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Muscle lactate transport studied in sarcolemmal giant vesicles

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Lactate transport was studied in giant (median diameter 6.3 μm) sarcolemmal vesicles obtained by collagenase treatment of rat skeletal muscle. The lactate transport displayed stereospecificity, had a high temperature coefficient, and could be inhibited up to 90% with known transport inhibitors (PCMBS and cinnamate). In equilibrium exchange experiments, the L-lactate flux demonstrated saturation kinetics with $K_m = 23.7$ mM and $V_{max} = 108$ pmol cm^{-2} s^{-1}. With lactate present on only one side of the membrane, (zero trans conditions), V_{max} was reduced to 48 pmol cm^{-2} s^{-1}. The flux rate displayed transacceleration. The lactate flux was coupled to a parallel H^+ flux. Under equilibrium exchange conditions, the carrier-mediated lactate flux was not pH-dependent. In the zero trans experiments, H^+ on the trans side acted as an inhibitor. The loaded form of the carrier reorients faster than the unloaded form, and the protonated form with no lactate bound reorients slowly or is immobile. When compared to intact muscles, the giant sarcolemmal vesicles retain their transport characteristics both qualitatively and quantitatively.

Introduction

All skeletal muscles seem to possess a lactate transport system. Facilitated diffusion of lactate has been observed in frog muscles [1,2], perfused rat hindlimb [3], mouse diaphragm [4], and isolated mouse soleus and extensor digitorum longus muscles [5,6,7]. In these studies, lactate transport was characterized by means of inhibitors. However, it is difficult to perform studies of transport kinetics with isolated or perfused whole muscles because of compartmentalization and lactate metabolism. Some of these problems have been solved by the use of small ($\ll 1$ μm) vesicles made from purified sarcolemmal membranes. These vesicles have recently been used to study certain aspects of lactate transport [8,9]. Giant sarcolemmal vesicles, obtained with collagenase treatment, have been used for patch-clamp studies of ion channels. These studies require only a limited number of vesicles [10–12]. The aim of the present study was to investigate whether these giant vesicles could be obtained in an amount sufficient to carry out transport studies. Furthermore, the aims were to characterize these vesicles, and to perform lactate flux measurements in order to obtain kinetic transport parameters. Therefore, flux measurements were carried out with different lactate concentrations and at different pH values under equilibrium exchange, zero trans (net efflux), and infinite cis conditions.

Materials and Methods

Isolation of vesicles. The hindlimb muscles from male Wistar rats were isolated and split lengthwise. The muscles were incubated for one hour at 34°C in 140 mM KCl, 10 mM Mops (pH 7.4), collagenase (Sigma type VII) 150 units/ml, and the proteinase inhibitor aprotinin (0.01 mg/ml, Sigma A1153). The muscles were then washed with KCl/Mops and 10 mM EDTA. Percoll (final concentration 16%) and aprotinin were added to the suspension of vesicles and cell fragments. A three-layer step density gradient was used to isolate the vesicles: The upper layer consisted of 1 ml KCl/Mops. The middle layer consisted of 3 ml 4% Nycodenz (Nycomed AS, Norway) in KCl/Mops. 5 ml of the vesicle suspension was placed at the bottom of the 10 ml centrifugation tube. After centrifugation (40 $\times g$ for 45 min) at room temperature the vesicles were harvested from the interface of the two upper solutions. The vesicles were then diluted with KCl/Mops and

Abbreviations: CIN, α-cyano-4-hydroxycinnamic acid; PCMBS, *p*-chloromercuriphenylsulfonic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; Mops, 4-morpholinepropane-sulfonic acid; iBCLA, isobutylcarbonyllactylanhydride.

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spun down (830 × g for 30 min). Eventually, the vesicles were inspected by phase contrast microscopy. The protein concentration was determined by the method of Lowry et al. [13].

Similar vesicles derived from the sarcolemma [14] have been used in patch-clamp studies of rectifying ion channels [10–12]. Based on the patch-clamp studies, it is possible to evaluate the orientation of the membrane, and it was found to be exclusively 'right-side-out' (in contrast to 'inside-out'). Therefore, in the present study the terms 'efflux' and 'influx' have the same meaning as for intact muscles.

