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Lactate efflux from sarcolemmal vesicles isolated from rainbow trout *Oncorhynchus mykiss* white muscle is *via* simple diffusion

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Summary

Lactic acid is produced as an end product of glycolysis in rainbow trout white muscle following exhaustive exercise. The metabolically produced lactic acid causes an intramuscular acidosis that must be cleared, either *via* net transport out of the muscle or by conversion to glycogen, thereby replenishing the muscle energy store. Trout muscle has been shown to retain lactate and utilise it as a substrate for *in situ* glycogen resynthesis. The giant sarcolemmal vesicle preparation was used to characterise the potential for lactate loss from white muscle of rainbow trout. Minimal lactate loss was expected due to the requirement within the intramuscular compartment of lactate for glycogen resynthesis. The sarcolemma was found to be highly resistant to lactate loss, with efflux rates approximately 500-fold lower than influx rates [0.049±0.006 nmol mg^{-1} min^{-1} (*N*=21) *versus* 26.4±6.3 nmol mg^{-1} min^{-1} (*N*=5), respectively, at 25 mmol l^{-1} lactate concentration]. Lactate efflux was linear over the range 10–250 mmol l^{-1} lactate, and greatest under conditions when intravesicular pH was lower than extravesicular pH, but was unaffected by α-cyano-4-hydroxycinnamate, a known inhibitor of lactate transport. These results suggest that lactate is relatively impermeant to the trout white muscle membrane and any lactate loss occurs *via* passive diffusion. This resistance to lactate diffusion can explain why trout muscle retains lactate post-exercise, despite transmembrane gradients that should favour net efflux.

Key words: sarcolemmal vesicle, lactate transport, white muscle, lactate, lactic acid, rainbow trout, *Oncorhynchus mykiss.*

Introduction

The metabolic consequences of high-intensity, burst-type activity to salmonids, such as rainbow trout *Oncorhynchus mykiss*, has been the subject of much research over the last 50 years (for a recent review, see Kieffer, 2000). The response of the white muscle fibers has been particularly well characterised. In brief, the early stages of muscle contraction are fuelled by phosphocreatine (PCr) and adenosine triphosphate (ATP) hydrolysis. Concurrent with the drop in PCr and ATP levels, glycogenolysis becomes activated, reducing glycogen levels in the working muscle by as much as 90% (Parkhouse et al., 1988; Milligan, 1996; Richards et al., 2002). Glycogenolysis causes an increase in intracellular lactate and H^+, with intramuscular pH (pH_i) dropping from a resting pH of approximately 7.4 to 6.8 post-exercise (Milligan and Wood, 1986; Parkhouse et al., 1988). This intracellular acidosis may inhibit muscle performance, and clearance of the metabolically produced lactic acid is required if muscle performance is to be recovered. In salmonids it is estimated that 80–90% of the lactate produced is retained within the muscle to be used as a substrate for *in situ* glycogenesis (Milligan, 1996; Kieffer, 2000).

The mechanism for retaining lactate within the muscle is unknown. A substantial lactate concentration gradient exists between the white muscle intra- and extracellular compartments post-exercise. For example, immediately post-exercise, white muscle lactate can peak at levels in excess of 50 mmol kg^{-1}, where extracellular levels are rarely greater than 5–8 mmol l^{-1} kg^{-1} (Wang et al., 1997). Unlike the immediate rise within the muscle, blood lactate rises slowly post-exercise, peaking 2–4 h after the cessation of exercise at maximal levels of about 16 mmol kg^{-1}, which is still less than levels in white muscle. In addition, extracellular pH is approximately 0.8 units higher than intramuscular pH, establishing an outwardly directed H^+ diffusion gradient, which should promote lactic acid efflux. Cumulatively, the pH and lactate concentration gradients create a large diffusion potential that should drive lactate out of the muscle. Yet despite this, only 15–20% of the lactate produced appears in the blood (Turner et al., 1983; Milligan and Wood, 1986).

