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Carbonic anhydrase facilitates CO_2 and NH_3 transport across the sarcolemma of trout white muscle

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Henry, Raymond P., Yuxiang Wang, and Chris M. Wood. Carbonic anhydrase facilitates CO_2 and NH_3 transport across the sarcolemma of trout white muscle. *Am. J. Physiol.* 272 (*Regulatory Integrative Comp. Physiol.* 41): R1754–R1761, 1997.—An isolated, perfused tail preparation was used to study the role of carbonic anhydrase (CA) in CO_2 and NH_3 transport across the sarcolemma of white muscle in the rainbow trout. Tissue was perfused with either control saline or saline containing the CA inhibitors quaternary ammonium sulfanilamide (QAS) or acetazolamide (Az). Inhibition of extracellular CA by QAS reduced CO_2 efflux by ~30% and caused a significant increase in intracellular P_{CO_2}. Inhibition of total muscle CA activity (extracellular and intracellular) by Az also caused a reduction in CO_2 efflux, but selective inhibition of intracellular CA only had no effect. Inhibition of both extracellular and intracellular CA activity resulted in increases in total intracellular ammonia concentrations, intracellular NH_3 partial pressure (P_{NH_3}) and an increased P_{NH_3} gradient across the sarcolemma. This suggests that both extracellular and intracellular CA function in normal NH_3 transport out of the muscle. We suggest that CA in the extracellular boundary layer facilitates CO_2 transport via the catalyzed hydration of CO_2, thus maintaining the PCO_2 gradient across the sarcolemma. H ions produced by that reaction serve to protonate excreted NH_3, which helps maintain the P_{NH_3} gradient. Thus CO_2 and NH_3 excretion are linked by the action of CA.

CO_2 excretion; ammonia excretion

THE ENZYME CARBONIC anhydrase (CA) is very widely distributed, both among species and among tissues, cell types, and organelles within individual organisms (e.g., Refs. 1, 5, 18, 32). Furthermore, there is a large body of experimental evidence supporting the role of CA in a variety of physiological and biochemical processes from intermediary metabolism to respiration. For many of these processes the function of CA has been studied primarily on the systemic level. The roles of erythrocyte and vascular CA have been well documented in the transport of CO_2 in blood and its excretion across the gas exchange organs in mammals and lower vertebrates (e.g., Refs. 8, 23, 26).

The limiting step in CO_2 transport, however, is believed to occur at the cellular level. The biochemical reactions that produce metabolic carbon dioxide liberate CO_2 gas (15, 20, 33), but because muscle contains a significant amount of CA activity, intracellular CO_2 is considered to be in instantaneous equilibrium with HCO_3^-. Although CO_2 gas is freely permeable across biological membranes, HCO_3^- is not (6, 24, 31); therefore, transport ultimately depends on the conversion of HCO_3^- to molecular CO_2 (dehydration) in the boundary layer on the "upstream" side of the membrane and the subsequent rate of rehydration in the boundary layer on the "downstream" side (16). These potentially rate-limiting steps are obviated by the presence of CA, which maintains a virtual instantaneous chemical equilibrium among the CO_2 species. It has been suggested that one of the selective pressures for the widespread tissue distribution of CA could be a universal role in CO_2 transport/excretion across the membranes of metabolically active cells (20).

One example of a highly active cell type is skeletal muscle. Originally believed to be absent from muscle, CA is now known to be present in both skeletal and cardiac fibers (9, 14, 15). In mammalian skeletal muscle, the CA II and III isozymes are present in the cytoplasm, with the distribution being correlated with metabolic fiber type. There is also a CA II-like isozyme associated with the sarcolemma (SL) of all fiber types as well as CA activity localized to the sarcoplasmic reticulum (SR). Muscle CA has been suggested as functioning in contraction, Ca^{+2} transport by the SR, neuromuscular transmission, energetics and metabolism, and facilitated CO_2 transport (10, 14, 28).

Outside of mammals, very little is known about the distribution and function of muscle CA. Among lower vertebrates, one report indicates that frog white muscle contains a CA type II isozyme (28), and there is also evidence that fish red muscle has a CA type III isozyme (27). CA function in lower vertebrate muscle has not been directly examined; however, studies on fish gills have shown that acidification of the external boundary layer facilitates the excretion of both CO_2 and NH_3 (37, 39). An analogous situation could exist in fish muscle. CA, associated with the SL and oriented toward the external surface of the cell, would acidify the extracellular boundary layer through the hydration of respiratory CO_2. This study reports on the role of cytoplasmic and SL CA in the cellular transport of CO_2 and NH_3 in white muscle of the trout.

MATERIALS AND METHODS

Experimental animals. Rainbow trout, *Oncorhynchus mykiss* (0.8–1.1 kg), were obtained from Spring Valley Trout Farm (Petersburg, Ontario). Fish were maintained in 800-liter fiberglass cylindrical tanks with flowing dechlorinated tap water at 5–12°C (ionic composition in meq/l as follows: 0.6 Na^+, 0.8 Cl^{-1}, 1.8 Ca^{+2}, 0.5 Mg^{+2}, and 0.04 K^+), pH 8.0. Trout were fed high-protein floating pellets (Aquaculture Zeigler Brothers) three times per week, but before an experiment the fish were starved for 5–7 days to achieve standard metabolic status. Animals were transferred to individual, darkened acrylic flow-through chambers and left undisturbed for 12–24 h before being used.

Isolated tail perfusion. Trout were anesthetized with MS-222 (0.5 g/l neutralized with NaOH) added to the water in the

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isolation box. This resulted in loss of equilibrium and opercular ventilation within 1 min without struggling or any disruption in resting metabolism and acid-base status (36). The tail was cut off at the point of the anus and weighed before perfusion. Animals were then killed by a blow to the head.