Incubation. In most experiments the vesicles were loaded with L-[^{3}H]lactate (5 μCi/ml, purchased from Amersham), unlabelled lactate and [^{14}C]sucrose. The latter was used as an extravesicular marker. After 30 min of incubation the vesicles were spun down and the sedimented vesicles were ready for the efflux studies. In the zero trans and infinite cis experiments sucrose was used to compensate for any changes in osmolarity.

Efflux experiments. The efflux studies were started when 10–25 μl of vesicles (30 mg protein/ml) were transferred to 15 ml of efflux medium. During the efflux period, 10–15 vesicle-free samples were obtained using a syringe mounted with a 0.25 μm filter. The ^{3}H and ^{14}C activities were determined with a Tri Carb 2000CA liquid scintillation counter. The ^{14}C counts were used to compensate for the extravesicular lactate. Since a slow (when compared to lactate efflux) release of [^{14}C]sucrose was observed, the first samples were used for calculation of the external lactate space.

Calculations. Since the efflux is dependent on the surface/volume ratio, the efflux from a mixture of vesicles with different diameters is not monoexponential. The efflux curve is the sum of monoexponentials:

$$y = \sum[1 - \exp(-t \cdot k \cdot (S_d/V_d))] \cdot F_d \cdot V_d$$

(y = external accumulated lactate, t = time, d = diameter, V_d = vesicle volume, S_d = surface, F_d = frequency of vesicles with the diameter d, k = rate constant). The experimental data were fitted to the sum of exponentials by means of the non-linear least-squares regression method. The initial efflux rate was calculated from the curve fit. With this method the initial efflux rate is based on all samples (10–15) instead of the fewer (2–3) samples, which can be obtained within the approximately linear first part of the efflux curve. For the calculations of flux per cm^2 membrane the distribution of vesicular diameters (shown below) and the vesicular lactate space as determined in the individual experiments were used. The vesicular lactate space was calculated as the difference between the total (internal + external) lactate space and the sucrose space (= external space).

Chemicals. The inhibitor of erythrocyte lactate transport isobutylcarbonyllactylanhydride (iBCLA) was synthetised according to Johnson et al. [15], and the activity of the compound was tested in lactate transport studies with human erythrocytes. The activity was identical to the one reported by Donovan and Jennings [16]. In experiments with iBCLA, albumin (1 mg/ml) was added.

Results

Characterization of the vesicles

The vesicle diameter varied from 2 to 43 μm, with a median of 6.3 μm (Fig. 1). Exclusively spherical vesicles were seen. The mean vesicular lactate space made up 79.5% of the vesicular volume. SDS gel electrophoresis of the solubilized vesicles showed that actin made up the main fraction of the material present within the vesicles, whereas myosin was nearly absent.

Stereospecificity and inhibitor sensitivity

The experiments were carried out under equilibrium exchange conditions. Vesicles were preloaded with 30 mM unlabelled L-lactate or D-lactate (pH 7.4), and ^{3}H-labelled L- or D-lactate. The efflux solution contained 30 mM unlabelled L- or D-lactate. The efflux of labelled L- or D-lactate was followed for 10 min (Fig. 2). In the experiment depicted in Fig. 2, the efflux of labelled L-lactate proceeded with a calculated rate constant $k = 0.053$ s^{-1}, whereas the rate constant for the efflux of labelled D-lactate was 0.025 s^{-1}. If the vesicles were preincubated with 0.5 mM PCMBS + 10 mM CIN, the efflux of labelled L-lactate proceeded with a rate constant of 0.009 s^{-1}. In similar experiments in which the L-lactate concentrations was low (1–5 mM) and the efflux was not saturated, the non-inhibitable fraction of