Our understanding of lactate transport mechanisms in fish muscle has grown considerably in the past 5 years. In early studies, Turner and Wood (1983), using an isolated perfused

trout trunk, reported that lactate efflux was enhanced, relative to controls, by the anion transport inhibitor 4-acetoamido-4′-isothiocyanstilbene-2,2′-disulphonic acid (SITS). Their interpretation of this observation was that lactate diffuses out of the muscle and subsequently taken back up, and this re-uptake was inhibited by SITS. More recently, Wang et al. (1997), using an isolated perfused tail-trunk preparation of rainbow trout, reported that both lactate influx and efflux are carrier-mediated. They estimated that about 33% of lactate influx was *via* a lactate$^-$/H^+ symporter (or monocarboxylate transporter; MCT), which was inhibited by α-cyano-4-hydroxycinnamate (CIN), about 45% of the influx was *via* a lactate$^-$/$Cl^-HCO_3^-$ antiporter, and the remainder *via* passive diffusion. Efflux is thought to be have both a free diffusional component, of both lactic acid and the lactate$^-$ anion, and a carrier-mediated component, probably a monocarboxylate transporter. Interestingly, lactate efflux appeared to be concurrent with lactate influx. Simultaneous uptake and release of lactate has been observed in mammalian heart muscle (Chatham et al., 2001).

The mechanism of lactate uptake was examined using sarcolemmal vesicles isolated from rainbow trout white muscle (Laberee and Milligan, 1999). The findings were similar to those of Wang et al. (1997) in that lactate uptake was saturable, mediated in part by a low-affinity, high capacity carrier that is partially inhibited by pyruvate, which is consistent with a monocarboxylate transporter. However, lactate uptake by white muscle sarcolemmal vesicles was not inhibited by CIN; rather both CIN and the anion-transport inhibitor, 4-acetoamido-4′-isothiocyanstilbene-2-2′-disulphonic acid (SITS) appeared to stimulate lactate uptake. The model emerging from these studies is that lactate uptake is carrier-mediated and its subsequent efflux is inhibited by SITS and CIN, suggesting that efflux is also carrier mediated. Thus, it would appear that in trout muscle lactate uptake and efflux occur *via* separate pathways.

In mammalian muscle, lactate uptake and efflux occur *via* two separate pathways, uptake mediated by MCT1 and efflux *via* MCT4 (Bröer et al., 1998; Dimmer et al., 2000; Fox et al., 2000). Both MCTs are found in mammalian fast-twitch, glycolytic fibers, which are both producers and consumers of lactate, as is fish white muscle (Bonen, 2001). MCT 1 has a high affinity for lactate (K_m approx. 3.5 mmol l^{-1}), but low specificity (K_m for pyruvate approx. 1 mmol l^{-1}) (Bröer et al., 1998) and its abundance is positively correlated with muscle oxidative capacity (Pilegaard et al., 1999; Bonen et al., 2000; Juel, 2001). The MCT4 isoform has a relatively low affinity for lactate (K_m 28–35 mmol l^{-1}; Dimmer et al., 2000; Fox et al., 2000) but a high substrate specificity (K_m for pyruvate approx. 150 mmol l^{-1}; Fox et al., 2000) and its abundance is positively correlated with glycolytic capacity (Bonen, 2001).

While fish white muscle clearly takes up lactate from the extracellular space, *via* a carrier with MCT1-like properties (Wang et al., 1997; Laberee and Milligan, 1999), the mechanism of efflux is unknown. It is unlikely that the mechanism for lactate efflux in fish muscle is similar to that in mammalian muscle because of the different metabolic fates of lactate in the two groups. In mammals, lactate is exported from the glycolytic fibers to the blood *via* the MCT4 and subsequently taken up by oxidative tissues, *via* the MCT1 (Bonen, 2001; Juel, 2001). In contrast, in trout muscle, lactate is retained within the muscle and used as a substrate for *in situ* glycogenesis. Thus, our primary objective was to use the giant sarcolemmal vesicle preparation to characterise lactate efflux from trout white muscle. A second objective was to combine what is known about lactate transport in mammalian muscle with our findings to develop a model to explain the mechanism of lactate retention within trout white muscle.

Materials and methods

Experimental animals

Male and female rainbow trout *Oncorhynchus mykiss* Walbaum weighing 150–300 g were purchased at Rainbow Springs Trout Farm (Thamesford, Ontario, Canada) throughout the year. They were held in a 1000 liter circular tank with continuous flow of dechlorinated tapwater maintained at 14±1°C, and subjected to a 12 h:12 h light:dark cycle. Fish were fed to satiation every day with commercial trout pellets. On the day of the experiment, fish were killed by anaesthetic overdose [2 g tricaine methane sulfonate (MS-222) and 2 g of $NaHCO_3$ in 10 liters of dechlorinated tapwater] immediately prior to muscle sampling.

