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YUXIANG WANG,[1,2] RAYMOND P. HENRY,[3] PAMELA M. WRIGHT,[2]
GEORGE J. F. HEIGENHAUSER,[2] AND CHRIS M. WOOD[2]
[2]*Department of Biology and Department of Medicine, McMaster University, Hamilton, Ontario, Canada L8S 4K1;* [3]*Department of Zoology, Auburn University, Auburn, Alabama 36849–5414; and* [1]*Division of Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Science, University of Miami, Miami, Florida 33149*

Wang, Yuxiang, Raymond P. Henry, Pamela M. Wright, George J. F. Heigenhauser, and Chris M. Wood. Respiratory and metabolic functions of carbonic anhydrase in exercised white muscle of trout. *Am. J. Physiol.* 275 (*Regulatory Integrative Comp. Physiol.* 44): R1766–R1779, 1998.—Electrical stimulation of a trout saline-perfused trunk preparation resulted in metabolic and respiratory responses comparable to those occurring after exhaustive exercise in vivo. Recovery of intracellular acid-base status and glycogen resynthesis were faster than in vivo. Intracellular carbonic anhydrase (ICF CA) blockade elevated intracellular P_{CO_2} relative to untreated postexercise controls, whereas extracellular CA (ECF CA) blockade did not, in contrast to previous work with muscle at rest. ECF CA blockade had only a transient effect on postexercise CO_2 and ammonia efflux. The relatively small pool of membrane-associated CA appears to be overwhelmed by exercise-induced CO_2 production in muscle. Transmembrane ammonia efflux appears to shift from diffusion primarily as NH_3 at rest, which is facilitated by ECF CA, to movement predominantly as NH_4^+ after exercise, which is independent of CA. The postponed recovery of intracellular pH caused by either or both ECF and ICF CA inhibition was consistent with reduced metabolic acid and lactate excretion from muscle. Creatine phosphate resynthesis was delayed by CA inhibition, whereas ATP replenishment was not affected. Delayed glycogen recovery indicates that HCO_3^--dependent pathway(s) may be involved in glyconeogenesis.

acid-base regulation; carbonic anhydrase inhibition; ammonia; energy-rich phosphates; glycolytic metabolism; benzolamide; acetazolamide

CARBONIC ANHYDRASE (CA) catalyzes the reversible hydration/dehydration of CO_2 as shown by

$$CO_2 + H_2O \xleftrightarrow{CA} H^+ + HCO_3^-$$

The chemical species in the catalyzed reaction involve both a respiratory end product (CO_2) and a potential metabolic precursor (HCO_3^-). Because of this, CA has been studied as an enzyme of respiratory CO_2 transport and excretion and as a metabolic enzyme that channels HCO_3^- into various synthetic pathways (for reviews, see Refs. 16, 18, 41).

Nowhere is this multifunctional aspect of CA more apparent than in skeletal muscle, where CA is compartmentalized to the cytoplasm, the sarcolemma, the sarcoplasmic reticulum, and the mitochondria (reviewed in Refs. 16, 18). In mammals, CA has been shown to facilitate the excretion of CO_2 across the sarcolemma in both type I (red, slow twitch, oxidative) and type II (white, fast twitch, glycolytic) fibers (11, 12). This prevents the buildup of CO_2 in the intracellular fluid and the occurrence of an intracellular respiratory acidosis that would disrupt normal physiological and metabolic functions. This pattern appears to hold for lower vertebrates as well. Sarcolemmal CA appears to facilitate the movement of CO_2 out of resting trout white muscle, and both sarcolemma and cytoplasmic CA appear to be involved in the efflux of NH_3 (20).

This is interesting from an evolutionary point of view because the prevailing paradigm has always been that the aquatic medium, due to its high capacitance for CO_2, did not exert any selective pressure on the development of physiological mechanisms for CO_2 transport and excretion. The fact that a relatively large fluid compartment such as blood or water could act as a virtually unlimited sink for CO_2 was considered the most important factor in shaping the mechanisms of CO_2 excretion in aquatic vertebrates (41). Similar arguments have been made for NH_3 excretion. (e.g., Ref. 3). However, membrane-associated CA oriented toward the extracellular fluid has now been shown to be important in facilitating both CO_2 and NH_3 fluxes across the sarcolemma, even in the presence of relatively small P_{CO_2} and NH_3 pressure (P_{NH_3}) gradients, in the latter case by providing H^+ ions for diffusion trapping of NH_3 as NH_4^+ (19). Those findings are in general agreement with the prevailing idea in mammalian physiology that the relatively small amount of membrane-associated CA functions in directional transport and the much larger pool of cytoplasmic CA functions primarily in maintaining instantaneous equilibrium among the chemical species of CO_2 in the intracellular fluid (ICF; Ref. 24). Our earlier study (20) of the transport function of CA in a lower vertebrate was performed on a resting perfused trout muscle preparation with a low metabolic rate. Sarcolemma-associated CA makes up only ~5% of the total muscle CA activity in this preparation, with the cytoplasmic pool comprising almost all of the remaining balance. Therefore, it would be interesting to know if that particular subcellular fraction of CA remains the func-

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tionally important component in CO_2 excretion under conditions of maximal CO_2 production (i.e., exercise) or whether the larger pool of cytoplasmic CA becomes more significant.

It would also be interesting to know whether the role of either or both fractions of muscle CA becomes more important after strenuous exercise, when NH_3 production in muscle becomes greatly elevated because of adenylate deamination (28, 40). Our recent work suggests that muscle cell membrane permeability to ammonia alters markedly after strenuous exercise, such that its NH_4^+ permeability increases relative to its NH_3 permeability at this time (43, 45). As a result, ammonia distribution becomes more dependent on the membrane potential (which sets the electrochemical gradient governing NH_4^+ movements) and less dependent on the pH gradient (which sets the P_{NH_3} gradient governing NH_3 movements). This appears to help retain ammonia in muscle for adenylate resynthesis (see Refs. 49, 50). If this is the case, then the importance of CA in promoting ammonia efflux by NH_3 diffusion trapping might become less important after exercise.

With respect to the role of CA in metabolism, the liver has been the major organ of focus in mammals because it is the primary site of ureagenesis and gluconeogenesis (along with the kidney); skeletal muscle has been less thoroughly studied. However, in mammals it is known that CA inhibition alters skeletal muscle contractile properties, utilization of metabolic substrates, and accumulation of metabolic intermediates and end products, especially during exercise (9, 11, 19). However, detailed studies on the exact role(s) of CA in these processes are lacking. In the relatively few lower vertebrates that have been investigated, the hepatic Cori cycle appears to be much less important than in mammals, and significant gluconeogenesis occurs in several other tissues, including skeletal muscle (4, 8, 14, 15). In teleost fish (in contrast to most higher vertebrates), there is now strong but circumstantial evidence that glyconeogenesis occurs in white muscle, but the exact metabolic pathway involved has never been elucidated (26, 29, 37, 48). Three potential routes for lactate (Lac) reprocessing in fish have been proposed, two of which involve a carboxylation step that would suggest the involvement of CA (i.e., carboxylation of pyruvate either via pyruvate carboxylase or via the reversal of malic enzyme; Refs. 14, 26, 30). The third involves the conversion of pyruvate to phospho*enol*pyruvate via the reversal of pyruvate kinase (37) and therefore would be independent of HCO_3^- and presumably independent of CA activity as well. Evidence as to the involvement or noninvolvement of CA in the process of glyconeogenesis from Lac would certainly help clarify the pathway(s) involved.

Another potential involvement of CA is in phosphagen metabolism. In mammals, CA inhibition has been reported to cause the breakdown of creatine phosphate (PCr) and an increase in ADP and inorganic phosphate (9, 11). Whether the breakdown of energy-rich phosphates is the result of respiratory acidosis remains unclear. In strenuously exercised trout white muscle, PCr is severely depleted, whereas ATP is less affected, but the former recovers much more quickly in parallel to the clearance of postexercise respiratory acidosis (43). Possibly, elevated P_{CO_2} acts as an end product inhibitor of pyruvate decarboxylation, thereby setting an upper limit to the rate of the tricarboxylic acid cycle and PCr resynthesis.

In this study, we have modified our isolated-perfused trout tail trunk preparation (19, 45) so that it can be exercised in situ. The preparation has been used to study the transport and metabolic roles of CA in white muscle under conditions of both exercise and postexercise recovery. Specifically, with respect to transport, the question of what roles sarcolemma and/or cytoplasmic CA play in facilitating the transport of CO_2 and NH_3 across the trout muscle cell membrane has been addressed under conditions of greatly increased CO_2 and NH_3 production. With respect to metabolism, the potential roles of the two muscle CA pools in Lac clearance, glycogen (Gly) resynthesis, and phosphagen dynamics after exercise have all been investigated. The results demonstrate the importance of multiple functions of both muscle CA pools in the exercise and postexercise physiology of the rainbow trout.

MATERIALS AND METHODS

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were purchased from Spring Valley Trout Farm, Petersburg, ON, Canada. The fish (initially 400–600 g) were held in two 800-liter fiberglass aquariums for 4–6 mo to grow to the desired size (700–1,000 g) before the experiment. Dechlorinated Hamilton tap water (meq/l; 0.6 Na^+, 0.8 Cl^-, 1.8 Ca^{2+}, 0.5 Mg^{2+}, 0.04 K^+; pH 8.0, temperature 5–14°C) was supplied to the fish holding tanks. The trout were fed with high-protein floating pellets (Aquaculture Zeigler Brothers) three times a week. However they were not fed for 5 days before an experiment, during which time they were kept at 15 ± 1°C. Metabolic rate and ammonia production in fish are extremely sensitive to feeding status; we have found that 5 days of starvation is sufficient to achieve stable O_2 consumption and ammonia production rates in trout of this size, without significant depletion of muscle Gly reserve (C. M. Wood, unpublished data). Before perfusion, the experimental fish were anesthetized with a high concentration of MS-222 (0.5 g/l neutralized with NaOH); fish usually lost equilibrium and ceased ventilation within 1 min without struggling. The tail portion was then severed by section at a point immediately posterior to the anus and weighed. In addition, in the control series (see below), an initial muscle sample was excised from a point immediately anterior to the point of section (see below). The fish was then killed by a blow on the head.