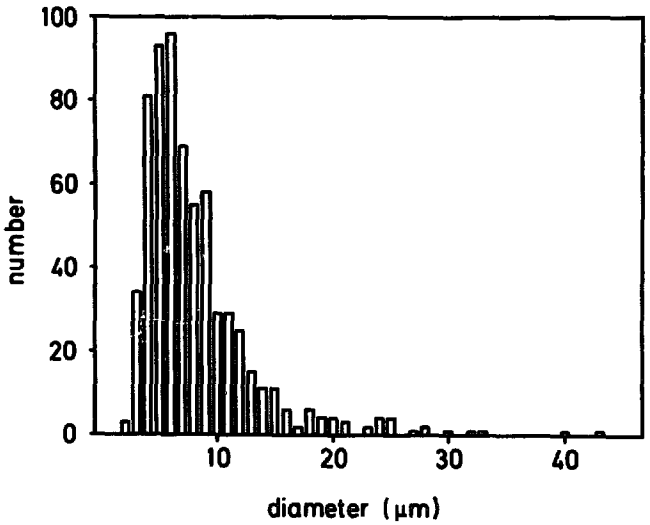

Fig. 1. Distribution of vesicle diameters as measured in the light microscope. Data are based on 700 measurements from six preparations.

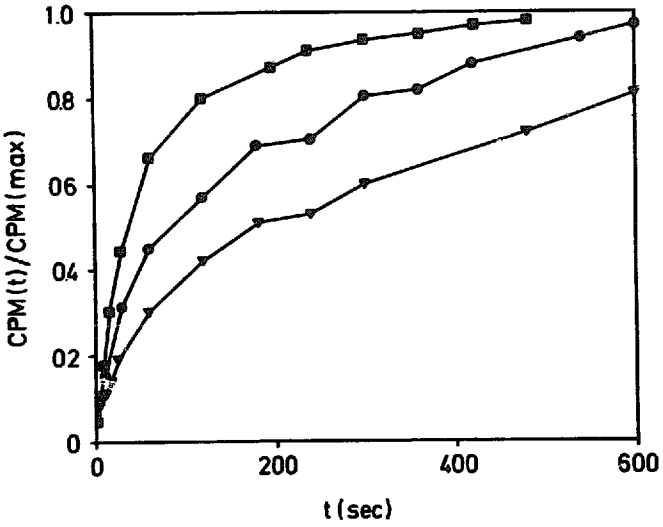

Fig. 2. Time course of lactate efflux; stereospecificity and inhibitor sensitivity. *y*-axis: the accumulated external lactate (in cpm) was calculated relative to the amount of tracer at equilibrium (cpm(max) ($t > 15$ min). *x*-axis: efflux time in seconds. Upper curve (■): efflux from vesicles loaded with 30 mM L-lactate. Middel curve (●): efflux from vesicles loaded with 30 mM D-lactate. Lower curve (▼): efflux from vesicles loaded with 30 mM L-lactate and preincubated for 30 min with 10 mM cinnamate+0.5 mM PCMBS. The depicted values are from three single series.

the efflux made up approximately 10% of the total efflux. DIDS (50 μM) had a negligible effect (< 10%), while iBCLA (0.5 mM) had no effect.

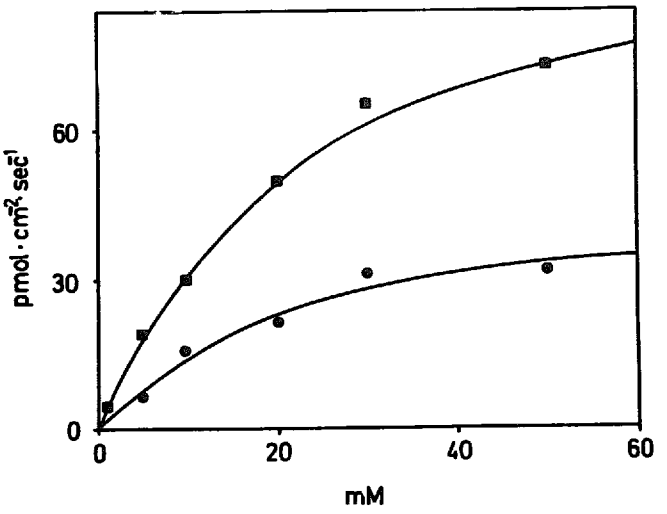

Fig. 3. Rate of initial carrier mediated lactate efflux plotted as a function of the external lactate concentration. The carrier mediated flux = total flux subtracted the non-inhibitable flux. Upper values (■): equilibrium exchange (internal and external concentration identical), pH 7.4. Lower values (●): zero trans experiments (no lactate in the external medium), pH 7.4. Each point represents the calculated carrier-mediated initial flux based on the best fit to the values from two series of experiments with a total of 20 or more samples. The lines depict the best linear fit to the data using an Eadie-Hofstee plot. Correlation coefficients are 0.99 and 0.93, respectively.