Giant sarcolemmal vesicle preparation

Sarcolemmal vesicles were prepared following the protocol of Laberee and Milligan (1999). All chemical solutions used in the experiments were made up in buffer containing 140 mmol l^{-1} KCl and 5 mmol l^{-1} 3-[*N*-morpholino]propanesulphonic acid (Mops) (Sigma, Oakville, ON, Canada), adjusted to pH 7.4 (KCl/Mops buffer).

Vesicle yield and vesicular integrity

The protein content of each vesicle preparation was assayed using the Bradford method (Bradford, 1976) using 10 µl of the vesicle solution, with bovine serum albumin (Fraction V, Boehringer Mannheim, Germany) as a standard. Lactate efflux is expressed per unit of vesicle protein.

Membrane integrity was routinely examined using the Trypan Blue exclusion method. Following vesicle isolation, a 0.4% solution of Trypan Blue was added to resuspended vesicles in a 1:25 ratio and the vesicles counted using a haemocytometer under a compound microscope. Vesicles were considered disrupted or incompetent if the intravesicular space was coloured with Trypan Blue. Preparations with <90% intact vesicles were discarded.

Vesicle orientation, number and size

The orientation of the sarcolemmal vesicle membranes (i.e. the percentage of vesicles in the right-side out orientation) was determined using the ^{3}H-ouabain binding method (Nørgaard, 1983). In brief, the extracellular domain of the Na^+/K^+-ATPase

was labelled with vanadate-facilitated ^{3}H-ouabain in both intact and deoxycholate-ruptured vesicles. Following isolation, vesicles were resuspended in sucrose solution containing 250 mmol l^{-1} sucrose, 1 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} vanadate (Sigma, Oakville, ON, Canada) and 5 mmol l^{-1} Mops, pH 7.4, in 1.7 ml microfuge tubes. Deoxycholate (Fisher Scientific; Toronto, Canada) was added to one set of tubes to a final concentration of 0.8 mg ml^{-1}, to permeate the membrane (permeated vesicles) and expose any intracellular ouabain binding sites (i.e. those vesicles that were inside-out). The other set had an equivalent volume of sucrose solution added as a control (intact vesicles). Tubes were shaken on an orbital shaker at 0.45 ***g*** for 30 min at 20°C. The vesicle solutions were centrifuged at 15 000 ***g*** for 30 s and the supernatant removed. Vesicles were then resuspended in sucrose solution containing 0.08 μCi ^{3}H-ouabain (ICN Radiochemicals; Montreal, Canada) and incubated for 30 min on the orbital shaker at 0.45 ***g***. The permeated vesicles permit access of ^{3}H-ouabain to the inside of the membrane. Any excess binding of ^{3}H-ouabain in permeated vesicles compared to intact vesicles indicates that the vesicular membrane is oriented inside-out. Both samples were transferred to 2.0 ml filter tubes containing 0.45 μm cellulose-nitrate filters. The incubation tubes were rinsed four times with a total of 100 μl of sucrose solution and the wash was added to the filter tube in order to retrieve the entire sample. The filter tubes were centrifuged at 15 000 ***g*** for 30 s to draw the solution through the filter while trapping the ^{3}H-ouabain-labelled vesicles on the filters. Each filter was washed four times with 100 μl of sucrose solution and centrifuged after each wash to draw the fluid through the filter. The filters and the filtrate (consisting of both the original incubation media plus the washes) were collected into scintillation vials and 5 ml of ReadySafe (Beckman; Missassauga, Canada) scintillation fluid were added to all vials. The vials were counted on a Packard 1900TR Liquid Scintillation Counter with automatic quench correction. The counts on the filters of the ruptured *versus* intact vesicles were compared and used to determine the proportion of vesicles in the right-side-out orientation.

Sarcolemmal vesicle diameters were measured by phase contrast microscopy and an average vesicle volume was calculated, assuming the vesicles to be a sphere. The number of vesicles that correlate with a known amount of protein (i.e. *x* number of vesicles present in *y* μg of protein) was calculated by counting vesicle samples in known volumes with a known protein content.