Catheters (Clay-Adams PE-90) were implanted into the caudal artery and vein and secured via ligature around the vertebral column. The tail was then submerged in a constant temperature saline bath (15 ± 0.5°C) and immediately perfused at a rate of 2 ml·min^{-1}·100 g tail wt^{-1}. Salmonid Cortland saline (38) plus 3% bovine serum albumin (BSA; fraction V, Sigma) and Na-heparin (50 IU/ml Sigma) was used as the basic perfusion medium. The perfusate was gassed with 0.25% CO_2, balance O_2 (Wosthoff gas-mixing pumps, Bochum, Germany), for at least 60 min in a Plexiglas disk oxygenator to achieve stable pH, P_{O_2}, and P_{CO_2} levels. The perfusate was drawn through a heat exchange coil (15°C) by a peristaltic pump (Gilson Minipuls 3) and delivered to the tail via a windkessel to dampen pressure oscillations. Perfusion pressure was monitored by a Narco Bio-Systems RP-1500 transducer and Gilson ICT-5H polygraph recorder. All tubing was stainless steel (for more details of the perfusion method, see Ref. 34).

Experimental protocol. At the time when the tail was cut off, a muscle sample was taken from the area dorsal to the lateral line and immediately anterior to the point of the cut. This was freeze-clamped with aluminum tongs precooled in liquid N_2 and held in liquid N_2 for analysis at a later time.

The isolated tail was perfused for 30 min to maximize the removal of red blood cells. At that time (T_{30}), venous followed by arterial perfusate samples (2 ml) were collected via the sampling ports with the use of gas-tight Hamilton syringes. For control, resting preparations, the tail was perfused for another 30 min (T_{60}) with nonheparinized saline/BSA, and a second set of venous and arterial perfusate samples were taken. A final muscle sample was taken from the perfused tail at T_{60}, freeze-clamped, and stored in liquid N_2 for later analysis.

Three treatments were used to examine the role of muscle CA in CO_2 and NH_3 transport. First, the tail was perfused with quaternary ammonium sulfanilamide (QAS), a membrane-impermeable CA inhibitor (17). A stock solution of QAS (100 mM) was made in saline; 25 ml was then added to 500 ml of saline/BSA in a second oxygenator to make a working concentration of 5 mM. After the standard 30-min perfusion to remove red blood cells, the tail was perfused for another 30 min with the perfusate containing QAS. This would selectively inhibit any SL-associated CA that has an extracellular orientation. The collection of arterial and venous perfusate samples and muscle samples was performed as in the controls.

The second treatment was designed to inhibit the total complement of CA in muscle. Approximately 16 h before being used in an experiment, trout were lightly anesthetized and injected with acetazolamide (Az) via caudal puncture. A 10 mM Az stock solution in saline was used. The fish was weighed, and total extracellular fluid (ECF) volume was calculated as 25% of body weight. The appropriate volume of Az stock was injected to produce an initial circulating concentration of 100 μM. This was done to give the Az adequate time to permeate across the SL (the sulfonamide inhibitors are known to require times in the range of hours to fully equilibrate across biological membranes; see Ref. 15). In this experiment, both the initial and the second 30-min perfusates contained 100 μM Az to maintain maximal CA inhibition. Arterial and venous perfusate samples were collected at T_{30} and T_{60}, and initial and final muscle samples were taken as described above.

The third treatment was designed to inhibit intracellular (cytoplasmic) CA only. Fish were preinjected with Az and perfused for the initial 30 min with Az as above. In this case, however, the tails were perfused with control saline for the second 30 min to wash out the Az from the extracellular space. This would presumably restore activity to any CA associated with the extracellular surface of the SL while leaving intracellular CA inhibited due to the slow permeability of Az. Again, perfusate and muscle samples were taken as above.

Sample analysis. Arterial and venous perfusate samples were analyzed for pH, total CO_2, P_{O_2}, protein, and water content immediately on sampling. Perfusate pH was measured using Radiometer electrodes (G297-G2 capillary pH and K497 calomel reference electrodes) thermostatted to the experimental temperature and connected to a Radiometer PHM 84 pH meter. P_{O_2} was measured using a thermostatted Radiometer O_2 electrode (E5046-O) and PHM 72 acid base analyzer. Total CO_2 was determined for 100-μl samples on a Corning 965 total CO_2 analyzer. Total protein and water content were measured with the use of a refractometer (American Optical).

The remaining volume of perfusate was frozen in liquid N_2 and stored at −80°C for later analysis. These samples were thawed, deproteinized in 8% perchloric acid (PCA), and analyzed for total ammonia concentration (T_{Amm}) enzymatically at 340 nm (LKB UltraspecPlus 4053 spectrophotometer) via the glutamate dehydrogenase method (Sigma 171-A).

Frozen muscle tissue was ground to a fine powder under liquid N_2 in a precooled mortar and pestle. Extraneous skin and bone were removed. Intracellular pH (pH_i) was measured using the freeze-clamp method of Portner et al. (25). Approximately 200 mg of the powder was then mixed with 1 ml of a metabolic inhibitor cocktail consisting of 150 mM KF and 6 mM nitrilotriacetic acid (Na salt); mixing was accomplished with the use of an Omni 1000 portable homogenizer with 5-mm generator rotating at 15,000 revolutions/min. The homogenate was then centrifuged at 9,000 *g* for 30 s in a sealed 1.5-ml microfuge tube. Duplicate 50-μl samples were used, and pH was measured as described above. Duplicate 200-μl samples were used to measure total intracellular CO_2 concentration, also by the method described above.

Approximately 100 mg of the powder was added to 1 ml of 8% PCA to deproteinize the homogenate. T_{Amm} was analyzed in the supernatant enzymatically via the glutamate dehydrogenase method as modified by Kun and Kearney (21).

For the measurement of tissue lactate concentration, ~20 mg of the lyophilized muscle powder was extracted in 1 ml of cold, 8% PCA for 1 h, then centrifuged at 9,000 *g* for 2 min. The supernatant was neutralized with tris(hydroxymethyl)-aminomethane (Tris) buffer and analyzed for lactate spectrophotometrically using the lactate dehydrogenase NAD/NADH assay (35).

Water content was determined by drying fresh tissue (0.5–1.0 g) to a constant weight at 80°C.