Equilibrium exchange experiments

To study equilibrium exchange, experiments were carried out with 1, 5, 10, 20, 30, and 50 mM L-lactate at external pH values of 7.4 and 6.5. Identical series of experiments were carried out with vesicles preincubated with 0.5 mM PCMBS and 10 mM CIN. It is assumed that the difference between the total flux and the non-inhibitable flux is identical to the carrier-mediated flux. When plotted against the lactate concentration the calculated initial carrier-mediated lactate efflux displayed saturation kinetics (Fig. 3). Using an Eadie-Hofstee plot and a linear fit to the data, the half-saturation constant, K_m ($\pm$S.D.), and the maximal efflux, V_{max}, were calculated to be 23.7 ± 2.6 mM and 108 ± 19 pmol cm^{-2} s^{-1} at pH 7.4, and 23.1 ± 4.9 mM and 158 ± 22 pmol cm^{-2} s^{-1} at pH 6.5, respectively.

Zero trans experiments. In these experiments the vesicles were preincubated with the protonophore CCCP (50 μM) in order to prevent the build up of a pH gradient during lactate efflux. Two equilibrium exchange experiments with 30 mM lactate demonstrated that CCCP per se had no effect on the efflux. The vesicles were loaded with 5, 10, 20, 30, or 50 mM L-lactate and the efflux to a lactate-free solution was studied. The volume of the external solution was more than 1000-times the vesicular volume, which secured zero trans conditions. The initial carrier-mediated efflux (net efflux) was lower than the one obtained for the corresponding concentration values under equilibrium exchange conditions. The calculated K_m and V_{max} were 20.9 ± 5.0 mM and 48 ± 10 pmol cm^{-2} s^{-1}, respectively (Fig. 3).

Infinite cis experiments. In these experiments the vesicles were preincubated with CCCP and loaded with a high (infinite) concentration of L-lactate (50 mM). The lactate concentration on the outside (trans) was varied from 0 mM to 50 mM. With no lactate on the outside the experimental conditions are identical to the ones for zero trans experiments with 50 mM lactate in the vesicles. However, with 50 mM lactate on the outside, the experimental conditions are identical to the ones for equilibrium exchange experiment with 50 mM lactate in the vesicles. The initial efflux rates were plotted as a function of the external (trans) lactate concentration as shown in Fig. 4. The half-saturation constant K_m for the effect of external (trans) lactate was 13.4 ± 5.9 mM.

pH dependency. Equilibrium exchange experiments with 20 mM lactate were performed at pH 7.8, 7.4, 7.0, 6.8, and 6.5 (Fig. 5). The collagenase treatment was carried out at pH 7.4, whereas the rest of the procedures took place at different pH values; the vesicles were therefore exposed to the new pH value for more than 90 min. It must however be noted that the internal pH could not be controlled. The rate of the non-inhibitable part of the exchange flux was doubled when pH was

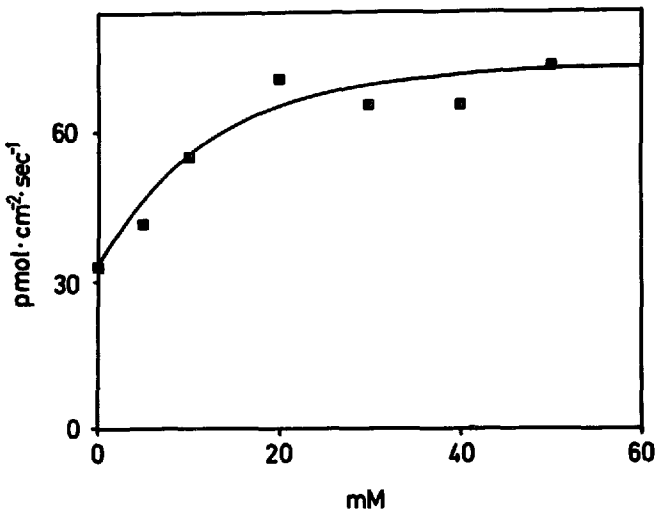

Fig. 4. Infinite cis experiments. Rate of initial carrier mediated lactate efflux as a function of the external (trans) concentration of lactate. The internal (cis) lactate concentration was 50 mM in all experiments (pH 7.4). Each point represents the calculated initial flux rate based on one serie with 12 samples. The line depicts the best linear fit to the data using an Eadie-Hofstee plot, $r = 0.98$.

reduced from 7.8 to 6.5. The total lactate exchange increased by 31%, which was mainly due to an increase in the non-inhibitable fraction.