Vesicle loading with ^{14}C-lactate

Lactate uptake by sarcolemmal vesicles at 25 mmol l^{-1} external [lactate] was first measured following the protocol of Laberee and Milligan (1999) to establish that the vesicles do take up lactate and to estimate the required loading times for efflux experiments. Once lactate uptake was established, sarcolemmal vesicles were incubated in a reaction mixture containing a known concentration of lactate in KCl/Mops solution (see above) plus 18.5 kBq ^{14}C-lactate (ICN Radiochemicals; universally labelled, specific activity=1.11×10^{12} d.p.m. mol^{-1}) in 1.7 ml microfuge tubes. Tubes were shaken at 0.45 ***g*** on an orbital shaker at room temperature for 30 min. The samples were then pelleted by centrifugation at 15 000 ***g*** for 30 s. The supernatant from each sample was collected into 20 ml scintillation vials. The pellets were washed twice with 500 μl of cold 25 mmol l^{-1} lactate in KCl/Mops (pH 7.4) to remove ^{14}C-lactate loosely bound to or trapped between the vesicles. Within 5–10 s of washing, the samples were centrifuged at 15 000 ***g*** for 30 s to pellet the vesicles. In all experiments one sample was snipped off immediately into a scintillation vial to estimate total lactate loaded after 30 min of incubation. All other samples were resuspended in 1 ml of efflux medium (see below) and sampled at times specific to the experimental conditions.

Any facilitated efflux from the vesicles was arrested by the addition of 1 ml of ice-cold 2.5 mmol l^{-1} $HgCl_2$ (STOP solution) at designated times, followed by centrifugation at 15 000 ***g*** for 30 s to pellet the vesicles. $HgCl_2$ alters the sulphydryl groups of proteins and has been shown to block both the influx and efflux of lactate in a variety of cell types (Grimditch et al., 1985; Roth and Brooks, 1990; Laberee and Milligan, 1999). To assess the effectiveness of the $HgCl_2$ STOP solution in inhibiting lactate efflux from this preparation, preloaded vesicles were immediately resuspended in ice-cold STOP solution and efflux monitored after 1 min. The supernatants and vesicle pellets were collected into scintillation vials and 10 ml scintillation fluid was added to all vials. Each sample was shaken and left overnight in the dark and counted on a Packard 1900TR Liquid Scintillation Counter with automatic quench correction

Time course and concentration dependence of lactate efflux

These experiments were performed under zero-*trans* conditions, i.e. all substrate and label were initially internal to the vesicles. The relative time course of lactate efflux at 25 mmol l^{-1} internal concentration was monitored from 10 to 600 s, sampling from the same sample at various times. At the given time, a 20 μl sample of the vesicle suspension was taken, added to 1 ml ice-cold STOP solution and immediately centrifuged at 15 000 ***g*** for 30 s. Both the supernatant and the vesicular pellets were separately collected into scintillation vials and counted as described.

The concentration of cold lactate pre-loaded into the vesicles was varied from 5 to 250 mmol l^{-1}. At the higher lactate concentrations, the vesicles were placed in efflux medium containing the appropriate concentration of sucrose to prevent osmotic shock.

To verify that vesicles were in fact loading with lactate, vesicles were osmotically shocked to burst them and the amount of lactate lost was measured. Vesicles were prepared and preloaded as previously described. Following washing, the pellet was resuspended in 1 ml of hypo-osmotic, lactate-free, 50 mmol l^{-1} KCl/Mops (pH 7.4). After 1 min, 1 ml of ice-cold STOP solution was added to the sample, which was then centrifuged at 15 000 ***g*** for 30 s. Both the supernatants and the vesicular pellets were collected into scintillation vials. In a parallel series of experiments, vesicular integrity was assessed after 1 min of hypo-osmotic exposure to ensure vesicles were, in fact, ruptured. Using the Trypan Blue exclusion method

previously described, less than 10% of the vesicles remained intact after 1 min of hypo-osmotic exposure.

pH and Na^+ dependence

When the intravesicular pH (pH_i) is greater than extravesicular pH (pH_e), a gradient is established that should diminish the potential for lactate efflux (Wang et al., 1997). Vesicles were prepared, preloaded with 25 mmol l^{-1} lactate and washed as described. The vesicular pellet was resuspended in 1 ml of lactate-free KCl/Mops at either pH 7.4 (pH_i=pH_e) or pH 5.1 (pH_i>pH_e).

