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Kinetics of lactate and pyruvate transport in cultured rat myotubes

Lena von Grumbckow [a,*], Peter Elsner [b], Ylva Hellsten [a], Bjørn Quistorff [b], Carsten Juel [a]

[a] *Copenhagen Muscle Research Center, August Krogh Institute, University of Copenhagen, Universitetparken 13, DK-2100 Copenhagen, Denmark*

[b] *Department of Medical Biochemistry and Genetics, The Panum Institute, University of Copenhagen, Copenhagen, Denmark*

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Abstract

Skeletal muscle transport of lactate and pyruvate was studied in primary cultures of rat myotubes, applying the pH-sensitive fluorescent indicator 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein. The initial rate of decrease in intracellular pH (pH_i) upon lactate or pyruvate incubation was used to determine total transport (carrier mediated and diffusion). Both lactate and pyruvate transport could be inhibited by a combination of 0.5 mM 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, 5 mM mersalyl and 10 mM α-cyano-4-hydroxycinnamate. The kinetic parameters, K_m and V_{max}, for carrier-mediated transport of lactate were 9.9 ± 1.1 mM and 0.69 ± 0.02 mmol l^{-1} s^{-1}, respectively. For pyruvate, K_m and V_{max} were 4.4 ± 1.3 mM and 0.30 ± 0.05 mmol l^{-1} s^{-1}, respectively. The diffusion component of the total transport was 0.0040 ± 0.0005[S] ($n = 4$) and 0.0048 ± 0.0003[S] ($n = 4$) for lactate and pyruvate, respectively. Furthermore, it was observed that the two monocarboxylate transporter isoforms present in mature skeletal muscles, MCT1 and MCT4 (formerly called MCT3 (M.C. Wilson, V.N. Jackson, C. Heddle, N.T. Price, H. Pilegaard, C. Juel, A. Bonen, I. Montgomery, O.F. Hutter, A.P. Halestrap, Lactic acid efflux from white skeletal muscle is catalyzed by the monocarboxylate transporter isoform MCT3, J. Biol. Chem. 273 (1998) 15920–15926)), were also expressed in primary culture of myotubes.

Keywords: Skeletal muscle; Cultured cell; Lactate transport

1. Introduction

During high intensity exercise there is a significant production of lactate by anaerobic glycolysis in skeletal muscle, leading to a cellular lactate accumulation of up to 50 mM in cell water and a corresponding acidification of more than 0.5 pH unit [21]. A large amount of the lactate formed is released to the blood and it is generally assumed that a fraction is taken up by liver and kidney [3] for conversion to glucose. Another fraction of the lactate is oxidized in resting muscles, in less active muscles, or in the previously active muscles during the recovery period [3]. In addition, it has been debated whether or not a significant fraction of the lactate may actually be converted to glycogen via muscle gluconeogenesis [1].

The sarcolemma forms a barrier for the movement of lactate out of the cells and into the muscle fibers. Lactate transport across the plasma membrane has

* Corresponding author. Fax: +45 35321567.

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been studied in numerous types of mammalian tissues including isolated muscle, and in muscle model systems [22,34], but there are only few studies of pyruvate transport [4,37,41]. Lactate and pyruvate transport has never been studied previously in intact primary muscle cells in culture.

Lactate and pyruvate cross the sarcolemma via a family of monocarboxylate transporters (MCT), which may have differing substrate and inhibitor specificities [34]. Under various physiologic conditions, the MCT isoforms are believed to mediate 50–90% of the total lactate transport [6]. As cultured muscle cells have a high potential for lactate formation the culture medium glucose concentration could affect the medium lactate concentration and, thus, the lactate transporter expression. Therefore, in the present study the effect of two glucose concentrations (5.05 and 22.7 mM) on the medium lactate concentration was investigated. Since the lactate transport involves co-transport of a proton, the uptake into cells will be accompanied by a slight acidification, which can be detected by using the pH-sensitive probe 2′,7′-bis-(carboxyethyl)-5(6)-carboxyfluorescein (BCECF).

The aim of the present study was: (1) to measure the transport kinetic parameters K_m and V_{max} and the diffusion component for lactate and pyruvate; (2) to evaluate cultured primary skeletal muscle cells as a model system for transport studies of lactate and pyruvate using the BCECF technique; (3) to identify the MCT isoforms present in cultured myotubes.

2. Methods

2.1. Materials

The fluorescent H^+ indicator BCECF was from Molecular Probes (Eugene, OR, USA). Culture dishes (150 mm) and Thermanox coverslip were purchased from Nunc (Denmark). Cell dishes (12 wells) were obtained from Corning (USA). Dispase II and DNase I were obtained from Boehringer Mannheim (Germany). Trypsin was obtained from Gibco BRL (USA). Matrigel was purchased from Becton Dickinson (USA). Horse serum and fetal calf serum were purchased from Biological Industries (Israel). The inhibitors and all other chemicals were obtained from Sigma Chemical (USA). The antibodies against MCT1 and MCT4 were a gift from Prof. A. Halestrap (Bristol, UK).