In zero trans experiments the vesicles were preloaded with lactate at pH 7.4 and, at the onset of the efflux period, transferred to external solutions with pH values

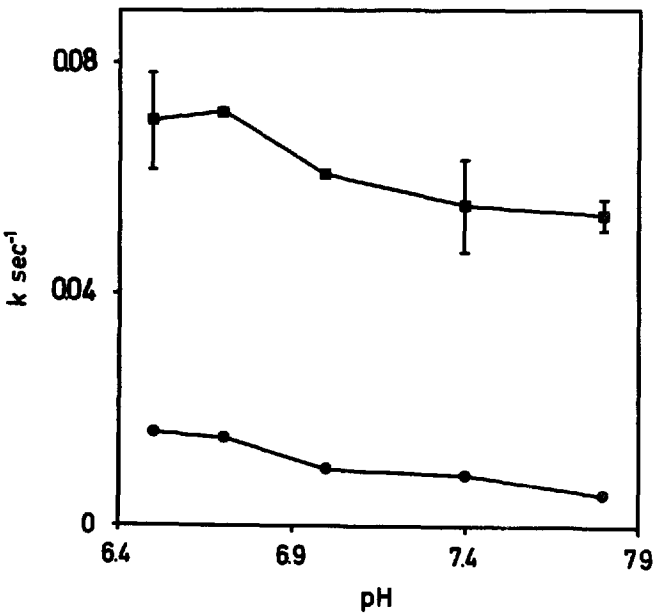

Fig. 5. pH dependency-equilibrium exchange. The rate constant (k) for lactate efflux as a function of the pH in the outer medium. The data points represent mean of 1–5 series with at least 10 samples in each series. If more than one series were completed the value shown represents the mean ± S.D. Upper curve (■): untreated vesicles. Lower curve (●): values from vesicles preincubated with 0.5 mM PCMBS + 10 mM CIN.

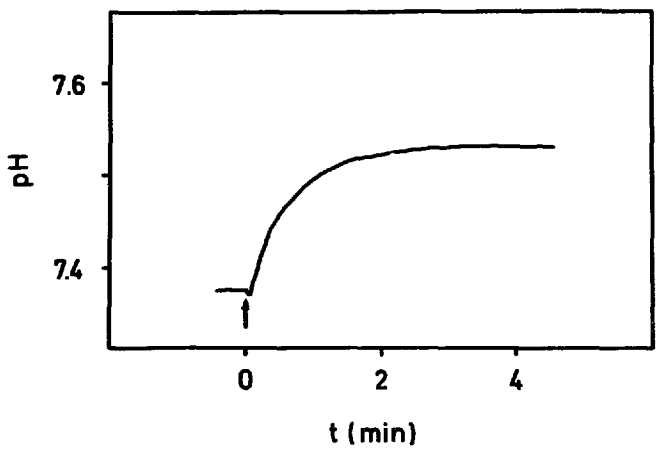

Fig. 6. Extravesicular pH increase produced by lactate influx. The vesicle suspension (pH 7.4, 0.1 mM Mops) was incubated (arrow) with lactate (pH 7.4) to a final concentration of 5 mM. pH was measured continuously with a small electrode in the 600 µl suspension which was magnetically stirred.

7.8, 7.4. and 6.5. At an external pH of 7.4, the rate coefficient for the tracer efflux was $0.0306 \pm 0.0059\ s^{-1}$ (±S.D., $n = 6$). If the vesicles were transferred to a solution with a pH of 6.5, k was reduced to $0.0228 \pm 0.0043\ s^{-1}$ ($n = 6$), whereas if the pH was increased to 7.8, the rate constant increased to $0.0366 \pm 0.0068\ s^{-1}$ ($n = 6$). With $k = 0.009\ s^{-1}$ for the non-inhibitable part of the flux, the changes in the carrier-mediated fluxes were −36% and +28%, respectively.