Carbonic anhydrase assay. Frozen tissue was shipped on dry ice to Auburn University via overnight carrier and stored at −80°C until analysis. For assay of control and inhibitor-treated samples, ~250 mg of muscle was thawed at 0°C and added to 2 ml of buffer (in mM: 75 mannitol, 225 sucrose, 10 Tris-PO_4, pH 7.40). The tissue was homogenized for 30 s with the use of an Omni 1000 motor-driven portable homogenizer with 10-cm generator. The homogenate was then mildly sonicated (35 W for 15 s, Heat Systems Microson) and centrifuged at 10,000 *g* for 20 min at 4°C (Sorvall RC5-B). The

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supernatant was analyzed for CA activity using the electrometric delta pH method described by Henry (19).

A second set of muscle was used to assay for cytoplasmic (soluble) and microsomal (membrane-associate) CA activity. Approximately 5 g of red and white muscle were added to 20 ml of homogenization buffer. The muscle was minced into small pieces using scissors and homogenized in a motor-driven Teflon/glass homogenizer. The homogenate was centrifuged at 7,500 *g* for 20 min at 4°C (Sorvall RC5-B) to remove large fragments, nuclei, and mitochondria. The supernatant, containing microsomes and cytoplasm, was then centrifuged at 100,000 *g* for 60 min at 4°C (Beckman L8M). The resultant pellet and supernatant were assayed as described above. Raw CA activity (μmol $CO_2 \cdot$ml sample$^{-1} \cdot$min^{-1}) was multiplied by the total sample volume (in ml) to yield total CA activity in the fraction.

Calculations and statistics. Values for ECF volume (ml/g) and intracellular fluid (ICF) volume (ml/g) of white muscle were taken from Wang et al. (34); these were obtained from estimates of muscle water content and the Cl-K space.

Oxygen consumption ($\dot{M}O_2$) and CO_2 efflux ($\dot{M}CO_2$) were calculated from the rate of perfusion and the differences in gas content between arterial and venous perfusate samples using the Fick principle. For O_2 the equation is as follows

$$\dot{M}O_2(\text{mmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}) = R_{\text{perfusion}} \times \alpha O_2 \times \Delta PO_2$$

where the rate of perfusion (R) was adjusted to 1.2 l$\cdot$kg$^{-1}\cdot$h^{-1}, ΔPO_2 is the partial pressure difference between arterial and venous samples (Torr), and αO_2 (1.77 μmol$\cdot$l$^{-1}\cdot$Torr^{-1}) is the solubility coefficient of O_2 at 15°C (2). For the analogous equation involving $\dot{M}CO_2$, ΔTCO_2 was used because arterial and venous total CO_2 concentrations were measured directly.

Ammonia efflux was also calculated using the Fick principle. The distribution of the gaseous (NH_3) and ionized (NH_4^+) forms was calculated from a modification of the Henderson-Hasselbach equation

$$NH_3 = \frac{T_{Amm} \times \text{antilog}(pH - pK)}{1 + \text{antilog}(pH - pK)}$$

Ammonium was calculated as the difference between the measured value for total ammonia and the calculated value for NH_3 gas

$$NH_4^+ = T_{Amm} - [NH_3]$$

where pH was measured and values for pK were taken from Cameron and Heisler (3). The partial pressure of NH_3 (P_{NH_3}) gas was calculated from the following equation

$$P_{NH_3} = \frac{[NH_3]}{\alpha NH_3}$$

where αNH_3 is the solubility coefficient at the appropriate ionic strength for ammonia taken from Cameron and Heisler (3).

All data were reported as means ± SE; *n* values are given in parentheses. Significant differences between means were evaluated by one-way analysis of variance (ANOVA). If the ANOVA indicated significance ($P < 0.05$), then post hoc comparisons were performed using Duncan's multiple-range and critical-range tests. Student's paired *t*-test was used to evaluate differences within treatment groups between T_{30} and T_{60} values. Simple, unpaired *t*-tests were used to evaluate differences between control and inhibitor-treated preparations.

Table 1. *Total CA activity in cytoplasmic and microsomal fractions of perfused red and white muscle from rainbow trout*

Sample	CA Activity	
	μmol CO_2/min	%Total activity
White muscle (4.51 ± 0.5 g sample)		
Cytoplasm	1,068 ± 241	95.6 ± 1.9
Microsomes	47.3 ± 8.6	4.4 ± 0.3
Red muscle (5.02 ± 0.5 g sample)		
Cytoplasm	1,046 ± 215	94.6 ± 2.1
Microsomes	56.4 ± 7.8	5.4 ± 0.6

Values are means ± SE ($n = 4$). CA, carbonic anhydrase.

RESULTS

Muscle carbonic anhydrase activity. Both the cytoplasmic and microsomal fractions of red and white muscle had measurable amounts of CA activity. There was no significant difference in CA activity between the two fiber types with regard to either fraction ($P > 0.05$, *t*-test; Table 1). Most of the CA activity was found in the cytoplasm, with the microsomal fraction accounting for only ~5% of the total. For white muscle, cytoplasmic CA showed a high sensitivity to Az in vitro, and microsomal CA was sensitive to QAS; activity in both fractions was completely abolished in vitro by the inhibitor concentrations used in the perfusion experiments (data not shown).

The in vivo administration of the different inhibitor protocols produced a pattern of inhibition, measured in vitro, that was generally consistent with the properties of the inhibitors. Perfusion with QAS resulted in only a 40% inhibition of muscle CA (Table 2), which is consistent with the drug's reported membrane impermeability (17). The comparatively high inhibition, relative to the low amount of CA in the microsomal fraction of the muscle, is probably a result of residual QAS in the ECF that gained access to cytoplasmic CA during homogenization. The procedure did not allow for the inhibitor to be washed out of the tail before the tissue sample was taken for analysis. Perfusion with Az, followed by washout with saline (Az_{ICF}), resulted in ~78% CA inhibition (Table 2). Again, according to the distribution of CA activity, this value was lower than expected; however, there is a significant amount of dilution of the sample during the assay, which could result in dissociation of the Az from the enzyme and consequently give

Table 2. *CA activity in resting white muscle in response to perfusion with saline (control), Az followed by saline washout (presumptive ICF inhibition), without washout (presumptive total CA inhibition), and perfusion by QAS (presumptive ECF inhibition)*

	Control	Az_{ICF}	Az_{tot}	QAS
CA activity, μmol $CO_2\cdot g^{-1}\cdot min^{-1}$	910 ± 143	202 ± 12	138 ± 12	542 ± 47
%Inhibition	0	77.8	84.8	40.4

Values are means ± SE ($n = 5$). Az, acetazolamide; ICF, intracellular fluid; QAS, quaternary ammonium sulfanilamide; ECF, extracellular fluid; tot, total.