Experimental design and protocols. An isolated tail trunk perfusion preparation developed earlier by our laboratory was employed in this study (refer to Ref. 45 for a detailed description). In brief, Cortland salmonid saline with 3% (wt/vol) BSA (fraction V, Sigma) was used as the basic perfusate. The perfusate was saturated with oxygen, whereas P_{CO_2} was kept constant at ~2 Torr (i.e., ~0.25% CO_2, balance O_2). Perfusate pH was adjusted to the desired level by varying $NaHCO_3$ concentration. Immediately after the tail trunk was cut off, catheters (Clay-Adams PE-90) were implanted into the caudal artery and vein and a ligature around the vertebral column was applied to secure them in place. The perfused tail trunk was placed in a thermocontrolled (15 ± 1°C) saline

bath, and the perfusate was also equilibrated to the same temperature before entering the trunk. The standard perfusion rate was 2 ml·min^{-1}·100 g^{-1} tail weight. For the first 30 min, a heparinized perfusate (50 IU/ml at pH 7.9; to simulate resting arterial pH values) was employed to purge red blood cells and to stabilize the preparation. After the initial 30 min, heparin-free perfusate was used. Venous (outflow) followed by arterial (inflow) perfusate samples [2 ml, representing *time 0* (*T0*)] were then collected through sampling ports with the use of gas-tight Hamilton syringes.

Immediately after the initial 30-min perfusion and perfusate sampling, exhaustive exercise was induced by the direct electrical stimulation of trunk muscle while perfusion at pH 7.9 continued. Shielded bipolar platinum electrode pads were placed on both sides of the tail trunk and held in place by a gauze bandage. Muscle was stimulated for 4 min at 100 V, 20-ms pulse duration, and 10 pulses/s frequency, with the polarity being altered at 15-s intervals to exercise the muscle in an undulatory manner similar to that of natural swimming. Muscle fatigue and exhaustion were noted as a lack of response to the stimulation toward the end of the 4-min exercise period.

After stimulation, the tail trunk was perfused with saline adjusted to a pH of 7.4 to simulate in vivo postexercise arterial acid-base conditions (43). Venous and arterial perfusate samples were then taken at 5 (*T5*), 15 (*T15*), and 30 (*T30*) min postexercise as described above. Part of the arterial and venous perfusate samples was used to measure pH, total CO_2 (T_{CO_2}), P_{O_2}, protein, and water content immediately on sampling. Aliquots (300 µl) of each were deproteinized in 600 µl of 6% perchloric acid (PCA) and stored at −70°C for later analysis of ammonia (Amm) and Lac concentrations, while the remainder of the sample was used to measure Na^+, K^+, and Cl^- concentration.

Four experimental series were performed: a control and three experimental treatments (Table 1). In all series, a final muscle sample (3–5 g; in the dorsal area above the lateral line) was excised from the perfused tail trunk at the end of each 60-min experiment, representing *T30* in the protocol. The tissue samples were freeze-clamped quickly with aluminum tongs prechilled in liquid N_2 and then stored in liquid N_2 for later analysis of pH_i, muscle tissue water content, and T_{CO_2}, Lac, Amm, Gly, ATP, PCr, Na^+, K^+, and Cl^- concentrations.

Table 1. *Experimental design for resting and exercised fish*

	Control	Total CA Block	ICF CA Block	ECF CA Block
Resting	No inhibitor	AZ_{tot} (100 µM) Preinjection	AZ_{ICF} (100 µM) Preinjection	QAS_{ECF} (5 mM) No preinjection
		Without ECF Washout	With ECF Washout	
Postexercise	No inhibitor	AZ_{tot} (100 µM) Preinjection	AZ_{ICF} (100 µM) Preinjection	BZ_{ECF} (10 µM) No preinjection
		Without ECF Washout	With ECF Washout	

Resting series were performed in our earlier study (22, 25, 36). AZ, acetazolamide, QAS, quarternary ammonium sulfanilamide; BZ, benzolamide; ICF, intracellular fluid; ECF, extracellular fluid; tot, total; CA, carbonic anhydrase.

The control series employed a drug-free perfusate (pH 7.9 before and during exercise, pH 7.4 thereafter) throughout the experiment. In the control series only, at the start of each experiment, immediately after the tail was cut off, a muscle tissue sample (3–5 g, same location) was taken from a point just anterior to the section to serve as a reference for resting in vivo levels of tissue metabolites and electrolytes. Also, in the control series only, two extra sets of preparations were run (i.e., additional to the preparations that ended at *T30*). These were terminated at 5 (*T5*) and 15 (*T15*) min after exercise, so that muscle samples could be taken at these times to establish a more detailed profile of the postexercise changes in muscle pH_i and metabolic status.

In the first experimental treatment, a CA inhibitor of low membrane permeability, benzolamide (BZ), was used to selectively block CA activity in the extracellular fluid (ECF) (BZ_{ECF}). Immediately after the initial 30-min perfusion to wash out red blood cells and the 4 min of exercise, the drug-free perfusate (pH 7.9) was changed to one containing 10 µM BZ (pH 7.4) for another 30 min. A perfusate BZ concentration of 10 µM (~1,000 × the inhibition constant) was applied to achieve ECF CA inhibition while avoiding ICF CA inhibition (13). Arterial and venous perfusate samples (*T5*, *T15*, *T30*) and muscle samples (*T30*) were collected as described above. Unlike our study on resting fish where quaternary ammonium sulfanilamide (QAS) was used to inhibit the ECF-oriented CA (20), BZ was chosen in the present study because, in our preliminary tests, QAS was found to retard the response of the tail trunk to the electrical stimulation. Furthermore, because CA inhibition in skeletal muscle is known to inhibit various mechanical and metabolic aspects of contraction (see introduction), BZ was not introduced to the tail trunk until the exercise routine was completed.

The second experimental treatment used the common, more membrane-permeable CA inhibitor acetazolamide (AZ) to inhibit ICF (cytoplasmic) CA only (AZ_{ICF}) by a previously established protocol (20). At 12–16 h before the experiment, fish were anesthetized, weighed, and injected via caudal puncture with sufficient stock solution of 100 mM AZ to produce an initial extracellular concentration of 100 µM, assuming an ECF volume (ECFV) of 25% of the fish's weight. The fish were left for 12–16 h in individual, darkened acrylic boxes served with aerated flowing water, thereby allowing the AZ sufficient time to equilibrate between the ECF and the muscle ICF. For the first 30 min of control perfusion, the perfusate contained 100 µM AZ (pH 7.9) to maintain full ICF inhibition. However, AZ was not used in the 30-min postexercise perfusate (pH 7.4) so as to wash the AZ out of the extracellular space. In this case, ECF CA activity would be restored, whereas the inhibition to ICF CA would remain due to the slow membrane permeability of AZ (20). Perfusate samples were taken at *T5*, *T15*, and *T30*, and muscle samples were taken at *T30*.

The third experimental treatment evaluated the effect of total CA inhibition (ICF and ECF CA blockade, AZ_{tot}). Again, the fish were first preinjected with AZ as described above. In contrast, the tail trunks were perfused with 100 µM AZ for the entire 60 min of perfusion (i.e., for 30 min preexercise at pH 7.9 and for 30 min postexercise at pH 7.4). Our previous study demonstrated that this protocol produced the highest level of CA inhibition (84.8%) of all three treatments (20). Perfusate samples were taken at *T5*, *T15*, and *T30*, and muscle samples were taken at *T30*.

Finally, all data for resting conditions, i.e., perfusate and muscle acid-base status, muscle metabolites, and metabolite fluxes, were obtained from muscle and perfusate samples

taken during our earlier study on resting tail trunks (20). These preparations were perfused for the same period of time (but at pH 7.9 throughout), subjected to either a control perfusion (no drugs) or a similar treatments with CA inhibitors, and sampled at *T30* (but without exercise). Extracellular CA inhibition was achieved using QAS. However, ion flux rates were not measured in our previous study on resting preparations. Therefore *T0* values collected before the exercise regimen were used as the reference points for the postexercise ion flux data. Note that because ECF CA blockade was applied only after exercise, the reference point is not parallel for this treatment only.

Analytic protocols. Perfusate pH was measured with a Radiometer glass capillary electrode (G297-G2 and K497 calomel reference electrode) thermostatted to 15 ± 1°C and registered on a Radiometer PHM84 pH meter. P_{O_2} was determined using a thermostatted Radiometer P_{O_2} electrode (E5046) connected to PHM72 meter. Total O_2 content was calculated based on the measured P_{O_2} (Torr) and the solubility coefficient αO_2 (1.77 μmol·l^{-1}·Torr^{-1}) at 15°C (2). T_{CO_2} was measured on 100 μl perfusate sample using a total CO_2 analyzer (Corning 965). P_{CO_2} and the concentration of HCO_3^- ($[HCO_3^-]$) were calculated by rearrangement of the Henderson-Hasselbalch equation with appropriate constants (αCO_2 and pK′) for trout plasma at 15°C (2). Perfusate ion concentrations ($[Na^+]$, $[K^+]$, and $[Cl^-]$) measurements were conducted on a specific electrode (AVL 983-S Electrolyte Analyzer). The perfusate Lac and total Amm (T_{Amm}) concentrations were determined by the Sigma L-lactate dehydrogenase and glutamate dehydrogenase assays (both NAD/NADH coupled), respectively. The perfusate total protein and water content were determined with a refractometer (American Optical). The nonbicarbonate buffer capacity ($\beta = -5.54 \pm 0.15$ mM $[HCO_3^-]$ pH unit) of the perfusate was determined in a previous study (45).