When the pH of the intravesicular compartment is less than that of the extravesicular space, a pH gradient is established such that more lactate is in the non-ionic, potentially diffusible form inside the vesicles. This condition sets up the potential for lactate to diffuse out of the vesicles down the pH gradient, and thus should enhance lactate efflux. Vesicles were prepared in KCl/Mops (pH 7.4) and pre-loaded with 25 mmol l^{-1} lactate in KCl/Mops (pH 5.1) to yield an average intravesicular pH of 5.8±0.7 (*N*=6). Intravesicular pH was measured on vesicular pellets lyzed using the freeze–thaw method (Zeidler and Kim, 1977). The lyzed pellets were injected into a Radiometer pH microelectrode maintained at 20°C and linked to a Radiometer PHM 73 blood gas monitor. The lactate-loaded vesicular pellet was washed and then resuspended into 1 ml of lactate-free KCl/Mops, pH 7.4, such that pH_i<pH_e, and sampled as per the standard protocol.

To assess potential extracellular Na^+-dependent lactate efflux, vesicles were prepared, preloaded and washed as described. The vesicles were resuspended into 1 ml of lactate-free 75 mmol l^{-1} NaCl/Mops + 75 mmol l^{-1} KCl/Mops, pH 7.4 and sampled as described.

Inhibitor series

All inhibitor series were run over a transport period of 1 min at an internal lactate concentration of 25 mmol l^{-1}. Controls were always run concurrently with inhibitors and contained an equivalent amount of DMSO. Vesicles were prepared, preloaded and washed as described. The vesicles were then resuspended in 1 ml of either 5 mmol l^{-1} 4-acetoamido-4′-isothiocyanstilbene-2,2′-disulphonic acid (SITS) or 5 mmol l^{-1} α-cyano-4-hydroxycinnamate (CIN) in 0.5% DMSO (w/v) neutralised with 5 mmol l^{-1} $NaHCO_3$ in KCl/Mops, pH 7.4, and sampled as described.

Statistical analysis

The values presented are means ± 1 standard error of the mean (S.E.M.). A one-way analysis of variance (ANOVA) was used for comparison between treatments, followed by a Tukey-HSD multiple comparison analysis.

Results and Discussion

Yield, vesicular integrity and orientation

The mean vesicle protein concentration used in each sample of the efflux experiments was 25.61±2.06 μg μl^{-1} (*N*=195), which corresponded to approximately 666 vesicles. 1 μg of vesicle protein represented on average 26±3 vesicles (*N*=11), as determined with a haemocytometer under phase-contrast microscopy. The mean vesicular diameter was 5.22±0.25 μm (*N*=11), yielding an estimated vesicular volume of 55.9±0.006 μm^3, which was within the range reported for giant vesicles isolated from both mammalian and trout muscle (Pilegaard et al., 1993; Laberee and Milligan, 1999).

As determined by Trypan Blue exclusion, 93.9±1.71% (*N*=68) of the vesicles in a preparation excluded the dye, and thus were considered to be intact. Vesicular integrity was monitored throughout the course of the experiments and any preparation in which less than 90% of the vesicles were intact was discarded. These estimates of vesicle viability are consistent with those reported by Laberee and Milligan (1999) for vesicles isolated from trout muscle.

To estimate whether vesicles were oriented with the correct side of the membrane facing outwards (i.e. the *in vivo* extracellular surface facing outwards), the Na^+–K^+ pumps were labelled with ^{3}H-ouabain. The vanadate-facilitated ouabain binding site of the Na^+–K^+ pump is located on the extracellular surface of the membrane (Hansen, 1979), so if all vesicles are in the correct orientation, ^{3}H-ouabain binding should be the same in intact and deoxycholate-treated vesicles. Deoxycholate has been shown to disrupt membrane integrity in a variety of different cell types, including giant sarcolemmal vesicles isolated from mammalian muscle (Pilegaard et al., 1993). In the present study, examination of deoxycholate-treated vesicles incubated with Trypan Blue by phase-contrast microscopy indicated that most vesicles incorporated the dye, in comparison to controls, indicating that vesicular integrity was ruptured. The ^{3}H-ouabain binding to intact vesicles was 169.9±48.7 d.p.m.$^{-1}$ μg protein^{-1} (*N*=10), which was not different from that seen in deoxycholate-treated vesicles (173.3±38.5 d.p.m.$^{-1}$ μg protein^{-1}, *N*=10), indicating that the vesicles were in the correct orientation. Thus, the terms ‘efflux’ and ‘influx’ as applied to the vesicles in this study retain their *in vivo* meaning.

