced rate of oxygen consumption and rapid pH recovery in the presence of DMA at pHe 7.9 suggest that a more ATP-efficient carrier such as the $Na^+/H^+/Cl^-/HCO_3^-$ exchanger may take over in these circumstances (see Introduction). An increase in H^+-ATPase activity would have increased energy demand instead.

At pHe 7.5 in the presence of DMA, an extended delay of more than 5–10 h was evident despite phospho-L-arginine depletion, suggesting metabolic acidification or that a loss of base from the intracellular space had occurred (Fig. 4). During the actual period of pH recovery at pHe 7.5, the rate of phospho-L-arginine degradation was reduced (Fig. 5), but it was still large enough to account for approximately 50 % of the rate of pH recovery finally observed.

DIDS was more effective than DMA in delaying pH recovery at both pHe 7.9 and 7.5, indicating that Na^+/H^+ exchange or the H^+-ATPase was not able to compensate fully for the inhibition of $Na^+/H^+/Cl^-/HCO_3^-$ exchange. These findings indicate that $Na^+/H^+/Cl^-/HCO_3^-$ exchange can more effectively compensate for the inhibition of Na^+/H^+ exchange than *vice versa*.

All our measurements were made in the presence of constant levels of extracellular pH but with changing levels of bicarbonate or P_{CO_2}. In the tissue model chosen, bicarbonate is not effective in changing the rate of energy turnover (see Reipschläger and Pörtner, 1996), but it may influence the set point of pHi regulation through its influence on Na^+-dependent Cl^-/HCO_3^- exchange. In barnacle muscle fibres, the affinity of this transport system for bicarbonate, determined as K_m, proved to be pHe-dependent, amounting to 4 mmol l^{-1} at pHe 8.0 and rising to 10 mmol l^{-1} at pHe 7.4 (Boron et al., 1981). These findings strongly suggest that accumulation of extracellular bicarbonate is a precondition for the recovery of pHi during hypercapnic extracellular acidosis (see Pörtner et al., 1998) and emphasize the relevance of extracellular acid–base regulation for the maintenance of intracellular pH.

Changes in rates of oxygen consumption and pHi under normocapnia

The results obtained by ^{31}P-NMR are supported by the inhibitor-induced changes in tissue oxygen consumption rates and pHi studied at two values of extracellular pH. In the light of the slow changes in pHi observed using ^{31}P-NMR, it must be emphasized that pHi values determined at the end of oxygen consumption analyses, i.e. approximately 2 h after inhibitor application, may not reflect steady-state values and will depend upon the initial contribution of the transporter to pHi maintenance and upon the degree of inhibition. If no pH change or only an insignificant trend was observed at this time, a long-term change cannot be excluded. The changes observed will also reflect the remaining overall rate of other transport mechanisms. In support of this idea, smaller effects of inhibitors on rates of oxygen consumption, together with more pronounced pH changes, are in accordance with reduced overall rates of proton-equivalent ion exchange during acidosis (Figs 2, 7).

This observation suggests that energy turnover associated with acid–base regulation decreases during extracellular acidosis. The effect of ouabain was smaller at pH 7.2 than at pH 7.9, indicating a reduced rate of overall ATP-dependent ion exchange. The reduced effects of DIDS and DMA on rates of oxygen consumption suggest that both Na^+/H^+ exchange and Na^+-dependent Cl^-/HCO_3^- exchange are active at a lower rate at pHe 7.2. In contrast, bafilomycin treatment did not reveal a significantly pHe-dependent change in H^+-ATPase activity. The effect of DMA on pHi was insignificant at both pHe values, confirming that Na^+-dependent Cl^-/HCO_3^- exchange may take over under conditions of acidosis (see above).