One portion of each freeze-clamped muscle sample was stored in liquid N_2 and later pulverized in liquid N_2 with an insulated mortar and pestle. An aliquot (~200 mg) of this muscle tissue powder was used to measure intracellular pH (pH_i) and T_{CO_2} (Ti_{CO_2}) with the homogenization technique (33). Intracellular P_{CO_2} and $[HCO_3^-]$ were calculated using the Henderson-Hasselbalch equation as outlined above, after appropriate correction of Ti_{CO_2} values for trapped ECF T_{CO_2} (see below). A second aliquot (~100 mg) of tissue powder was deproteinized in 1 ml 8% PCA and stored in liquid N_2 for tissue ammonia analysis by a modified glutamate dehydrogenase method (23). A third, much larger aliquot was lyophilized for 64 h and used for tissue PCr, ATP, Gly, Lac, and ion analysis. Muscle PCr, ATP, and Gly concentrations were analyzed by standard enzymatic techniques (1). Tissue Lac was determined by the same enzymatic assay described above. For tissue Na^+, K^+, and Cl^- measurements, the freeze-dried tissue powder (~20 mg) was extracted in 1N HNO_3 (1 ml) at 50°C for 48 h. The supernatant was used for $[Na^+]$ and $[K^+]$ analysis by flame atomic absorption spectrometry (Varian AA-1275). Tissue $[Cl^-]$ was measured by coulometric titration (Radiometer CMT10). A common standard was used to calibrate all the ion measurements in both perfusate and muscle tissue.

A second portion of muscle tissue (nonpulverized; 200–300 mg) was used to determine the muscle tissue water content by drying at 70°C in an oven for over 48 h to constant weight.

In addition, in the BZ series, another portion of nonpulverized muscle tissue was shipped to Auburn University on dry ice and used for muscle CA activity measurement. For the other groups, muscle CA activities were taken from measurements on identical protocols in our previous study (20). For this assay, frozen tissue (~250 mg) was homogenized for 30 s in 2 ml buffer (in mM: 75 mannitol, 225 sucrose, 10 Tris-PO_4, pH 7.4) with the use of an Omni 1000 motor-driven homogenizer and then sonicated (35 W, 15 s, Heat Systems Microson) and centrifuged at 10,000 *g* for 20 min at 4°C (Sorvall RC5-B). The supernatant was analyzed for CA activity at 4°C by the electrometric pH method (17).

Calculations. Metabolic acid (ΔH_m^+) added to the perfusate from the perfused muscle trunk was calculated according to Milligan and Wood (27)

$$\Delta H_m^+ = [HCO_3^-]_a - [HCO_3^-]_v - \beta \times (pH_a - pH_v)$$

where the subscripts a and v denote measured values in arterial and venous perfusate.

Flux rates of Lac, T_{Amm}, ΔH_m^+, total CO_2, O_2, and ions (Na^+, K^+, and Cl^-) were calculated according to the Fick principle using the perfusion rate (1.2 l·kg^{-1}·h^{-1}) and the differences in concentration between arterial inflow and venous outflow.

Intracellular fluid volume (ICFV) and ECFV were calculated based on water content, $[K^+]$, and $[Cl^-]$ in whole muscle tissue and venous perfusate using the "Cl^- − K^+ space" concept of Conway (5) described earlier (45). All intracellular metabolite and electrolyte concentrations were corrected for trapped ECF content in whole muscle tissue and expressed per unit ICF water, whereas the concentrations in the perfusate were expressed per unit ECF water.

Statistical analyses. Data are presented as means ± SE (*n*), where *n* represents the number of preparations in each treatment group. The *F* test was performed to identify any significant differences in variance between the treatment and control groups or between the resting and postexercise groups in each treatment, then appropriate *t*-tests were performed to assess whether differences existed between the tested groups ($P \leq 0.05$). One-way ANOVA and Duncan's multiple-range and critical range test were performed for multiple comparisons with reference to the control ($P \leq 0.05$).

RESULTS

The exercise protocol introduced by electrical stimulation resulted in vigorous muscle contraction in the control group and in all experimental groups, with the muscle exhausting gradually toward the end of the 4-min exercise regimen. The BZ treatment was not applied until immediately after the end of exercise. The two AZ treatment groups did not show any signs of earlier exhaustion or less muscle contraction as suggested in studies on higher vertebrates (10, 11). The perfusion pressure increased by ~5 cmH_2O immediately after exercise, but returned to the original level (~10 cmH_2O) within 10 min.

AZ_{ICF} and AZ_{tot} treatments, applied exactly as in the present study, resulted in 77.8 and 84.8% inhibition, respectively, of CA activity in the perfused tail trunk muscle (20); for methodological reasons, these are believed to be conservative estimates. In contrast, BZ treatment resulted in only a 38.0% inhibition of CA activity, which is almost identical to the 40.4% inhibition rate reported earlier for the other ECF CA inhibitor QAS (20).

Acid-base status and gas exchange. At rest, the pH_i of perfused white muscle was ~7.28, comparable to the in vivo value of 7.25 ± 0.02 ($n = 8$) measured at the time of death, and after exercise decreased by ~0.6 units to

6.73 at *T5* in the control series (Fig. 1). This change, occurring after 4 min of in situ electrical stimulation of the perfused tail trunk, was comparable to reported values in the white muscle of intact trout under commonly used in vivo exercise protocols, such as 6 min of hand chasing (27, 37, 39, 43). However, in contrast to the in vivo situation where either a further decrease or no change in pH_i occurs by 30 min, pH_i recovered relatively quickly to 6.82 at *T15* and 7.07 at *T30* in the control group. This value was still significantly lower than the initial in vivo resting pH_i and the resting values in perfused preparations.

In the three experimental groups treated with CA inhibitors, muscle pH_i at rest (data reported in 20) remained at the control resting levels. Postexercise pH_i measurements were taken only at *T30*; in comparison with corresponding resting values, pH_i remained much more depressed at this time in all three treatments (Fig. 1). AZ_{ICF} produced the greatest depression of postexercise pH_i (6.89), whereas BZ_{ECF} produced the least depression (6.99). In all three cases, these differences were significant relative to the control pH_i at *T30*.

Before exercise (*T0*), outflowing perfusate pH (pH_v) was ~0.1 unit below the inflowing pH_a of ~7.90 in both control and experimental series. The resting arterial-venous (a-v) difference in pH (*T30*) showed the same pattern (Fig. 1). In the control series, this difference increased after exercise to ~0.3 units below the new inflowing pH_a of 7.40. These values remained stable at *T5*, *T15*, and *T30*. In all three CA inhibition treatments, the outflowing perfusate pH_v values were significantly higher compared with the corresponding control values at *T15* and *T30*, and also at *T5* in the BZ_{ECF} treatment only. Thus the $pH_a - pH_v$ difference decreased significantly at these times relative to the control series, especially in BZ_{ECF} series. These higher pH values in the outflowing perfusate reflected the slower correction of intracellular acidosis in the three treatment groups (Fig. 1).

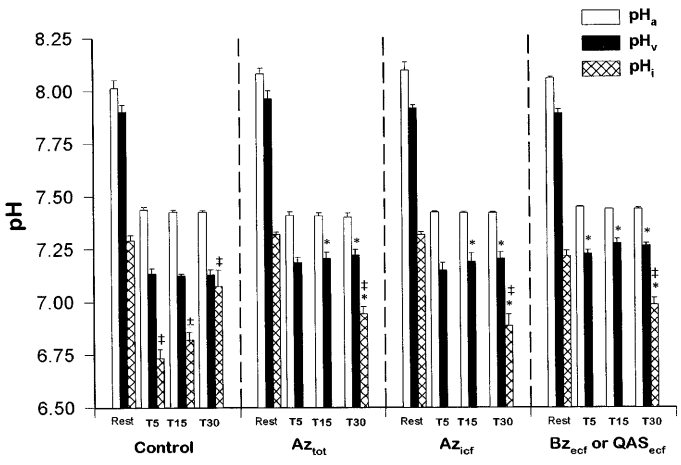

Fig. 1. pH in the inflow saline (arterial, pH_a), outflow saline (venous, pH_v), and intracellular fluid (pH_i) of white muscle of isolated perfused tail trunk preparations at rest and after exercise [*time 5*, *15*, and *30* min (*T5*, *T15*, and *T30*)] in control and 3 carbonic anhydrase (CA) inhibition groups. Values are means ± SE; $n = 5$ or 6 for each treatment group. Rest values were taken at *T30* of perfusion in the resting preparation (20). For the resting series, extracellular fluid (ECF) CA inhibition was achieved by using quaternary ammonium sulfanilamide (QAS); for the exercise series, benzolamide (BZ) was employed for ECF CA inhibition. AZ, acetazolamide; ICF, intracellular fluid; tot, total. * Significant difference ($P \leq 0.05$) from corresponding control values. ‡Significant difference from the corresponding rest value ($P \leq 0.05$).

Before exercise (*T0*), the outflowing perfusate P_{CO_2} (Pv_{CO_2}) was ~3.5 Torr relative to an inflowing P_{CO_2} (Pa_{CO_2}) of ~2 Torr (Fig. 2*A*). After exercise, Pv_{CO_2} increased ~2.5-fold at *T5*, declining only slightly by *T30* in the control group (Fig. 2*A*). CA blockade did not affect Pv_{CO_2} at rest, and the same general postexercise pattern was seen in all three treatment groups. However, the postexercise Pv_{CO_2} elevation was significantly attenuated throughout the recovery period by the BZ_{ECF} treatment and at *T30* by the AZ_{ICF} treatment (Fig. 2*A*), in general agreement with the pH_v data (Fig. 1).