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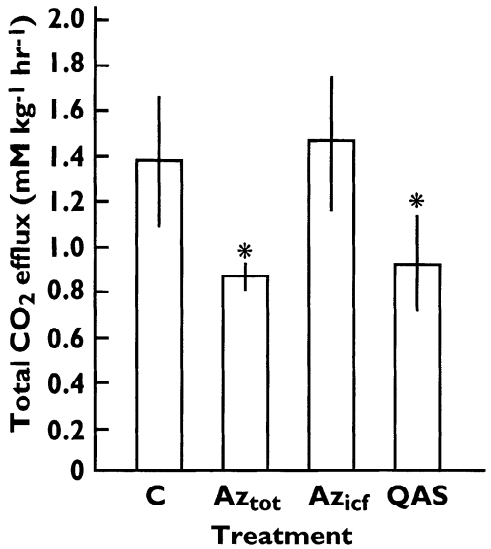

Fig. 1. Total CO_2 efflux from resting, perfused tail white muscle. Means ± SE, n = 5 or 6, temperature = 15°C. C, tails perfused with control saline; Az_{tot}, fish preinjected with acetazolamide (Az) and tails perfused with Az-saline for the full 60-min experiment; Az_{icf}, fish preinjected with Az and tails perfused with Az-saline for the first 30 min, followed by perfusion with control saline for the second 30 min of the experiment; QAS, tails perfused with saline containing quaternary ammonium sulfanilamide. See MATERIALS AND METHODS for details of inhibitor concentrations. ifc, Intracellular fluid. *Statistically significant compared with control.

an artificially low percent inhibition. For perfusion with Az for the entire experiment (Az_{tot}), there was still some measurable CA activity (15%). Although these values represent very conservative estimates of actual in vivo inhibition, it is possible that there was some residual intracellular CA activity in the two Az treatments.

CO_2 efflux and intracellular acid-base status. Two of the three experimental treatments caused a reduction in CO_2 efflux compared with saline-perfused tails. Perfusion with QAS (ECF inhibition only) and Az without washout (Az_{tot}, ECF and presumptive ICF inhibition) both resulted in an ~35% reduction in CO_2 excretion from white muscle (Fig. 1). Presumptive intracellular CA inhibition only (Az_{ICF}) did not have a significant effect.

None of the treatments altered $\dot{M}O_2$ of the muscle preparation (Table 3). This indicates that perfusion with the various inhibitors did not change metabolic CO_2 production. Changes in respiratory quotient occurred for the QAS and Az_{tot} perfusions, but these were due to reductions in CO_2 efflux.

Table 3. *$\dot{M}O_2$, $\dot{M}CO_2$, and RQ in isolated tail preparations perfused with BSA/saline alone (control), saline plus QAS, saline plus Az followed by saline washout (Az_{ICF}), and saline plus Az (Az_{tot})*

Sample	$\dot{M}O_2$	$\dot{M}CO_2$	RQ
Control	0.80 ± 0.04	1.41 ± 0.19	1.74 ± 0.31
QAS	0.85 ± 0.13	0.92 ± 0.21	1.28 ± 0.14
Az_{ICF}	0.93 ± 0.04	1.45 ± 0.31	1.86 ± 0.36
Az_{tot}	0.89 ± 0.07	0.88 ± 0.07	1.19 ± 0.02

Values are means ± SE in mM·kg^{-1}·h^{-1} (n = 5 or 6; temperature, 15°C). $\dot{M}O_2$, oxygen uptake; $\dot{M}CO_2$, carbon dioxide efflux; RQ, respiratory quotient; BSA, bovine serum albumin.

CA inhibition had a less pronounced effect on the intracellular acid-base status of white muscle. None of the three treatments either significantly lowered intracellular pH (pH_i) or significantly elevated intracellular total CO_2 concentrations. Perfusion with QAS resulted in a significant elevation of intracellular P_{CO_2} by ~3 Torr (Fig. 2). An increase of ~1 Torr was seen for total CA inhibition (Az_{tot}), but the high variability in that group resulted in there being no statistically significant change.

No treatment altered the resting concentrations of lactate in the tail muscle preparation (data not shown).

Ammonia efflux and intracellular ammonia levels. Ammonia efflux from white muscle was highly variable, even within a single experimental group. Inhibition of CA putatively associated with the SL and having an

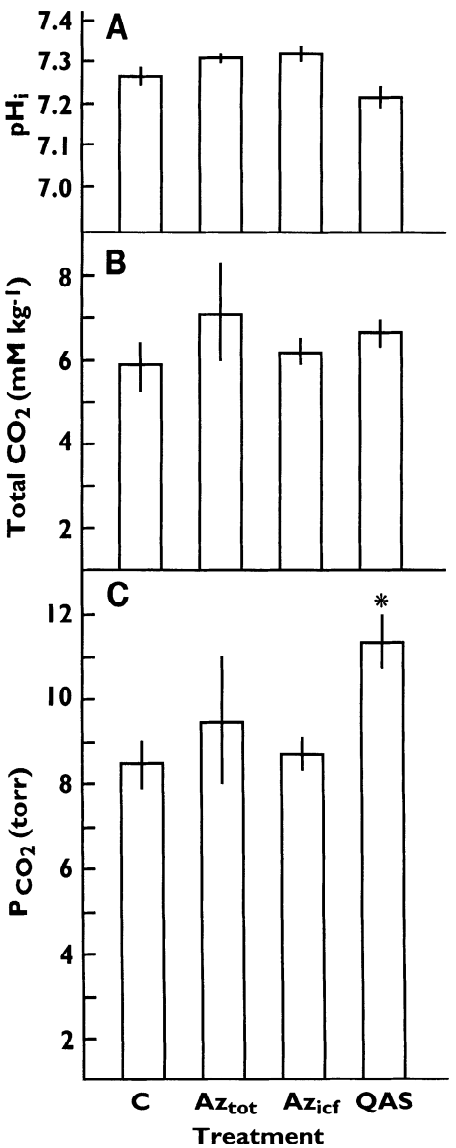

Fig. 2. Intracellular pH (pH_i; *A*), total CO_2 concentration (*B*), and P_{CO_2} (*C*) from resting, perfused tail muscle. Means ± SE, n = 5 or 6, temperature = 15°C. Treatments are same as described in Fig. 1. *Statistically significant compared with control.