H^+/lactate coupling

Incubation of a vesicle suspension (low buffer capacity, 0.1 mM Mops, 140 mM KCl (pH 7.4)) with lactate at the same pH was followed by a gradual alkalinization of the external solution (Fig. 6). The time course of the pH increase was similar to the time course of the lactate flux (Fig. 2). The mean ratio between the amount of acid removed from the outer medium, and the amount of lactate taken up by the vesicles, was 0.68 ± 0.06 ($n = 6$). The alkalinization could be inhibited by PCMBS and CIN (data not shown).

Temperature effect

The effects of temperature were investigated with 30 mM L-lactate in the equilibrium exchange and zero trans experiments at 22°C and 35°C. The Q_{10} for the total lactate transport (carrier mediated + simple diffusion) was 2.2 under both types of experimental conditions.

Discussion

The collagenase method resulted in the formation of large vesicles, with which it was possible to study lactate transport and which could probably be used to study other transport systems.

Stereospecificity

The rate constant for D-lactate efflux was half that of L-lactate, but still twice that of the inhibited L-lactate transport. It can not be decided from these experiments if the L-lactate carrier is partly stereospecific, or if D-lactate is transported via another system.

Inhibitor sensitivity

In accordance with the results of studies done with small vesicles [8], the small or negligeable effects of DIDS and iBCLA on lactate flux demonstrated that the carrier is different from the inorganic anion exchanger and the monocarboxylate carrier present in erythrocytes. The effects of the inhibitors PCMBS and CIN showed that in muscles the lactate flux is predominantly carrier-mediated. At low concentrations of lactate, this fraction made up approximately 90% of the total flux. At higher lactate concentrations the carrier system was saturated such that with 50 mM lactate and under equilibrium exchange conditions, the carrier-mediated flux will be reduced to approximately 70% of the total flux.

pH-dependency

In perfused rat skeletal muscle the influx of lactate was increased 35% when external pH was decreased from pH 7.4 to 6.8, whereas a pH increase to 7.7 decreased the influx by 19% [3]. Similar results have been obtained with isolated mouse muscles [7].

In the experiments with giant vesicles the lactate equilibrium exchange increased with lower pH. The main part of this effect was due to an increased non-inhibitable flux, which is consistent with the assumption that this component of the flux is due to simple diffusion of undissociated lactic acid. The carrier-mediated equilibrium exchange was independent of pH, demonstrating that H^+ is not rate-limiting if lactate is available on both sides of the membrane. In the zero trans experiments an increased H^+ concentration on the trans side significantly reduced the lactate efflux, probably because the protonated form of the carrier reorients slowly when compared to the unloaded form.

In their experiments with small vesicles, Roth and Brooks [9] reported that the lactate net influx (zero trans) was dramatically increased with an inward directed H^+ gradient, and inhibited with an outward H^+ gradient, whereas parallel changes in internal and external pH had no effect on the lactate influx. It seems that H^+ is stimulatory if lactate is present, but inhibitory if lactate is not available. The explanation might be that H^+ binds first, and if lactate is not bound, the protonated carrier is not able to reorient itself. Furthermore, if pH is changed on both sides of the membrane, the stimulatory effect of H^+ on the cis side may be counteracted by the inhibitory effect on the trans side.

The pH changes during lactate fluxes (Fig. 6) demonstrated that H^+ and lactate are transported in the same direction and with the same time course, i.e. a lactate gradient can cause H^+ transport against a developing uphill H^+ gradient. (OH^- transport in the opposite direction can not be ruled out, but is unlikely [17]). In our experiments, the mean H^+/lactate stoichiometry was 0.68. The possible involvement of a Na^+/H^+ exchange system was excluded because of the Na free medium. However, the ratio was probably influenced by the simple diffusion of lactic acid, which varied with the H^+-gradient. It has been reported for the small vesicles [8] that the cotransport is electroneutral (1 : 1), which is probably also the case for the giant vesicles.