In this perfused trunk preparation, resting muscle had an intracellular P_{CO_2} of 7.31 ± 0.63 Torr ($n = 8$) and an intracellular T_{CO_2} of 6.21 ± 0.61 mM ($n = 8$), both marginally higher ($0.10 \leq P \leq 0.05$) than the in vivo values measured at the time of death ($P_{CO_2} = 5.54 \pm 0.76$ Torr, $n = 8$; $T_{CO_2} = 4.69 \pm 0.61$ mM, $n = 8$). These resting values in the perfused muscle were unaffected by AZ_{ICF} or AZ_{tot} treatment, but extracellular inhibition significantly elevated intracellular P_{CO_2} without a significant change in T_{CO_2} (Fig. 2, *A* and *B*; data reported in Ref. 20).

Compared with resting muscle, exercise in the control series resulted in a 75% increase in intracellular P_{CO_2} to ~13 Torr at *T30* (Fig. 2*A*), but intracellular T_{CO_2} was not significantly elevated (Fig. 2*B*). Relative to the control group at *T30*, intracellular P_{CO_2} was slightly elevated by CA blockade in all three treatment groups (significant only in the AZ_{ICF} treatment; Fig. 2*A*), whereas intracellular T_{CO_2} remained unchanged (Fig. 2*B*). T_{CO_2} efflux in the control group increased by more than twofold immediately after exercise and declined slightly (28%) during the following 30 min of recovery (Fig. 3). BZ_{ECF} inhibition significantly depressed this postexercise T_{CO_2} efflux by ~25%, an effect that was significant at both *T5* and *T30*. The T_{CO_2} efflux rates after exercise were not affected by AZ_{ICF} and AZ_{tot} inhibition (Fig. 3).

In comparison with the control resting O_2 consumption rate (M_{O_2}), the stimulation protocol did not induce a significant increase in M_{O_2} (Table 2). Relative to appropriate resting M_{O_2} values or to simultaneous control values, the AZ_{tot} group exhibited a small decrease and the AZ_{ICF} and BZ_{ECF} group exhibited essentially no change in M_{O_2}. Notably, the postexercise elevation in T_{CO_2} efflux significantly exceeded the increase in M_{O_2} in all groups (Fig. 3, Table 2). The nonstoichiometric changes in T_{CO_2} efflux and O_2 uptake led to a "respiratory quotient" of >1.0. This result is in agreement with our previous observations in resting perfused muscle and suggests that the entire T_{CO_2} efflux was not directly derived from oxidative metabolism in mitochondria (20).

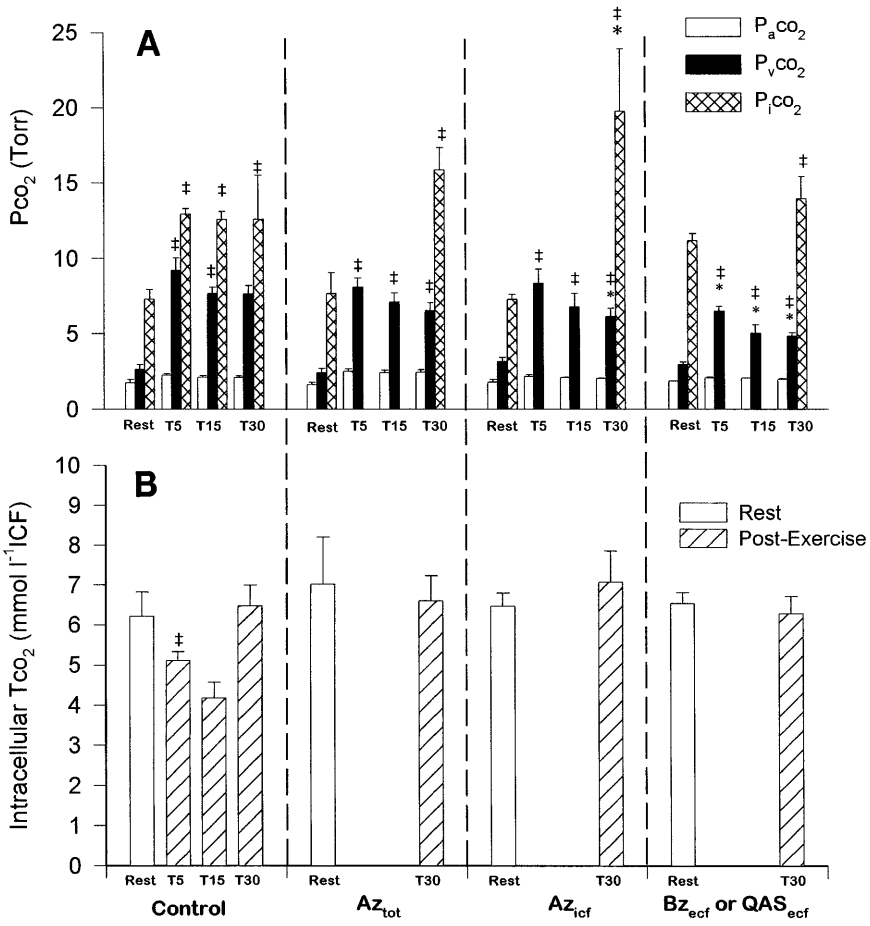

Fig. 2. *A*: calculated CO_2 partial pressure (Pco_2) in inflow saline (Pa_{CO_2}), outflow saline (Pv_{CO_2}), and white muscle intracellular (Pi_{CO_2}) at rest and after exercise (*T5*, *T15*, and *T30*) in the control and 3 CA inhibition groups. Values are expressed as Torr (7.5 Torr = 1 kPa). *B*: measured intracellular total CO_2 (Ti_{CO_2}) in trout white muscle at rest and *T30* postexercise. Rest values were taken at *T30* of perfusion in the resting preparation (20). For the resting series, ECF CA inhibition was achieved by using QAS; for the exercise series, BZ was employed for ECF CA inhibition. *Significant difference ($P \leq 0.05$) from the corresponding control values. ‡Significant difference from the corresponding rest values ($P \leq 0.05$). Other details as in legend of Fig. 1.

At rest, ΔH^+_m flux in the control group was slightly negative, indicating metabolic acid uptake by the muscle (Fig. 4). With exercise, ΔH^+_m flux shifted to positive values at *T15* and *T30*, indicating an unloading of metabolic protons from the muscle. At rest, AZ_{tot} and AZ_{ICF} CA inhibition had no effect on the direction of ΔH^+_m, but extracellular CA inhibition resulted in ΔH^+_m efflux. In contrast, CA blockade, in all treatments, significantly altered this pattern of postexercise ΔH^+_m flux, which remained negative (i.e., inwardly directed) throughout the 30-min recovery period (Fig. 4). This inhibition of metabolic proton efflux was greatest in the AZ_{tot} treatment and least in the BZ_{ECF}, a difference that

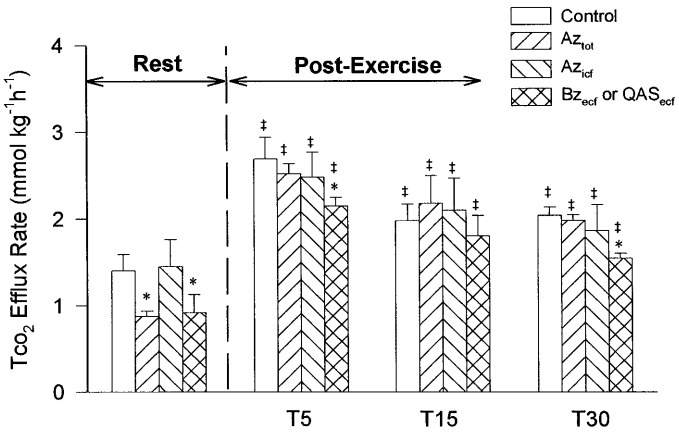

Fig. 3. Total CO_2 efflux rate (T_{CO_2}; mmol·kg^{-1}·h^{-1}) from the perfused tail trunk muscle in the control and 3 CA inhibition groups at rest and after (*T5*, *T15*, and *T30*) exercise. Rest values were taken at *T30* of perfusion in the resting preparation (20). For the resting series, ECF CA inhibition was achieved by using QAS; for the exercise series, BZ was employed for ECF CA inhibition. *Significant difference ($P \leq 0.05$) from corresponding control values. ‡Significant difference from corresponding rest values ($P \leq 0.05$). Other details as in legend of Fig. 1.

Table 2. *MO_2 and RQ of the perfused tail trunk at rest and after exercise*

	Control	AZ_{tot}	AZ_{ICF}	BZ_{ECF} or QAS_{ECF}
MO_2				
Rest	0.80 ± 0.04	0.89 ± 0.07	0.93 ± 0.04	0.85 ± 0.13
T5	0.85 ± 0.06	0.72 ± 0.02*†	0.84 ± 0.04	0.82 ± 0.04
T15	0.81 ± 0.06	0.74 ± 0.02*†	0.87 ± 0.05	0.77 ± 0.06
T30	0.80 ± 0.04	0.70 ± 0.00*†	0.90 ± 0.03*	0.78 ± 0.06
RQ				
Rest	1.74 ± 0.31	1.19 ± 0.02	1.86 ± 0.36	1.28 ± 0.14
T5	3.17 ± 0.22†	3.70 ± 0.34†	3.05 ± 0.43†	2.54 ± 0.12†
T15	2.47 ± 0.18†	3.01 ± 0.49	2.31 ± 0.38†	2.26 ± 0.13†
T30	2.58 ± 0.27†	2.81 ± 0.11	2.06 ± 0.32	1.79 ± 0.10*

Values are means ± SE. Oxygen consumption rate (M_{O_2}) values are in mmol·kg^{-1}·h^{-1}. Rest values were taken at *time 30 min* (*T30*) of perfusion in the resting preparation (20). RQ, respiratory quotient; *T5*, *T15*, *time 5* and *15 min*, respectively. *Significantly different ($P \leq 0.05$) from corresponding control values; †significantly different ($P \leq 0.05$) from corresponding rest values.