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extracellular orientation (QAS treatment) resulted in an ~30% reduction in ammonia efflux, as did presumptive total muscle CA inhibition using Az (Az_{tot}); however, variability within and among groups (especially the control) was so high that these differences were statistically insignificant (Fig. 3).

The effects of CA inhibition were more pronounced on intracellular ammonia variables. All treatments resulted in elevated total intracellular ammonia concentrations, which were between double and triple the resting, control values (Fig. 4). Because of the very high variability in the Az_{tot} group, however, only the Az_{ICF} and the QAS treatments were statistically significant compared with the control. The high intracellular ammonia concentrations translated to an approximate doubling of the intracellular P_{NH_3} in all three of the inhibitor treatments (Fig. 4). Again, however, because of high variability, the increase in the Az_{tot} group was not significant, whereas the other two were. Finally, the P_{NH_3} gradient across the SL was increased from three- to fourfold, with the increases in the Az_{ICF} and QAS groups being statistically significant (Fig. 4).

DISCUSSION

Trout white muscle appears to be similar in its distribution of CA to white muscle to other lower vertebrates and to mammals. There is both cytoplasmic and microsomal CA activity, with the cytoplasmic fraction having the bulk (95%) of the total activity. This profile is similar to that of mammalian white skeletal muscle in which cytoplasmic CA activity accounted for 85% of the total and the remaining 15% was assigned to membranes and intracellular organelles (29). The high sensitivity to Az also suggests that, as in other species, trout white muscle contains a type II isozyme.

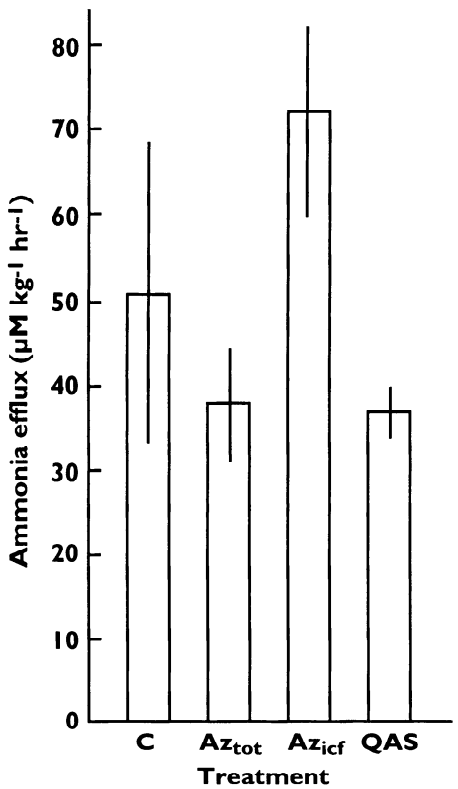

Fig. 3. Total ammonia efflux from resting, perfused tail muscle. Means ± SE, n = 5 or 6, temperature = 15°C. Treatments are the same as described in Fig. 1.

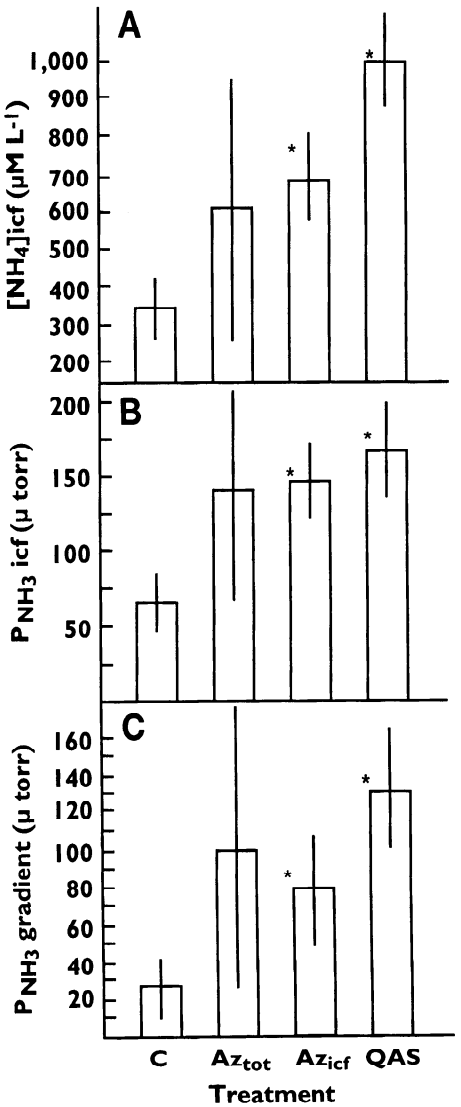

Fig. 4. Total intracellular ammonia concentration ($[NH_4]_{icf}$; A), intracellular partial pressure of NH_3 (P_{NH_3}; B), and trans-SL P_{NH_3} gradient (C) for resting, perfused tail muscle. Means ± SE, n = 5 or 6, temperature = 5°C. Treatments are the same as described in Fig. 1. *Statistically significant compared with control.

The reduction in muscle M_{CO_2} resulting from two of the inhibitor treatments (Az_{tot} and QAS, Fig. 1) strongly suggests that a portion of the microsomal CA activity is localized on the SL, with an extracellular orientation, and that this pool of CA functions in the facilitated transport of CO_2 out of the muscle tissue. In both treatments, inhibitor concentrations in the ECF were well over 100 times the inhibition constant (K_i) values for the enzyme (K_i values for microsomal CA were in the nanomolar range for Az and micromolar range for QAS; R. Henry, unpublished data), ensuring that any putative extracellular CA was fully inhibited.