An interesting result of our study is that inhibition of $Na^+/H^+/Cl^-/HCO_3^-$ exchange by DIDS did not cause a reduction in the rate of oxygen consumption but induced an increase that was greatest at pHe 7.9 and much reduced at pHe 7.2. When DIDS was applied in combination with DMA at pH 7.9, the increase was significantly reduced, an effect enhanced by the addition of bafilomycin. This suggests that inhibition of Na^+-dependent Cl^-/HCO_3^- exchange led to increased H^+ transport *via* the Na^+/H^+ exchanger and possibly also the H^+-ATPase. The higher ATP requirement of the latter two transporters probably caused, or at least contributed to, the increase in the rate of oxygen consumption. In support of this conclusion, DMA exerted a larger effect in the presence than in the absence of DIDS. Addition of bafilomycin A_1 revealed the stimulation of H^+-ATPase activity. The reduction in the rate of oxygen consumption was particularly large when bafilomycin was added to the combined effect of DIDS and DMA at pHe 7.9 (Fig. 6), leading to the complete reversal of the DIDS-induced increase in the rate of oxygen consumption. In this case, simultaneous inhibition of all three transporters was confirmed by a large and significant reduction of intracellular pH under control conditions and during extracellular acidosis. Under these conditions, the stimulatory pressure on pHi regulation must be considered high, and blockage of Na^+/H^+ exchange and H^+-ATPase may be less complete, as indicated by a rate of oxygen consumption comparable with that of controls and higher than expected from the reduced activity of all three transporters.

The increase in the rate of oxygen consumption after application of DIDS was not significant at pHe 7.2, in accordance with a reduced rate of Na^+-dependent Cl^-/HCO_3^- exchange during extracellular acidosis and a minor shift from Na^+-dependent Cl^-/HCO_3^- exchange to Na^+/H^+ exchange and/or H^+-ATPase. This led to a much larger reduction in pHi in the presence of DIDS than in the presence of DMA or bafilomycin A (Fig. 7A) and also explains the inability of pHi to recover under these conditions (Fig. 4). Moreover,

reductions in pHi were similar after application of either DIDS alone or of DIDS in combination with DMA or bafilomycin A_1. Together with the reduction in pHi after inhibition of the Na^+/K^+-ATPase by ouabain, these data suggest that Na^+-dependent Cl^-/HCO_3^- exchange plays an important role in pHi regulation during extracellular acidosis, albeit at a reduced rate. This confirms the conclusions derived from the analyses of pHi recovery by ^{31}P-NMR (see above).

A quantitative approach?

At first sight, the analysis of pH recovery and the change in the rate of oxygen consumption of the tissue after inhibition would allow quantification of the activity of ion transporters. However, neither approach provides a direct estimate since inhibition of one transporter may lead to a compensatory increase in the activity of another. The latter phenomenon has also been reported for kidney tubules (Fleser et al., 1995). It may occur whenever transporters have comparable functions.

Inhibition of the Na^+/K^+-ATPase by ouabain depressed aerobic tissue energy turnover by 25.2 % at pHe 7.90 and by 17.4 % at 7.20 (Fig. 7B). These values compare well with the 18 % depression observed in goldfish (*Carassius auratus*) hepatocytes (Krumschnabel et al., 1994). Trout hepatocytes show a somewhat smaller reduction of 12 %, and for these cells the depression in the rate of oxygen consumption has been shown to be in agreement with the actual activity of Na^+/K^+-ATPase (measured *via* rubidium uptake; Krumschnabel et al., 1996). The percentage change in the rate of oxygen consumption may be a minimum estimate of the change in transporter activity if H^+-ATPase was stimulated to compensate for some of the acidification elicited by ouabain.

Since Na^+/H^+ and $Na^+/H^+/Cl^-/HCO_3^-$ exchange are likely to compensate for each other during inhibition, the effects of the specific inhibitors on oxygen consumption can be considered as minimum effects. Considering that oxygen consumption decreases by 25.2 % in the presence of ouabain and by 13.7 % in the presence of DMA, the Na^+/H^+ exchanger appears to use more than 50 % of the Na^+ gradient provided by Na^+/K^+-ATPase at pHe 7.9 (Table 1). During acidosis (pHe 7.2), however, the ATP turnover of Na^+/K^+-ATPase dropped by approximately 42 %, to a similar extent as Na^+/H^+ exchange, revealing no significant change in the fractional contribution of Na^+/H^+ exchange or $Na^+/H^+/Cl^-/HCO_3^-$ exchange during acidosis. The effects of acidosis may not include a large enough shift in $Na^+/H^+/Cl^-/HCO_3^-$ exchange to become evident in the rate of oxygen consumption; however, the predominant efficiency of this carrier under acidosis is suggested by the large fall in pHi in the presence of DIDS during normocapnia (Fig. 7A) and the capacity of pHi to recover during hypercapnic acidosis in the absence (Fig. 2) and in the presence (Fig. 4) of DMA at elevated extracellular bicarbonate levels (see above and figure legends for the effective bicarbonate levels).