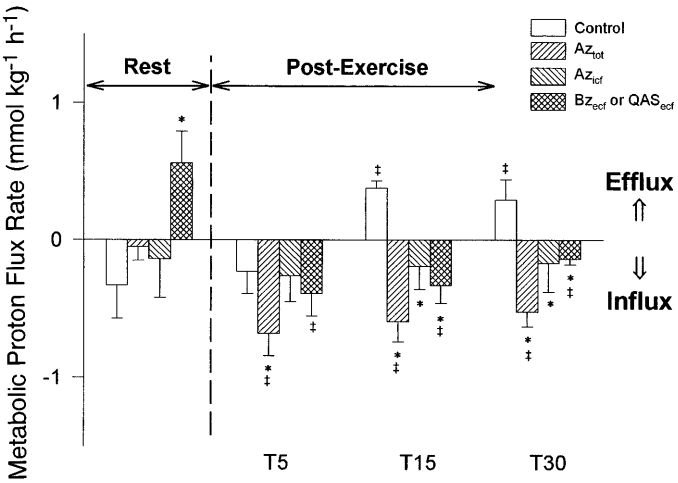

Fig. 4. Metabolic proton flux rate in the perfused tail trunk of the control and 3 CA inhibition groups at rest and after (*T5*, *T15*, and *T30*) exercise. Positive and negative flux rate values indicate efflux and influx, respectively. Rest values were taken at *T30* of perfusion in the resting preparation (20). For the resting series, ECF CA inhibition was achieved by using QAS; for the exercise series, BZ was employed for ECF CA inhibition *Significant difference ($P \leq 0.05$) from the corresponding control values. ‡Significant difference from the corresponding rest values ($P \leq 0.05$). Other details as in legend of Fig. 1.

was significant only at *T30*. The depressed ΔH_m^+ influx in the BZ_{ECF} group after exercise (e.g., *T30*) was consistent with the elevated ΔH_m^+ efflux caused by extracellular CA inhibition at rest. In general these effects agreed well with the lower values of pH_i and higher values of pH_v accompanying CA inhibition seen in Fig. 1.

Effects on ammonia metabolism. Ammonia efflux rates (Fig. 5) were variable within each treatment group but were generally lower than ΔH_m^+ flux rates (Fig. 4), or approximately 10% of T_{CO_2} efflux rates (Fig. 3). Ammonia efflux was unaffected by CA inhibition at rest, and the efflux rates increased by more than fivefold after exercise in the control series (Fig. 5). ECF CA blockade with BZ_{ECF} reduced this postexercise increase by ~50% at 15 min; however, the attenuation was only transient. AZ_{tot} and AZ_{ICF} also led to minor, nonsignificant reductions (Fig. 5). It was our intention to measure transmembrane ammonia gradients, as was done in the previous study at rest (20), but unfortunately, most of the muscle samples were lost in the failure of the ultra-cold freezer in which they were stored. As a result, intracellular ammonia was only measured in vivo at the time of death (323 ± 37 μM, n = 8) and at rest (*T30* of perfusion: 259 ± 57 μM, n = 8) and in the control group at *T15* (4,199.97 ± 632.91 μM, n = 5) and *T30* postexercise (2,031.82 ± 586.35 μM, n = 5).

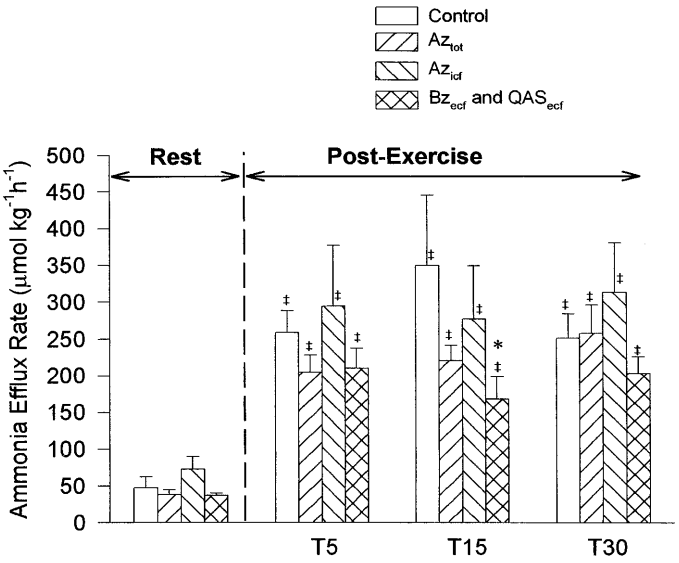

Fig. 5. Total ammonia efflux from the perfused tail trunk muscle in the control and 3 CA inhibition groups at rest and after (*T5*, *T15*, *T30*) exercise. Rest values were taken at *T30* of perfusion in the resting preparation (20). For the resting series, ECF CA inhibition was achieved by using QAS; for the exercise series, BZ was employed for ECF CA inhibition *Significant difference ($P \leq 0.05$) from corresponding control values. ‡Significant difference from corresponding rest values ($P \leq 0.05$). Other details as in legend of Fig. 1.

Effects on energy-rich phosphates metabolism. Resting muscle PCr and ATP concentrations in the perfused tail trunk measured at *T30* of perfusion were comparable to those measured in vivo at the time of death (Table 3). In parallel with experiments performed on intact fish in vivo (37, 43), muscle PCr in the perfused tail trunk was almost depleted by exhaustive exercise

Table 3. *Intracellular PCr, ATP, Gly, and Lac concentrations in resting and postexercise perfused trunk preparation subjected to different treatments*

	Control	AZ_{tot}	AZ_{ICF}	QAS or BZ_{ECF}
PCr				
In vivo	38.57±3.44			
Rest	39.26±1.78	16.88±0.95*	36.69±1.69	26.19±2.50*
T5	6.92±3.14‡	NA	NA	NA
T15	12.21±0.56‡	NA	NA	NA
T30	29.52±3.32‡	17.76±1.63*	11.93±3.06*†	22.81±1.71*
ATP				
In vivo	7.51±0.56			
Rest	10.75±0.13‡	9.94±0.55	11.04±0.32	10.45±0.47
T5	8.93±0.87	NA	NA	NA
T15	7.33±0.30	NA	NA	NA
T30	8.29±0.81†	8.83±0.44	7.42±0.45†	8.79±0.23†
Gly				
In vivo	NA			
Rest	39.17±2.6	30.55±2.33*	30.52±2.38*	40.08±2.99
T5	10.21±2.26†	NA	NA	NA
T15	7.53±2.39†	NA	NA	NA
T30	40.66±6.56	25.98±2.86*	24.45±4.39*	27.82±2.22*†
Lac				
In vivo	3.97±0.86			
Rest	3.04±0.59	4.54±0.65	3.82±1.61	4.42±1.52
T5	33.94±8.82†	NA	NA	NA
T15	39.09±6.01†	NA	NA	NA
T30	21.18±4.03†	20.77±5.61†	27.12±7.81†	21.32±3.82†

Values are means ± SE in mmol/l ICF. In vivo values were obtained from muscle in the control series at the time of death. Rest values were taken at *T30* of perfusion in the resting preparation (20). In vivo concentrations measured in the control fish before perfusion (i.e., at time of death) are included in comparison. *Significant difference between control and corresponding treatment values; †significant difference of postexercise values relative to resting value within each treatment group ($P \leq 0.05$), ‡significant difference between rest (perfused) and in vivo values in control series. NA, not available; PCr, creatine phosphate; Gly, glycogen; Lac, lactate.

and was largely but not completely recovered after 30 min (Table 3). In contrast, ATP levels were only moderately reduced after exercise, an effect that persisted at *T15* and *T30*.

CA inhibition had no effect on muscle ATP levels at rest or on the ~20% depression seen at *T30* (Table 3). However, the effects of CA inhibition on PCr metabolism were pronounced. Under resting conditions, total and ECF CA inhibition reduced PCr reserves to 43 and 67% of control resting levels, respectively. However, under ICF CA blockade, PCr concentrations remained at control resting levels (Table 3). In the postexercise state, the effects of CA inhibition were rather different. Muscle PCr concentrations at 30 min postexercise in the AZ_{tot} and BZ_{ECF} treatment groups remained significantly below the corresponding control levels (*T30*), and they did not differ from their initial resting values (Table 3). However, the AZ_{ICF} treatment, which had not affected PCr levels at rest, resulted in the largest depression at *T30* postexercise (Table 3). These results suggest that ECF CA activity may play a role in maintaining the resting muscle PCr levels, whereas ICF CA activity may be important for PCr recovery.

Effects on glycolytic metabolism and Lac efflux. Resting muscle Gly concentrations (~40 mM) measured at *T30* in the perfused tail trunk were high relative to most in vivo studies (reviewed in Ref. 3) but were internally consistent; unfortunately in vivo samples taken at the time of death in the present study for comparison were lost. Lac values in the perfused preparation at rest (3–4 mM) were comparable to in vivo measurements (Table 3). In the control series, the intensive exercise protocol triggered a massive Gly breakdown similar to that seen in many in vivo studies (27, 37, 43). Surprisingly, under these in vitro conditions, Gly recovered much faster than in the in vivo studies (Table 3). It took a mere 30 min for muscle Gly to be completely restored to resting levels in the perfused tail trunk, a process that usually takes many hours to complete in vivo. In concert with this change in muscle Gly, there was a >10-fold elevation in muscle Lac accumulation immediately after exercise in the control series (Table 3). However, based on the stoichiometry of 1 Gly = 2 Lac, the buildup of Lac accounted for only about one-half of the Gly depletion at *T5*. Muscle Lac burden was reduced by 50% during the 30-min recovery period; however, in contrast to Gly, it did not return to the resting level. By *T30*, Lac clearance accounted for only ~40% of Gly resynthesis. This lack of stoichiometric agreement between Gly and Lac dynamics indicates that part of glyconeogenesis may have originated from sources other than Lac (Table 3).