In mammalian systems, experimental approaches similar to those used here have previously shown the

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presence of CA activity on the SL, having an ECF orientation, and functioning in CO_2 hydration/dehydration. For example, in the isolated-perfused dog hindlimb preparation, injected ^{14}C-labeled HCO_3^- was rapidly converted to CO_2, which then freely diffused throughout the entire tissue space, resulting in a slow washout of the label (40). Treatment with Az reduced the CO_2 space to essentially the vascular space. In addition, the washout time for ^{14}C-labeled bicarbonate was greatly reduced by Az treatment in both perfused cat and rabbit hindlimb preparations (7, 12), indicating that, under normal conditions, the labeled HCO_3^- in the perfusate had access to an extracellular CA. Furthermore, experiments using the CA inhibitor Prontosil bound to dextran molecules of increasing molecular weights, showed that the ECF CA activity of muscle was inhibited only by those complexes that were not so large as to be restricted from crossing the capillary wall (11). Thus it was concluded that muscle CA was confined to the interstitial space (i.e., the ECF surface of the SL) and absent from the general vasculature. The similar results reported here represent the first evidence for the presence of an ECF-oriented CA in the SL of muscle in lower vertebrates. Of the two treatments that reduced CO_2 efflux from muscle, only one (QAS) caused a significant change in intracellular acid-base status. The resultant increase in intracellular P_{CO_2} supports the idea that extracellular CA localized on the SL plays a role in facilitating CO_2 transport out of muscle cells. This is probably accomplished by the rapid hydration of excreted CO_2, forming H^+ and HCO_3^- in the external boundary layer of the cell, thus helping to maximize the P_{CO_2} gradient across the membrane. The fact that the changes in intracellular acid-base status were not more uniform or greater in magnitude was not surprising. Geers and Gros (13) calculated that CA inhibition in mammalian skeletal muscle should produce a maximum depression of pH_i of 0.1 U based on a reduction of facilitated CO_2 transport alone. They also reported a range of pH depression from 0.1 to 0.8 U, but this was after a simulated bout of exercise via electrical stimulation and after a much longer time course of inhibition (2–4 h).

Resting trout white muscle has a rate of CO_2 production that is only one-tenth that of mammalian muscle [0.1 (calculated from Fig. 1) vs. 1.0 ml $CO_2 \cdot 100\ g^{-1} \cdot min^{-1}$ (Ref. 13)], and the perfused tail preparation was followed for a maximum of 1 h. CA inhibition at the systemic level of CO_2 transport (i.e, the red blood cells and the lungs or gills) results in a transient decrease in whole-organism CO_2 excretion until the CO_2 accumulates in the blood to a level where normal $\dot{M}_{CO_2}$ is restored but at a higher P_{CO_2} gradient. It is possible, therefore, that over the 30- to 60-min experimental time course used in trout, CO_2 was still accumulating in the intracellular space of the muscle and had not built up to a point where either normal tissue $\dot{M}_{CO_2}$ was restored or intracellular acid-base status was significantly perturbed. Furthermore, the intracellular space of muscle is extremely well buffered (β = 73.6 slykes, Ref. 22), adding to the difficulty of altering pH_i. This situation would be exacerbated if ammonia transport and CO_2 transport were linked at the cellular level, and an inhibition of CO_2 efflux also resulted in a reduction of ammonia excretion. If so, there would be a concomitant accumulation of CO_2 and NH_3, the latter being protonated to NH_4^+ and thus consuming an H^+ ion and ameliorating any potential intracellular respiratory acidosis.

The results of the QAS and Az treatments suggest that CO_2 and NH_3 transport are linked, and that extracellular CA inhibition affects both processes. Both QAS and Az_{tot} caused a 30% reduction in NH_3 excretion, although high variability in the control value made this difference statistically insignificant. Interestingly, changes in the intracellular ammonia variables were much more demonstrable. Total intracellular ammonia concentration increased two- to threefold after the three inhibitor treatments, although the increase for Az_{tot} was not statistically significant because of high variability. Additionally, the calculated intracellular P_{NH_3} tripled for all three treatments, with Az_{ICF} and QAS being significantly increased. The ultimate result was an increase in the P_{NH_3} gradient across the SL by four- to sixfold.

These results suggest that CA function is necessary for normal ammonia efflux from muscle tissue, and that both membrane-associated CA in the boundary layer of the ECF and CA in the ICF are involved. The role of CA on the SL and localized in the boundary layer of the ECF is probably similar for both CO_2 and NH_3 efflux. The hydration of excreted CO_2 produces protons that could then combine with excreted NH_3, forming NH_4^+ in the ECF boundary layer. The result would be a reduction in both molecular CO_2 and NH_3 in the external boundary layer that would help maintain the partial pressure gradients of both gasses across the SL.

If this is the case, then the mechanism of cellular ammonia transport (i.e., across the SL of muscle cells) appears to be similar to that which is believed to occur during systemic ammonia excretion (from blood to water) across a complex epithelium, the fish gill. Ammonia is believed to move across the gill primarily as NH_3 gas despite the fact that, at physiological pH, <3% of the total ammonia is in the gaseous form (4, 37). Diffusion appears to take place along a small gradient of 40–50 μTorr (3, 4). Protonation of excreted NH_3 in the external boundary layer of the gill has been shown to be important in maintaining normal ammonia excretion rates in fish (37, 39). Wang et al. (34) have also shown that removal of available protons in the ECF boundary layer reduces ammonia efflux in trout muscle. Therefore, acidification of the external boundary layer, and the resultant conversion of NH_3 to NH_4^+, appears to be an important component of facilitated ammonia diffusion at both the systemic and cellular levels (34, 37, 40).

One possible difference between extracellular acidification at the systemic (branchial) vs. cellular (SL) level may be in the source of the protons. CA has been suggested as being present on the external surface of the gill and playing a role in both systemic CO_2 and

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NH_3 excretion by generating protons through the catalysis of the hydration of excreted CO_2 (39). However, branchial ammonia excretion is reduced by amiloride (39) in the absence of any CA inhibitors, potentially reducing ammonia excretion by limiting extracellular boundary layer protons through the inhibition of Na^+-linked H^+ excretion. It is possible that direct excretion of H^+ ions could play a significant role in branchial exctracellular acidification.