Since the primary objective of this work was to examine lactate efflux, it was first necessary to demonstrate that the vesicles could be lactate loaded. Vesicles took up lactate at a rate of 26.4±6.3 nmol min^{-1} mg^{-1} protein (*N*=8) at an external lactate concentration of 10 mmol l^{-1}, which is comparable to that previously reported by Laberee and Milligan (1999).

Lactate efflux from sarcolemmal vesicles

Lactate efflux from sarcolemmal vesicles preloaded with 25 mmol l^{-1} lactate proceeded linearly for up to 70 s (Fig. 1), therefore all subsequent measurements of efflux were carried out for a preiod of 1 min. Lactate efflux increased with increasing intravesicular lactate concentrations from 10–250 mmol l^{-1} linearly, at rates approximately 500-fold slower than influx rates. For example, at a lactate concentration of 25 mmol l^{-1}, mean efflux was 0.049±0.006 nmol mg^{-1} protein min^{-1} (*N*=6; Fig. 2) compared to influx rates of 15–20 nmol mg^{-1} protein min^{-1} (Laberee and Milligan, 1999).

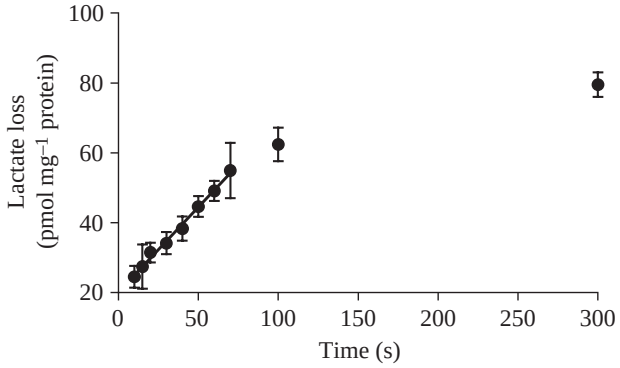

Fig 1. Lactate loss (pmol mg^{-1} protein) from rainbow trout white muscle sarcolemmal vesicles over time. Vesicles were loaded with 25 mmol l^{-1} lactate in 140 mmol l^{-1} KCl/Mops + 18.5 kBq ^{14}C-lactate. Values are means ± 1 S.E.M.; *N*=6 (10, 15, 20, 40, 50 and 70 s values); *N*=10 (30, 60 s values); *N*=5 (100, 300 s values).

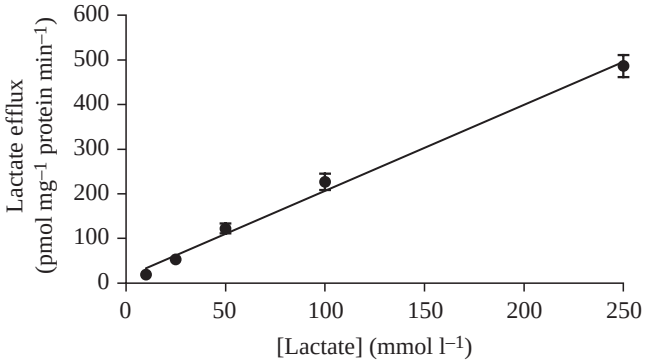

Fig 2. The effect of internal lactate concentration on lactate efflux from trout white muscle sarcolemmal vesicles. All efflux was measured over a period of 1 min. *N*=7 (50 and 100 mmol l^{-1} lactate concentrations); *N*=6 for 10 mmol l^{-1} lactate); *N*=4 (300 mmol l^{-1} lactate); *N*=12 (25 mmol l^{-1} lactate).

To determine whether these very slow efflux rates were due to poor loading of the vesicles, despite measuring apparently good lactate uptake rates, vesicles loaded at 25 mmol l^{-1} lactate were burst by osmotic shock and lactate loss measured. Over a 1 min period, mean lactate loss was 29.3±0.2 nmol mg^{-1} protein (*N*=8), which was approx. 500-fold greater than efflux rates from intact vesicles. This indicates that the slow efflux rates are real, and not an artefact of poor lactate loading. Rather, it appears that the trout white muscle sarcolemmal membrane is relatively impermeant to lactate.