Under resting conditions, AZ_{ICF} and AZ_{tot} treatments resulted in a significant decrease in muscle Gly storage, whereas extracellular blockade had no effect (Table 3). In parallel to the resting data, Gly in AZ_{tot} and AZ_{ICF} also remained significantly lower than the control value after 30 min of recovery from exhaustive exercise. However, these concentrations did not differ significantly from the respective resting values in each treatment group. In contrast, extracellular CA blockade (BZ_{ECF}), which was without effect at rest, resulted in a substantially lower muscle Gly concentration at *T30* postexercise in comparison to either the control series at this time or the corresponding rest value (Table 3).

Resting muscle Lac concentrations were not significantly affected by CA blockade. Similarly, there were no significant differences in muscle Lac among all four treatment groups 30 min after exercise (Table 3).

At rest, Lac flux rates (Fig. 6) from perfused muscle (net efflux) were comparable in magnitude but opposite in sign (net uptake) to those of metabolic protons (ΔH^+_m; Fig. 4) and were unaffected by CA blockade. After exercise, Lac efflux rates increased approximately fivefold to a stable value throughout the 30-min postexercise period in the control series (Fig. 6). All three CA-inhibition treatments significantly reduced this postexercise elevation by 20–50%. This attenuation was evident immediately after exercise and throughout recovery in AZ_{tot} and BZ_{ECF} groups but only at *T30* in the AZ_{ICF} group. Overall, extracellular CA inhibition with BZ_{ECF} was most effective in reducing postexercise Lac efflux (by ~50%). Although these changes in Lac efflux associated with exercise and CA inhibition are substantial on a relative basis, it should be appreciated that on an absolute basis they are small relative to the postexercise Lac burden in the muscle (Table 3). At most, measured Lac efflux could account for <15% of Lac clearance from muscle over the 30-min recovery period (Table 3), so it is not surprising that the effects of CA inhibition on efflux rate (Fig. 6) were not reflected in muscle Lac concentrations.

Effects of CA inhibition on muscle ionic balance. Ion and fluid levels in perfused trout muscle at rest (*T30*) were slightly altered relative to in vivo values measured at the time of death, with significant elevations in intracellular Na^+ and K^+ as well as in ECFV and a reduction in intracellular Cl^- concentration (Table 4). In the postexercise control series at *T30*, intracellular Na^+ and K^+ concentrations as well as

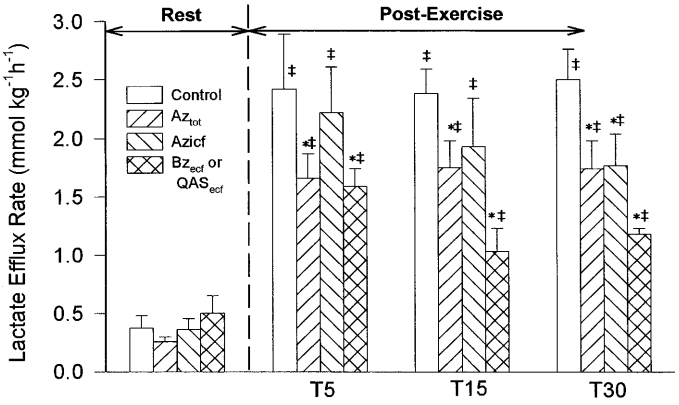

Fig. 6. Net lactate efflux from the perfused tail trunk muscle in control and 3 CA inhibition treatment groups at rest and after (*T5*, *T15*, *T30*) exercise. Rest values were taken at *T30* of perfusion in the resting preparation (20). For the resting series, ECF CA inhibition was achieved by using QAS; for the exercise series, BZ was employed for ECF CA inhibition. *Significant difference ($P \leq 0.05$) from corresponding control values. ‡Significant difference from corresponding rest values ($P \leq 0.05$). Other details as in legend of Fig. 1.

Table 4. *Intracellular Na^+, K^+, and Cl^- concentrations and ECFV and ICFV in resting and postexercise trout white muscle*

	Na^+	K^+	Cl^-	ECFV	ICFV
	mmol/l ICF			ml/g wet wt	
In vivo	5.95 ± 0.46	148.03 ± 3.67	7.09 ± 0.63	0.074 ± 0.004	0.662 ± 0.006
Rest	8.40 ± 0.52*	163.87 ± 4.45*	4.75 ± 0.43*	0.102 ± 0.005*	0.656 ± 0.008
Postexercise (*T30*)					
Control	13.82 ± 1.62†	174.66 ± 7.86†	4.07 ± 0.19	0.089 ± 0.006	0.682 ± 0.004†
AZ_{tot}	7.63 ± 4.44*	188.89 ± 12.03†	3.97 ± 0.32†	0.091 ± 0.016	0.670 ± 0.021
AZ_{ICF}	20.78 ± 1.92*†	198.05 ± 12.80†	3.91 ± 0.30†	0.085 ± 0.008†	0.669 ± 0.016
BZ_{ECF}	9.53 ± 0.92*	189.77 ± 6.79†	3.84 ± 0.15†	0.080 ± 0.006†	0.692 ± 0.010†

Values are means ± SE. In vivo values were obtained from muscle in the control series before the perfusion. Rest values were taken at *T30* of perfusion in the resting preparation (20). In postexercise groups, values were obtained from the control and 3 CA inhibition treatment groups at *T30*. ECFV and ICFV, ECF and ICF volume, respectively. *Significant difference between corresponding control and treatment values in postexercised fish or between rest (perfused) and in vivo values in resting fish ($n = 8$, $P \leq 0.05$); †significant difference from corresponding rest values (20).

ICFV were significantly elevated relative to the corresponding values at rest. CA blockade resulted in changes in intracellular Na^+ concentration in comparison with values in exercise control, whereas K^+ and Cl^- concentrations remained unchanged. Among the changes caused by CA inhibition, AZ_{tot} and BZ_{ECF} led to significant depressions in Na^+ concentration. In contrast, AZ_{ICF} caused Na^+ concentration to increase. Intra- and extracellular fluid volumes were not significantly different among control and CA inhibition groups, indicating that fluid shifts do not occur with CA inhibition.

Muscle ionic concentrations may not be a particularly sensitive measure of changes in the actual flux rates of ions. Ionic flux measurements themselves were quite variable (Fig. 7), reflecting the difficulty of measuring small changes in perfusate ion concentrations against high background concentrations. Nevertheless, clear trends were seen. At rest, ionic flux rates between the perfusate and the muscle were not significantly different from zero (Na^+, Cl^-) or slightly negative (a small net K^+ uptake; Fig. 7). After exercise, net Na^+, Cl^-, and K^+ fluxes all increased (i.e., net losses), with the increase in Cl^- flux appearing greatest at *T5* and the increases in Na^+ and K^+ appearing fairly consistent throughout the recovery period. The changes for both Na^+ and Cl^- were significant throughout recovery, whereas those for K^+ were significant only at *T5* (Fig. 7). CA inhibition had little effect on ionic flux rates at rest or on the basic pattern of changes after exercise, in agreement with the muscle concentration data (Table 4). However at *T30*, Na^+ losses were significantly reduced by ~40%, whereas Cl^- and K^+ losses were slightly elevated relative to the control series by all three blocking treatments. AZ_{ICF} also elevated Cl^- loss at rest.

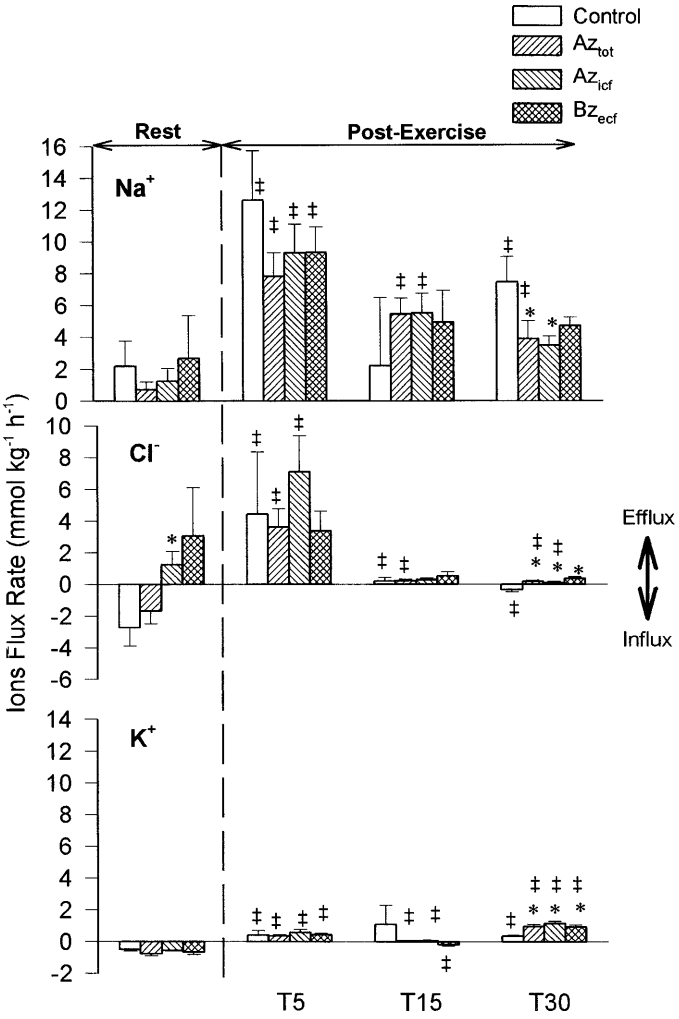

Fig. 7. Net Na^+, Cl^-, and K^+ flux rates in the perfused tail-trunk muscle in control and 3 CA inhibition treatment groups before and after (*T5*, *T15*, *T30*) exercise. Positive and negative flux rates indicate efflux and influx, respectively. In contrast to Figs. 1–6, rest values were taken from the same preparations as the postexercise values, before the initiation of exercise. AZ was present during the rest measurements in the AZ_{tot} and AZ_{ICF} series, but in the BZ_{ECF} series, BZ was added only immediately after the end of exercise. *Significant difference ($P \leq 0.05$) from corresponding control values. ‡Significant difference from the corresponding rest values ($P \leq 0.05$). Other details as in legend of Fig. 1.