This does not appear to be the case for ammonia excretion at the cellular level across the SL. Ammonia efflux is amiloride-insensitive (34), indicating that neither Na^+/NH_4^+ exchange nor protons excreted by Na^+/H^+ exchange were contributing to the acidification of the extracellular boundary layer fluid. The large increase in the transmembrane P_{NH_3} gradient in response to CA inhibition strongly suggests that the source of H^+ for extracellular acidification is the catalyzed hydration of respiratory CO_2 by CA associated with the SL membrane.

Interestingly, presumptive inhibition of CA in the ICF also appeared to cause a reduction in ammonia efflux, as evidenced by the increased P_{NH_3} gradient. This suggests that cytoplasmic CA is also important for normal efflux. The most probable mechanism for this would be for CA in the intracellular boundary layer to act as a proton shuttle between the ammonia and carbon dioxide pools. As NH_3 diffuses across the SL, protons would dissociate from NH_4^+ to reestablish the normal equilibrium between the two species. Those protons could then serve in the dehydration of HCO_3^- to molecular CO_2 to reestablish the equilibrium among those chemical species as molecular CO_2 diffuses out of the muscle cell. The action of CA would prevent the buildup of H^+ ions in the intracellular boundary layer that otherwise might shift the equilibrium among the chemical species of ammonia to NH_4^+, reduce the P_{NH_3} gradient, and impair NH_3 excretion. This would also be advantageous for CO_2 efflux, because the rapid consumption of H^+ in the catalyzed dehydration of bicarbonate would help maintain the necessary intracellular P_{CO_2} level to drive diffusion across the SL. It therefore appears that CO_2 and NH_3 excretion are linked not only on the systemic level (at the gill) but also at the cellular level, and that the molecular link between the two processes is the action of CA.

Perspectives

CO_2 transport across biological membranes is a complex process, involving both the dehydration of HCO_3^- to CO_2 gas and the actual diffusion of CO_2 across the membrane. CA has been shown, in model systems, to facilitate this process by preventing uncatalyzed dehydration of HCO_3^- in the boundary layer on the upstream side of the membrane from being rate limiting. This report represents the first direct experimental evidence in vivo of CA-facilitated CO_2 excretion across a biological membrane. Furthermore, CA on the extracellular surface (the downstream side) of the membrane also was shown to be functionally important in CO_2 transport. This could have evolved in response to the relatively low metabolic rates characteristic of ectothermic lower vertebrates, and the consequent transmembrane diffusion of CO_2 down a relatively small P_{CO_2} gradient. Having CA in the boundary layer on both sides of the membrane maximizes the transmembrane P_{CO_2} gradient. Membrane CA activity is low but appears to be present in excess of what is needed to maintain resting CO_2 excretion; it would be interesting to examine whether this putative excess plays a more important role under conditions of increased CO_2 production such as exercise. In addition, CA-catalyzed hydration of CO_2 in the extracellular boundary layer appears to facilitate transmembrane NH_3 transport.

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REFERENCES

1. **Bauer, C., G. Gros, and H. Bartels** (Editors). *Biophysics and Physiology of Carbon Dioxide*. New York: Springer-Verlag, 1980.
2. **Boutilier, R. G., T. A. Heming, and G. K. Iwama.** Appendix: physicochemical parameters for use in fish respiratory physiology. In: *Fish Physiology*, edited by W. S. Hoar and D. J. Randall. New York: Academic, 1984, p. 403–430.
3. **Cameron, J. N., and N. Heisler.** Studies of ammonia in the rainbow trout: physico-chemical parameters, acid-base behaviour and respiratory clearance. *J. Exp. Biol.* 105: 107–125, 1983.
4. **Cameron, J. N., and N. Heisler.** Ammonia transfer across fish gills: a review. In: *Proceedings in Life Sciences: Circulation, Respiration, and Metabolism*, edited by R. Gilles. Heidelberg, Germany: Springer-Verlag, 1985, p. 91–100.
5. **Dodgson, S. J., R. E. Tashian, G. Gros, and N. D. Carter** (Editors). *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*. New York: Plenum, 1991.
6. **Effros, R. M., G. Mason, and P. Silverman.** Role of perfusion and diffusion in $^{14}CO_2$ exchange in the rabbit lung. *J. Appl. Physiol.* 51: 1136–1144, 1981.
7. **Effros, R. M., and M. L. Weissman.** Carbonic anhydrase activity of the cat hind leg. *J. Appl. Physiol.* 47: 1090–1098, 1979.
8. **Forster, R. E., S. Nioka, R. P. Henry, S. J. Dodgson, and B. T. Storey.** Lung carbonic anhydrase. *Prog. Respir. Res.* 21: 41–46, 1986.
9. **Fremont, P., P. M. Charest, C. Cote, and P. A. Rogers.** Distribution and ultrastructural localization of carbonic anhydrase III in different skeletal muscle fiber types. In: *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*, edited by S. J. Dodgson, R. E. Tashian, G. Gros, and N. D. Carter. New York: Plenum, 1991, p. 241–246.
10. **Fremont, P., H. Riverin, J. Frenette, P. A. Rogers, and C. Cote.** Fatigue and recovery of rat soleus muscle are influenced by inhibition of an intracellular carbonic anhydrase isoform. *Am. J. Physiol.* 260 (*Regulatory Integrative Comp. Physiol.* 29): R615–R621, 1991.
11. **Geers, C., G. Gros, and A. Gartner.** Extracellular carbonic anhydrase of skeletal muscle associated with the sarcolemma. *J. Appl. Physiol.* 59: 548–558, 1985.
12. **Geers, C., and G. Gros.** Inhibition properties and inhibition kinetics of an extracellular carbonic anhydrase in perfused skeletal muscle. *Respir. Physiol.* 56: 269–287, 1984.
13. **Geers, C., and G. Gros.** Effects of carbonic anhydrase inhibitors on contraction, intracellular pH and energy-rich phosphates of rat skeletal muscle. *J. Physiol. (Lond.)* 423: 279–297, 1990.
14. **Geers, C., and G. Gros.** Muscle carbonic anhydrase: function in muscle contraction and in the homeostasis of muscle pH and PCO_2. In: *The Carbonic Anhydrases: Cellular Physiology and*

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Molecular Genetics, edited by S. J. Dodgson, R. E. Tashian, G. Gros, and N. D. Carter. New York: Plenum, 1991, p. 227–239.