Some considerations may illustrate the role of membrane permeability in determining the cost of acid–base regulation. With a total cost of ion regulation of approximately 1.8 μmol ATP g^{-1} h^{-1} (calculated from the sum of the effects of ouabain and bafilomycin A on the rate of oxygen consumption assuming glucose as a substrate) at pHe 7.9, H^+-ATPase and Na^+/H^+ exchange would consume approximately 0.65 μmol ATP g^{-1} h^{-1} each (as a minimum estimate for both, see above) in addition to the fraction required by $Na^+/H^+/Cl^-/HCO_3^-$ exchange, which is smaller than the remaining 0.5 μmol ATP g^{-1} h^{-1} since it shares this rate with other processes stimulating Na^+/K^+-ATPase activity. Assuming an average ATP requirement of 0.33 ATP per proton extruded, net proton extrusion would consume approximately 0.15 μmol ATP g^{-1} h^{-1}, which is approximately 10 % of the total energy allocated to acid–base regulation. This emphasizes the relevance of futile cycling in the control of intracellular pH, since approximately 90 % of the energy used by acid–base regulation is consumed just for pH maintenance (no net H^+ extrusion). The ratio of the cost of net proton extrusion to the cost of futile cycling remains more-or-less unchanged during acidosis, indicating that a reduction in proton leakiness during acidosis may coincide with the reduction in energy demand.

Conclusions and perspectives

Together with our previous work (Reipschläger and Pörtner, 1996), the present study confirms that acid–base regulation bears a significant cost and that the modulation of the cost of cellular acid–base regulation is part of an energy-saving strategy during metabolic depression under environmental stress. These observations also extend to the whole organism, in which a significant fraction of the decrease in metabolic rate induced by hypercapnia or anoxia will be associated with a reduction in the rate of acid–base regulation (Pörtner et al., 1991, 1998). The general importance of these relationships in animals is supported by unpublished data collected in tilapia (*Oreochromis alcalicus grahami*) from alkaline Lake Magadi which indicate that the cost of acid–base regulation can even exceed 50 % of baseline metabolic rate under extreme environmental conditions (C. M. Wood, personal communication).

At first sight, our findings conflict with the view derived from molecular studies of vertebrate systems that intracellular pH is a major modulator of Na^+/H^+ exchange (Bianchini and Pouysségur, 1994). This contradiction is only apparent because low pHi may stimulate the Na^+/H^+ exchanger but low pHe may limit its maximum activity, thus contributing to a reduction in metabolic rate. In *S. nudus* muscle tissue, the inhibition of both Na^+/H^+ exchange and Na^+-dependent Cl^-/HCO_3^- exchange and the predominant use of the more ATP-efficient carrier during extracellular acidosis contribute to the reduction in energy turnover that is reflected in a lower Na^+/K^+-ATPase activity. Comparable phenomena may also be involved in the protective effects of acidosis during anoxia in mammalian heart muscle, hepatocytes and renal cortex (Bing et al., 1973; Pentilla and Trump, 1974; Bonventre and Cheung, 1985).

It is of interest in this context that Neylon et al. (1990) and Little et al. (1995) reported that cellular ATP depletion

depresses the activity of the Na^+/H^+ exchanger but not that of Na^+- and HCO_3^--dependent mechanisms in mammalian vascular smooth muscle. The latter are also responsible for pH regulation in anoxic turtle heart (Shi et al., 1997). Taken together, these results suggest that the more efficient HCO_3^--dependent mechanisms may become more important under environmental or other stresses related to energy deficiency. Why are the more ATP-efficient transporters not exclusively preferred? The delayed recovery under the effects of acidosis suggests that the Na^+/H^+ exchanger and H^+-ATPase add flexibility to the baseline rate of acid–base exchange achieved by the Na^+-dependent Cl^-/HCO_3^- exchanger. This increase in flexibility would have to occur at the expense of a higher rate of futile cycling of acid–base regulatory mechanisms and, in consequence, an elevated rate of energy turnover.

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