DISCUSSION

Preparation. In previous studies on the effects of exercise on trout white muscle (44, 45), we first exercised the live fish by chasing them to exhaustion and then quickly killed the animal to make the perfused tail trunk preparation. A major improvement in the present study was the development of a method to exercise the resting perfused tail trunk in situ, thereby allowing

comparison of measurements before and after exercise. The direct stimulation protocol that we employed reproduced typical postexercise metabolic, respiratory, and acid-base responses shown in many in vivo studies (39, 43, 48). Clearly, the perfused tail trunk does not reflect the exact physiological condition in postexercise muscle in vivo, because it lacks red blood cells and hormones. It eliminates extraneous effects from the presence of CA in other organs and tissues such as the red blood cells, gills and liver, thereby facilitating specific studies on muscle. In particular, the preparation was designed to dissect out the role(s) of CA in the intracellular and extracellular space of fish white muscle with respect to gas exchange, acid-base regulation, and metabolism. An unavoidable consequence of the lack of red blood cell CA in particular is the fact that intracellular P_{CO_2} in the muscle is slightly elevated (e.g., 7.3 vs. 5.5 Torr at rest, probably more after exercise), because P_{CO_2} at the downstream end of the diffusion gradient from myocyte to capillary blood (or perfusate) must be higher in the absence of blood-based catalysis.

The available CA inhibition data indicate that the CA inhibitors were distributed in the fluid compartments for which the experimental protocol was intended (20). The lowest degree of muscle CA inhibition, 38%, was seen for the BZ_{ECF} treatment (comparable to 40% inhibition by QAS in resting muscle), whereas the AZ_{ICF} and AZ_{tot} treatments produced 78 and 85% inhibition, respectively, as in resting muscle. Because of the method by which the muscle tissue is processed for measurement of CA activity, these inhibition values are conservative estimates of total inhibition (20). CA inhibition in mammalian fibers has been reported to affect various aspects of muscle contraction (27, 39, 43, 44). The mechanical properties of trout muscle could not be measured directly in the perfused tail trunk, but no obvious differences in muscle contraction were observed between the control and CA inhibition treatments with AZ. Note in particular that QAS was used only at rest, and BZ_{ECF} treatment was started after the end of exercise so as to avoid any such complicating effects.

Another noteworthy aspect of the preparation was its ability to maintain high values of intracellular PCr and ATP and low values of Lac (Table 3) throughout perfusion at rest. These values were close to those measured in vivo at the time of death (Table 3; see Ref. 46 for a comparison of additional resting values for rainbow trout in the literature). Furthermore, after exercise in situ, perfused trunks in the control series rapidly corrected these values (also Gly) back toward resting levels. These observations suggest that the metabolic machinery is intact and that O_2 or nutrient supply is not a limiting factor.

As a guide to further discussion, Table 5 summarizes the major effects observed in exercised trout muscle resulting from separate inhibition of extra- and intracellular CA.

Effects on gas, acid-base, and Lac exchanges. In the case of a metabolically active tissue such as muscle, CA can be important in both gas transport and intracellular acid-base balance. At rest in trout muscle as well as mammalian muscle, cytoplasmic CA is believed to function primarily in maintaining an instantaneous equilibrium between CO_2 and HCO_3^-: as CO_2 diffuses across the sarcolemma out of the cell, it is immediately replaced from the much larger but less permeable intracellular HCO_3^- pool (18, 19). Membrane-associated CA on the extracellular surface of the sarcolemma is believed to function in the catalyzed hydration of excreted CO_2 to HCO_3^- in the extracellular boundary layer, maintaining the transmembrane P_{CO_2} gradient by preventing the buildup of molecular CO_2. CA inhibition in resting muscle results in lowered CO_2 efflux (20).

Table 5. *Summary of the major effects of CA inhibition on exercised trout white muscle in comparison with trout in the control postexercise preparation*

	ECF CA Inhibition	ICF CA Inhibition	Total CA Inhibition
pH_i	−	−	−
Pi_{CO_2}	NC	+	NC
T_{CO_2} efflux	−	NC	NC
Ti_{CO_2}	NC	NC	NC
M_{O_2}	NC	+	−
ΔH_m^+ efflux	reversed	reversed	reversed
Amm efflux	− (transient)	NC	NC
Lac efflux	−	−	−
Muscle Lac	NC	NC	NC
Muscle PCr	−	−	−
Muscle ATP	NC	NC	NC
Muscle Gly	−	−	−
Muscle Na^+	−	+	−
Muscle K^+	NC	NC	NC
Muscle Cl^-	NC	NC	NC
ECFV	NC	NC	NC
ICFV	NC	NC	NC
Na^+ efflux	NC	−	−
K^+ efflux	+	+	+
Cl^- efflux	+	+	+

All values are compared with the corresponding control. −, significant decrease; +, significant increase; NC, no change; reversed, a change of direction in the flux (i.e., from efflux to influx). Pi_{CO_2}, intracellular P_{O_2}; Ti_{CO_2}, total intracellular CO_2; ΔH_m^+, metabolic proton flux rate.

In exercised trout muscle, CO_2 and H^+ production rates are both much higher than at rest, with H^+ production driving HCO_3^- dehydration. CA facilitates rapid establishment of a new equilibrium, and elevated CO_2 excretion is achieved with very little elevation over resting intramuscular T_{CO_2} concentrations (Fig. 2*B*). In fact, after a 1-h recovery period, fish white muscle often exhibits a depression in intracellular HCO_3^- and T_{CO_2}, because the CA-catalyzed reactions are driven in the direction of dehydration as a result of the elevated levels of metabolic protons combined with the rapid removal of molecular CO_2 (27, 39, 43, 44).

One of the most apparent differences in the role of trout muscle CA at rest vs. postexercise appears to be an increase in the importance of the cytoplasmic CA pool in CO_2 efflux. When cytoplasmic CA was inhibited (AZ_{ICF}), there was a significant decrease in pH_i and a significant increase in the intracellular P_{CO_2} at 30 min postexercise (Figs. 1 and 2*A*). At the same time, T_{CO_2}

efflux and intracellular T_{CO_2} concentrations were not different from those in the control group (Figs. 2*B* and 3). These results indicate that with the cytoplasmic pool of CA inhibited, exercised muscle produces CO_2 faster than it can be excreted; CO_2 builds up in the intracellular space until a new steady state is established in which a higher intracellular P_{CO_2} is responsible for maintaining normal CO_2 excretion rates. The decreased pH_i, which is a result of both the respiratory acidosis and the accumulation of metabolic protons, pushes the equilibrium of the CO_2-HCO_3^--H^+ system toward increased P_{CO_2}. This factor appears to be important, because there was a pronounced ICF acidosis despite the very high intracellular buffer capacity of trout white muscle (27).

In contrast, CA inhibition did not cause an intracellular acidosis in trout white muscle at rest (20). Rather, CO_2 efflux was reduced, at least by extracellular CA inhibition, intracellular P_{CO_2} increased, and the low rate of production and accumulation of CO_2 in 30 min was insufficient to produce a new steady state. The large intracellular CA pool, which may be present in excess for CO_2 excretion at rest, becomes more important under conditions of peak CO_2 load. For respiratory gas exchange at the systemic level in mammals and lower vertebrates (e.g., within the red blood cell), it is generally accepted that CA is present in tremendous excess of requirements for resting CO_2 excretion. However, under periods of exercise, and therefore maximal CO_2 production, the high levels of CA activity become necessary (e.g., Ref. 38). An analogous situation may exist for individual tissues and cells as well: under conditions of high CO_2 production, the large CA pool in the cytoplasm becomes necessary to maintain outward CO_2 transport.

In support of this idea, ECF CA inhibition alone (BZ_{ECF}) did not result in a significant increase in intracellular P_{CO_2} at 30 min postexercise (Fig. 2*A*), although it did slightly depress the elevated total CO_2 efflux (Fig. 3). Conversely, selective inhibition of CA in the ECF in white muscle at rest by QAS did cause an increase in intracellular P_{CO_2} as well as a more marked depression of resting total CO_2 efflux (20). So with regard to CO_2 efflux, it appears that the relatively small pool of membrane-associated CA on the extracellular surface of the sarcolemma may be swamped by the high levels of CO_2 produced during exhaustive exercise and that the intracellular enzyme plays the dominant role at this time.

The additional intracellular acidoses in all of the CA inhibition treatments (Fig. 1) may have been primarily of metabolic origin, because in all of them there occurred a sustained influx of H_m^+ after exercise, in contrast to the net efflux seen in the control series at this time (Fig. 4). In the absence of CA catalysis, the efflux of metabolic protons (from Lac production or ATP breakdown) from the muscle cells through the interstitial fluid into the perfusate cannot be "neutralized" by HCO_3^- in the transit time available. As a result, pH in the poorly buffered interstitial fluid is lower. Metabolic proton efflux would be reduced because the efflux must fight a larger net electrochemical gradient, one which strongly favors influx. Thus net metabolic proton uptake occurs. Earlier we concluded that Lac efflux from postexercise trout muscle involves a Lac^--H^+ symport and the free diffusion of H-Lac (44, 47), so the reduction in net Lac efflux caused by all three CA inhibition treatments (Fig. 6) fits well with the reversal of ΔH_m^+ fluxes.