Lactate efflux was unaffected by $HgCl_2$ (STOP solution; Fig. 3A). Mercuric chloride is known to modify protein sulfhydryl groups and inhibit lactate uptake in trout sarcolemmal vesicles (Laberee and Milligan, 1999) as well as lactate efflux from vesicles isolated from rat muscle (Roth and Brooks, 1990; Pilegaard et al., 1993). Lactate efflux was unaffected by 5 mmol l^{-1} CIN, which is a competitive inhibitor of the H^+-lactate cotransporter in skeletal muscle (Poole and Halestrap, 1993; Roth and Brooks, 1993), but was stimulated

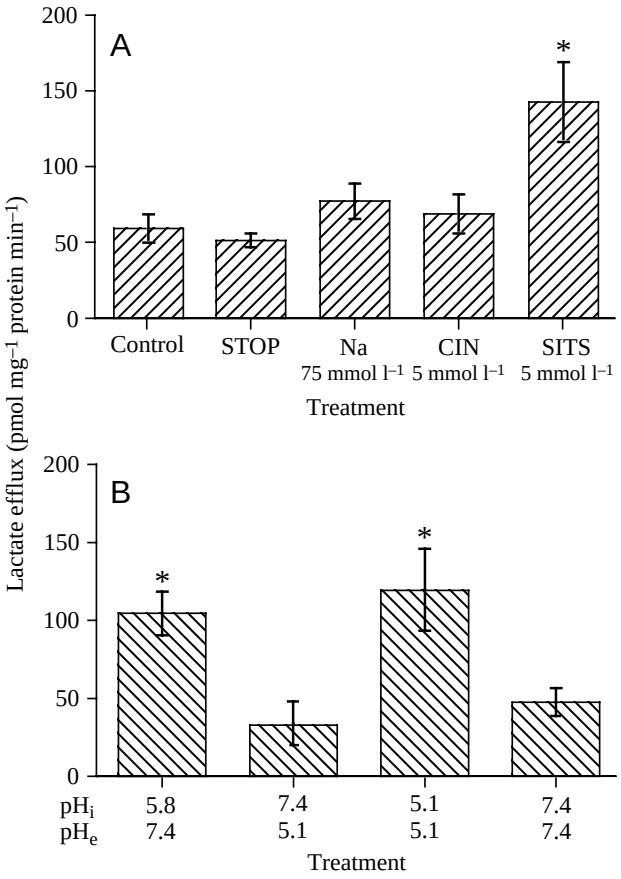

Fig 3. (A) The effects of various inhibitors on lactate efflux from trout white muscle sarcolemmal vesicles. All vesicles were loaded with 25 mmol l^{-1} lactate and 18.5 kBq ^{14}C-lactate. Values are means ± 1 S.E.M. Control (*N*=5): efflux was measured in KCl/Mops buffer; STOP (*N*=10), efflux in ice-cold, 2.5 mmol l^{-1} $HgCl_2$; 75 mmol l^{-1} Na (*N*=7): efflux in 75 mmol l^{-1} KCl, 75 mmol l^{-1} NaCl Mops buffer; 5 mmol l^{-1} CIN (*N*=6): efflux in KCl/Mops containing 5 mmol l^{-1} α-cyano-4-hydroxycinnamate; 5 mmol l^{-1} SITS (*N*=11): efflux in KCl/Mops containing 5 mmol l^{-1} 4-acetoamido-4′-isothyiocyanstilbene-2,2′-disulphonic acid. *A significant difference ($P<0.05$) from the control value. (B) The influence of pH gradient on lactate efflux from trout white muscle sarcolemmal vesicles. Values are means ± 1 S.E.M. $pH_i<pH_e$, *N*=11; $pH_i>pH_e$, *N*=7, $pH_i=pH_e=5.1$, *N*=5; $pH_i=pH_e=7.4$, *N*=6. *A significant difference ($P<0.05$) from the $pH_i=pH_e=7.4$ value.