Transport kinetics

The highest lactate transport capacity was obtained in equilibrium exchange experiments with high concentrations of lactate on both sides of the membrane. If lactate was only available on one side of the membrane (zero trans conditions), the efflux rates also displayed saturation kinetics, but with a more than 50% lower V_{max}. Therefore, if interpreted in terms of a model, one of the rate-limiting steps in the zero trans experiments is the translocation/reorientation of the unloaded carrier which moves slower than the loaded form. In line with these results it was found that if the concentration of lactate on the trans side was increased, the lactate efflux rate also increased. Thus, the system displayed trans-acceleration.

The model

The transport kinetic parameters obtained under these experimental conditions led to the development of a kinetic model for the lactate/H^+ transportsystem in skeletal muscle: (1) Lactate and H^+ are co-transported (Fig. 6). (2) The transport is electroneutral, probably 1 : 1 (Fig. 6 and [8]). (3) The loaded carrier reorients faster than the unloaded form (V_{max} equilibrium exchange $\gg V_{max}$ zero trans). (4) H^+ binds first, the intermediate form of the carrier with only H^+ bound is immobile or reorients slowly (H^+ on the trans face is inhibitory, the system displays transacceleration).

Lactate transport in giant vesicles; a comparison with small vesicles, intact muscles, and erythrocytes

The usefulness of the giant vesicles for transport studies can be evaluated by comparing the transport kinetic paramaters K_m and V_{max} with values from other lactate transport studies (Table I). The values obtained are in the same order of magnitude as for perfused whole muscles from the rat [3], which demonstrates that the membranes of the giant vesicles have retained the total transport capacity during the enzymatic treatment and isolation procedures. In studies with isolated mouse muscles and human knee extensor muscles values of K_m

TABLE I

K_m and V_{max} values for sarcolemmal vesicles, erythrocytes and intact skeletal muscles

	K_m (mM)	V_{max} pmol cm^{-2} s^{-1}	V_{max} mmol kg^{-1} s^{-1}	Ref.
Sarcolemmal giant vesicles				
Exchange (pH 7.4)	23.7 ± 2.6	108 ± 19	1.13	
(pH 6.5)	23.1 ± 4.9	158 ± 22	1.70	
Infinite cis (pH 7.4)	13.4 ± 5.9			
Zero trans (pH 7.4)	20.9 ± 5.0	48 ± 10	0.48	
Small vesicles				8
Uptake (zero trans)	40.1	< 3 (estimated)		
Erythrocytes				21
Equilibrium exchange	46	4.3		
Net efflux (zero trans)	10.5	0.97		
Perfused rat hindlimb				
Net uptake	21		0.14	3
Mouse muscles, single fibres (pH 6.2)			0.20	7
Human muscles				18
Total efflux (cellular lactate 41 mmol l^{-1} water)			0.10	

and V_{max} of the same order of magnitude have been reported [7,18]. For the small vesicles formed from purified sarcolemmal membranes a V_{max} of 139.4 nmol/mg protein per min has been reported [8]. With the giant vesicles and in the zero trans experiments, the V_{max} was 1019 nmol/mg protein per min, and higher if the actin content is subtracted from the total protein content. The time course of lactate entry into the small vesicles can also be estimated from Figs. 1 and 2 in Roth and Brooks [8], and seems to be similar to the time course for lactate efflux from giant vesicles (Fig. 2, this study). However, the surface to volume ratio is 0.9 μm^{-1} for the giant vesicles, whereas it is more than 12 μm^{-1} for the small vesicles since their diameter is less than 0.5 μm [19]. The calculated V_{max} for lactate transport in the small vesicles based on these assumption is < 3 pmol cm^{-2} s^{-1}, which is less than 10% of the capacity in the giant vesicles, and not higher than in human erythrocytes. Therefore, the membranes of the small vesicles seem to lose more than 90% of their transport capacity during vesicle preparation. It has also been reported that small vesicles formed from erythrocyte membranes have a reduced transport capacity (per cm^2) when compared to the intact cells [20]. As this is not the case for the giant vesicles, they seem to offer a better model system than small vesicles for the study of quantitative aspects of lactate transport.

Acknowledgements

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