However, it is important to understand that the ΔH_m^+ influx shown in all the CA inhibition treatments (Fig. 4) is equivalent to (and cannot be differentiated from) HCO_3^- efflux. The conventional wisdom is that cell membranes in both fish and mammals are not permeable to bicarbonate ions (40, 41), although HCO_3^- movement can still be carried out through carrier-mediated transport, i.e., via the Cl^-/HCO_3^- or Lac^-/HCO_3^- antiport. Earlier we concluded that the Lac^-/HCO_3^- antiport was not involved in Lac efflux after exercise in trout white muscle (44, 47), and the present Cl^- flux measurements reveal no differential effects of CA inhibition on net Cl^- exchange (Fig. 7). It appears more likely that the observed effects really do represent the movements of metabolic protons. An additional factor in some of the treatments was a retardation of glycogen resynthesis (Table 3), which would delay the biochemical removal of metabolic protons.

Regardless, the results clearly demonstrated that muscle CA not only plays an important role in facilitating CO_2 release from skeletal muscle, but also influences the metabolic acid movement across muscle cell membranes after exercise. This may be particularly true in the poorly vascularized fish white muscle, where interstitial fluid has relatively low nonbicarbonate buffer capacity and absolute CA activity is also low. Hence, limitations on the rate at which CO_2, H^+, and HCO_3^- come to equilibrium may very well affect their transport from muscle to extracellular space.

These results strongly suggest that CA is involved in both respiratory and metabolic acid-base regulation in trout white muscle. Unlike the present study, AZ infusion in vivo caused an increase in intracellular P_{CO_2} in white muscle of postexercise trout, whereas pH_i remained unchanged (7). A direct comparison between the intact fish and the isolated perfused tail trunk may not be very realistic. The perfused tail trunk is a highly simplified system in which white muscle is the sole target of CA inhibition; the intact fish in vivo is a multicompartment system in which CA inhibition can occur simultaneously at multiple sites. Indeed, the blocking protocol used by Currie et al. (7) favored inhibition of erythrocytic CA, causing an increase in P_{CO_2} throughout all compartments of the animal. It is unlikely that the 5- to 30-min period of AZ administration (7) would have been adequate to fully block CA in the muscle.

In control perfused trunk preparations, intracellular acidosis was corrected rapidly after exercise (Fig. 1) relative to in vivo studies (27, 37, 39, 43). This phenomenon is likely associated with a similarly fast glycogen repletion rate (Table 3; see below), which biochemically consumes metabolic acid. The rapid rate in the per-

fused tail trunk may reflect the absence of "stress" hormones. In trout in vivo, Pagnotta et al. (32) demonstrated that exercise-induced cortisol mobilization hinders both glycogen resynthesis from Lac and postexercise acid clearance. The adaptive significance is unclear. However cortisol "removal" by pharmacological intervention greatly accelerates the in vivo correction rate to a level comparable to that seen in vitro. It would be informative to confirm this conclusion by the addition of cortisol to the perfusate in the tail trunk preparation.

Effects on glycogen, energy-rich phosphate, and ammonia metabolism. Today, it is generally accepted that Lac release from muscle and transport to the liver for hepatic-based gluconeogenesis (the Cori cycle) are less important in fish than in mammals. Instead, most fish retain a large portion of the Lac synthesized during exhaustive exercise within white muscle, where it is later used as a carbon source for in situ glycogen resynthesis (14, 26, 48). The rapid restoration of Gly and partial clearance of Lac by the perfused trunk muscle in the present study (Table 3) further supports this view, However, it must be pointed out that the glucose in the perfusate (as in vivo) could have potentially provided the substrate for this glycogen replenishment, although there are multiple lines of evidence that oppose this interpretation (26, 48). What remains particularly unclear at present is the biochemical pathway by which Lac is converted back to glycogen in fish muscle; an involvement of CA for HCO_3^- supply might be expected in several of the proposed routes (see introduction). Regardless, we would not necessarily expect CA inhibition to have the same effects on Lac transport and metabolism in fish as in mammals. Geers et al. (9) reported that in mammalian muscle, CA inhibition with chlorazolamide led to marked increases in both muscle Lac concentration and efflux rate into blood, effects which were not seen in the perfused trout trunk (Table 3, Fig. 6) or in vivo (7). Indeed CA inhibition reduced Lac release from the perfused preparation, perhaps through the effect on proton gradient discussed earlier.

The depressed resting levels of Gly in the fish that had been pretreated with AZ (AZ_{tot} and AZ_{ICF} groups), but not in QAS-treated fish (Table 3), show that intracellular CA activity is essential to maintenance of normal glycogen stores and are consistent with findings in mammalian muscle (6, 34). More importantly, all three CA inhibition treatments slowed the rate of Gly resynthesis after exercise (Table 3). Interpretation is complex, however, because Lac clearance was not affected, and furthermore Gly resynthesis substantially exceeded Lac removal (Table 3), suggesting that other fuels were used. At any one time, the Gly store is a function of synthesis and degradation rates (i.e., glycolysis), so potential effects on the latter must also be considered. One possible interpretation is that the pathway of Gly resynthesis involves carboxylation of pyruvate via pyruvate carboxylase or via the reversal of malic enzyme rather than reversal of pyruvate kinase (see introduction). However, the lower pH_i accompanying CA inhibition (Fig. 1) may also be an important factor; Walsh and Milligan (42) summarized circumstantial evidence indicating that acidosis itself inhibits both glycogenesis and gluconeogenesis from Lac in trout tissues. Clearly, there is a need for more detailed analyses in a simpler system (i.e., in the test tube); the important lesson from the present study is that muscle CA plays a critically direct or indirect role and should be considered in such systems.

ATP concentrations in white muscle were not affected by CA inhibition either at rest or after exercise (Table 3). Curiously though, extracellular CA inhibition greatly lowered intracellular PCr at rest, whereas intracellular CA inhibition had no effect. This may be associated with the observation that at rest intracellular P_{CO_2} was elevated only by extracellular CA inhibition and not by intracellular blockade (20). However, intracellular CA inhibition, which did elevate intracellular P_{CO_2} after exercise, appeared to become extremely potent in delaying or preventing PCr recovery after exercise. This depletion or slower resynthesis of PCr may be due to end product inhibition on pyruvate dehydrogenase caused by P_{CO_2} buildup. In rat muscle, similar effects of CA inhibition (9, 11) and respiratory acidosis (35) in causing PCr depletion have been reported. Although AZ has previously been shown to result in substantial limitation of the carboxylation reaction in mammalian preparation (16), a similar effect of intracellular CA inhibition may also result in end product inhibition of decarboxylation reactions such as that catalyzed by pyruvate dehydrogenase. At least in mammals, the resynthesis of PCr has been shown to be dependent on the rate of ATP turnover supplied by the maximal activity of the TCA cycle (31). Therefore in end product inhibition of pyruvate dehydrogenase the rate-limiting step would be expected to reduce the rate of ATP synthesis and, hence, PCr regeneration. Additionally or alternately, the well-known effect of creatine kinase activation by low pH_i and ADP (21) may have been involved. Thus the intracellular acidosis resulting from CA inhibition may have increased creatine kinase activity. The increased availability of protons would shift the equilibrium of the $PCr + H^+ + ADP = Cr + ATP$ reaction to the right, thereby tending to deplete PCr reserves, as seen in mammalian muscle (9).

After exhaustive exercise, ammonia efflux from the perfused muscle was increased approximately fivefold, but no significant changes in this pattern were observed in the total and intracellular CA inhibition groups and the decrease of ammonia efflux in the extracellular CA inhibition group was only transient (Fig. 5). In the absence of intracellular ammonia measurements that would have allowed estimation of the relevant P_{NH_3} and NH_4^+ electrochemical gradients, interpretation is greatly limited. Nevertheless, the relative lack of effect of CA inhibition supports our general hypothesis that diffusion trapping of NH_3 becomes less important at this time because the muscle cell membranes become more permeable to NH_4^+ during postexercise recovery (see introduction). In other words, NH_4^+ permeability increases relative to NH_3 permeability

after exercise, perhaps in response to acidosis, thereby ensuring that the strong electrochemical gradient directed in the uptake direction will help retain ammonia in the cells for adenylate resynthesis (43, 45).

Perspectives

Extracellular and intracellular CA localized, respectively, to the sarcolemma and the cytoplasm in trout white skeletal muscle, are engaged directly or indirectly in many physiological processes, including acid-base balance, respiration, metabolism of phosphagens, glycolysis/glyconeogenesis, transmembrane relocation of ammonia and Lac, and perhaps ion regulation. Sarcolemma-associated CA plays a critical role in coordinating and promoting the transmembrane movements of ammonia and CO_2 at rest via the shuttling of H^+ ions between these two chemical pools in the interstitial fluid boundary layer. However, during postexercise recovery, when CO_2 and ammonia production rates greatly increase, its role in CO_2 export may be superceded by the increased importance of the much larger intracellular CA pool in this regard. In addition, the overall role of both CA pools in ammonia excretion appears to be diminished because of the increased importance of NH_4^+ permeability relative to NH_3 permeability at this time. However both pools contribute to establishing the acid-base conditions that favor a modest efflux of metabolic acid and Lac from the muscle to the extracellular fluid. The reduced restoration of glycogen in muscle caused by CA inhibition suggests that postexercise gluconeogenesis may occur via an HCO_3^--dependent pathway, although other interpretations are possible. Finally, the rapid postexercise recovery of metabolites and acid-base status in the perfused tail trunk, which at present is a hormone-free preparation, supports the idea that stress hormones such as cortisol exert negative effect on glycogen replenishment and Lac and metabolic acid clearance in postexercise trout white muscle in vivo. In future, the ability to manipulate and strictly control hormone levels and metabolic fuels in the perfusate of the tail trunk may provide a valuable approach for understanding the integration of these processes.

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Address for reprint requests: Y. Wang, Division of Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Science, Univ. of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149.

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