by 5 mmol l^{-1} SITS, which inhibits anion transport (Fig. 3A). These results are inconsistent with what has been reported previously for trout muscle. Both these compounds stimulated lactate influx in trout sarcolemmal vesicles (Laberee and Milligan, 1999), which was interpreted as reflecting an inhibition of efflux. Further, in an isolated perfused trunk preparation, 5 mmol l^{-1} CIN caused a 75% reduction in lactate efflux from trout muscle and SITS was without effect (Wang et al., 1997). These different effects of inhibitors in the perfused trunk and sarcolemmal vesicle preparations may reflect differences in the scale of the preparation. In the present study, efflux rates are very low, relative to those measured in

the perfused trunk, so that small effects caused by SITS or CIN are more likely to be seen. Furthermore, the perfused trunk model is a multiple membrane system and both SITS and CIN are non-specific inhibitors that may act at multiple sites (e.g. sarcolemmal membrane, mitochondrial membrane, blood vessel endothelium) (Halestrap, and Denton, 1974). The sarcolemmal vesicle, however, is a single membrane and the effect observed is specific to the sarcolemmal membrane. Since SITS is a non-specific anion transport inhibitor, it may have secondarily affected lactate movement by its actions on Cl^-/HCO_3^- exchange and alteration of the transmembrane pH gradient which, because of the smaller scale of the vesicle preparation compared to the perfused trunk, was a measurable phenomenon. Lactate efflux is sensitive to alterations in the transmembrane pH gradient, such that when intravesicular pH (pH_i) was acidic relative to extravesicular pH (pH_e), lactate efflux increased (Fig. 3B) as diffusive loss of lactic acid increased. Even though lactate efflux was stimulated when $pH_i<pH_e$, a situation seen *in vivo*, the absolute rate of loss was still quite low, in the pmol mg^{-1} protein min^{-1} range, compared to uptake rates in the nmol mg^{-1} protein min^{-1}. Taken together, these data suggest that the trout muscle sarcolemmal membrane is relatively impermeant to lactic acid, and that the putative monocarboxylate transporter identified by Wang et al. (1997) and Laberee and Milligan (1999) is probably responsible for lactate uptake only, and efflux is *via* passive diffusion and is minimal.

This model for lactate movement across the trout muscle membrane, in which the MCT-like transporter is responsible for facilitating lactate uptake only, is different from that described for mammalian fast-twitch muscle (see below), but consistent with what is known about muscle lactate metabolism in trout *in vivo*. During exhaustive exercise lactate is produced *via* glycogenolysis; consequently glycogen levels drop and lactate levels increase. Only a small fraction (approx. 10–15%; Turner and Wood, 1983; Turner et al., 1983) of the total lactate produced leaves the muscle, probably as a consequence of the resistance of the white muscle membrane to lactate loss. Since lactate is the primary substrate for glycogenesis, facilitated transport of the lactate out of the muscle would only serve to delay restoration of muscle glycogen stores. Exogenous lactate is taken up by MCT-like transporter and used by the trout white muscle, primarily as an oxidative substrate as opposed to glycogenic substrate (J. Kam and C. L. Milligan, unpublished observation).

In mammalian fast-twitch muscle fibers, monocarboxylate transporters are involved in both lactate influx and efflux across the muscle membrane (Juel, 2001; Bonen, 2001), with diffusion playing a minimal role. The different role for monocarboxylate transporters in mammals *versus* fish is not surprising, given the different metabolic fates of lactate. In mammals, the primary fate of muscle lactate is oxidation, *via* other tissues (including other muscle tissue; Brooks et al., 1999). Muscle lactate is also cleared fairly quickly (within 60–90 min in mammals compared to 4–8 h in trout) and glucose is the main glycogenic substrate. Mammalian fast-twitch fibers contain both the MCT1 (a high-affinity, low-capacity transporter) and MCT4 (a low-affinity, high-capacity transporter) isoforms, responsible for lactate uptake and efflux, respectively (Bröer et al., 1998; Bonen et al., 2000; Dimmer et al., 2000; Fox et al., 2000). It is hypothesised that these two transporters provide the muscle with an efficient method for transporting lactate when lactate levels are low (i.e. under resting or low activity conditions, when lactate is used oxidatively) or high (during intense exercise, to facilitate net efflux) (Bonen et al., 2000). Clearly, in trout muscle, the phenomenon of lactate-based *in situ* glycogenesis introduces a potential for redundancy if membrane transport systems facilitated lactate efflux. The use of lactate as a glycogenic substrate negates the need to release lactate for clearance purposes.

In conclusion, this study, in conjunction with the that of Laberee and Milligan (1999), suggests that in trout white muscle, a MCT-like transporter is responsible for facilitated lactate uptake only, with efflux occurring *via* passive diffusion. The 500–1000-fold lower rate of efflux than influx indicates the trout muscle is relatively impermeant to lactate and explains the phenomenon of lactate retention observed *in vivo*.

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