15. **Gros, G., and S. J. Dodgson.** Velocity of CO_2 exchange in muscle and liver. *Annu. Rev. Physiol.* 50: 669–694, 1988.
16. **Gutknecht, J., M. A. Bisson, and F. C. Tosteson.** Diffusion of carbon dioxide through lipid bilayer membranes: effects of carbonic anhydrase, bicarbonate and unstirred layers. *J. Gen. Physiol.* 69: 779–794, 1977.
17. **Henry, R. P.** Quaternary ammonium sulfanilamide: a membrane-impermeant carbonic anhydrase inhibitor. *Am. J. Physiol.* 252 (*Regulatory Integrative Comp. Physiol.* 21): R959–R965, 1987.
18. **Henry, R. P.** Multiple functions of crustacean gill carbonic anhydrase. *J. Exp. Zool.* 248: 19–24, 1988.
19. **Henry, R. P.** Techniques for measuring carbonic anhydrase activity in vitro. The electrometric delta pH and pH stat methods. In: *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*, edited by S. J. Dodgson, R. E. Tashian, G. Gros, and N. D. Carter. New York: Plenum, 1991, p. 119–125.
20. **Henry, R. P.** Multiple roles of carbonic anhydrase in cellular transport and metabolism. *Annu. Rev. Physiol.* 58: 523–538, 1996.
21. **Kun, E., and E. B. Kearney.** Ammonia. In: *Methods of Enzymatic Analysis*, edited by U. Bergmeyer. New York: Academic, 1971, p. 1802–1806.
22. **Milligan, C. L., and C. M. Wood.** Tissue intracellular acid-base status and the fate of lactate after exhaustive exercise in the rainbow trout. *J. Exp. Biol.* 123: 123–144, 1986.
23. **Perry, S. F.** Carbon dioxide excretion in fishes. *Can. J. Zool.* 64: 565–572, 1986.
24. **Perry, S. F., P. S. Davie, C. Daxboeck, and D. J. Randall.** A comparison of CO_2 excretion in a spontaneously ventilating blood-perfused trout preparation and saline-perfused gill preparations: contribution of the branchial epithelium and red blood cell. *J. Exp. Biol.* 101: 47–60, 1982.
25. **Portner, H. O., R. G. Boutilier, Y. Tang, and D. P. Toews.** Determination of intracellular pH and PCO_2 after metabolic inhibition by fluoride and nitrilotriacetic acid. *Respir. Physiol.* 81: 255–274, 1990.
26. **Roughton, F. J. W.** Recent work on carbon dioxide transport by the blood. *Physiol. Rev.* 15: 241–296, 1935.
27. **Sanyal, G., E. R. Swenson, and T. H. Maren.** The isolation of carbonic anhydrase from the muscle of *Squalus acanthias* and *Scomber scombrus*: inhibition studies. *Bull. Mt. Desert Island Biol. Lab.* 24: 66–68, 1982.
28. **Scheid, P., and W. Siffert.** Effects of inhibiting carbonic anhydrase on isometric contraction of frog skeletal muscle. *J. Physiol. (Lond.)* 361: 91–101, 1985.
29. **Siffert, W., and G. Gros.** Carbonic anhydrase C in white skeletal muscle tissue. *Biochem. J.* 205: 559–566, 1982.
30. **Tang, Y., and R. G. Boutilier.** White muscle intracellular acid-base and lactate status following exhaustive exercise: a comparison betweeen freshwater and seawater adapted rainbow trout. *J. Exp. Biol.* 156: 153–171, 1991.
31. **Tang, Y., H. Lin, and D. J. Randall.** Compartmental distributions of carbon dioxide and ammonia in rainbow trout at rest and following exercise, and the effect of bicarbonate infusion. *J. Exp. Biol.* 169: 235–249, 1992.
32. **Tashian, R. E., and D. Hewett-Emmett** (Editors). Biology and chemistry of the carbonic anhydrases. *Ann. NY Acad. Sci.* 429: 640, 1984.
33. **Walsh, P. J., and R. P. Henry.** Carbon dioxide and ammonia metabolism and excretion. In: *Biochemistry and Molecular Biology of Fishes*, edited by P. W. Hochachke and T. P. Mommsen. New York: Elsevier, 1991, vol. 1, p. 181–207.
34. **Wang, Y., G. J. F. Heigenhauser, and C. M. Wood.** Ammonia movement and distribution after exercise across white muscle cell membranes in rainbow trout. *Am. J. Physiol.* 271 (*Regulatory Integrative Comp. Physiol.* 40): R738–R749, 1996.
35. **Wang, Y., G. J. F. Heigenhauser, and C. M. Wood.** Lactate and metabolic H^+ transport and distribution after exercise in rainbow trout white muscle. *Am. J. Physiol.* 271 (*Regulatory Integrative Comp. Physiol.* 40): R1239–R1250, 1996.
36. **Wang, Y., M. P. Wilkie, G. J. F. Heigenhauser, and C. M. Wood.** The analysis of metabolites in rainbow trout white muscle: a comparison of different sampling and processing methods. *J. Fish Biol.* 45: 855–873, 1994.
37. **Wilson, R. W., P. M. Wright, S. Munger, and C. M. Wood.** Ammonia excretion in freshwater rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for Na^+/NH_4^+ exchange. *J. Exp. Biol.* 191: 37–58, 1994.
38. **Wolf, K.** Physiological salines for freshwater teleosts. *Prog. Fish Cult.* 25: 135–140, 1963.
39. **Wright, P. A., D. J. Randall, and S. F. Perry.** Fish gill water boundary layer: a site of linkage between carbon dioxide and ammonia excretion. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* 158B: 627–635, 1989.
40. **Zborowska-Sluis, D. T., A. L'Abbate, and G. A. Klassen.** Evidence of carbonic anhydrase activity in skeletal muscle: a role for facilitative carbon dioxide transport. *Respir. Physiol.* 21: 341–350, 1974.

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