2.2. Primary cultures of rat myotubes

Myotubes were prepared from 21 day old embryos of Wistar rats, as described by Kühl [24] and Daniels [5] with two modifications: for dissociation of tissue, 0.15% trypsin, 0.1% collagenase type I and 0.01% DNase I was used, and the cells were cultured in growth medium, DMEM (Dulbecco's modified Eagle's medium) containing 20% fetal calf serum. After tissue digestion and preplating, the cells were seeded in 150 mm 0.1% gelatine coated culture dishes as described by Daniels [5]. Between 46 and 50 h after seeding, the myoblasts were separated from the other cell types by dispase treatment [5,24]. Myoblasts were plated directly onto individual coverslips (13 mm), placed in the wells of a 12 well dish, at a density of approx. 7.5×10^4 cells/coverslip in 120 μl of growth medium. Plating and incubation was at 37°C in 8% CO_2 + 92% air. Thirty minutes after plating, the myoblasts were attached to the surface of the coverslips and an additional 680 μl growth medium was added to each well.

The coverslips were coated with 120 μl 1% Matrigel in DMEM the day before plating and incubated at 37°C. The Matrigel solution was removed by suction before plating.

The cells were confluent at day 2 after seeding and the growth medium was removed. Cell differentiation (the cells begin to fuse, forming multinucleated myotubes) was accelerated by replacing the growth medium with DMEM containing 10% horse serum (fusion medium). Three times per week half of the fusion medium in each well was replaced with fresh fusion medium until experiments were carried out. The cells on coverslips were used for experiments at days 12–14 after plating.

2.3. Creatine kinase activity, protein determination and medium lactate analysis

Coverslips prepared from the same cell batch preparation as used for lactate and pyruvate transport were used for analysis of CK activity and protein content of the myotubes. The cells were washed three times with ice-cold phosphate buffer, harvested by

scraping with a rubber policeman and then dissolved in 200 μl of glycyl-glycine buffer (5 mM EDTA, 150 mM KCl, 5 mM $MgSO_4$, 25 mM glycyl-glycine, 1 mM dithiothreitol and 0.02% albumin, adjusted to pH 7.5 with NaOH) [12]. Creatine kinase activity and protein content were measured on the extracts by the method of Passonneau and Lowry [32] and Lowry et al. [25], respectively.

The medium lactate concentration was determined with a lactate analyzer (Yellow Spring Instrument model 2700 Select Biochemistry Analyzer, Yellow Springs).

2.4. Determination of lactate and pyruvate transport

2.4.1. Loading of myotubes with BCECF for fluorometric measurements

For the transport experiments 12–14 day old myotubes were washed with a HEPES buffer (136 mM NaCl, 2.6 mM KCl, 1.8 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$, 0.36 mM $NaH_2PO_4 \cdot H_2O$, 5 mM HEPES, 5.55 mM glucose adjusted to pH 7.4 at 25°C by NaOH) [17] and subsequently exposed to the acetoxymethyl ester of BCECF (10 μM) dissolved in the same buffer. The washing procedure ensures that the internal lactate concentration prior to the transport experiment is low. The mechanism of loading relies on the fact that the acetomethyl group is cleaved intracellularly by cytoplasmic esterases, to produce the membrane-impermeable pH dependent fluorescent dye BCECF. Cells were incubated at 37°C for 60 min and washed twice in the HEPES buffer in order to remove any external BCECF, BCECF-AM and any accumulated lactate and pyruvate. The loaded cells were stored protected from light.

2.4.2. Measurement of monocarboxylate transport

A coverslip with BCECF loaded cells was placed diagonally in a cuvette with 2 ml HEPES buffer. The cuvette had magnetic stirring and was temperature controlled at 25°C. The fluorescence spectrophotometer was a dual wavelength Hitachi F-2000, operating at the excitation wavelengths 500 and 440 nm with emission measured at 535 nm for both excitation wavelengths. In order to convert the observed 500/440 nm ratio changes to changes in intracellular pH, a calibration curve was produced by stepwise addition of HCl or NaOH, which allows the calculation of pH changes as

$$\Delta pH = \alpha \cdot \Delta ratio \quad (1)$$

where α is the slope of the calibration curve. The calibration was performed in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which ensures H^+ equilibration across the membrane, according to the methods described by James-Kracke [17]. The calibration curve was found to be linear in the pH interval 6.1–7.4.

Change in pH_i was converted to H^+ flux by means of buffer capacity, β, calculated from the pH displacement obtained by addition of 10 mM butyrate [41].

$$\beta = 1/\Delta pH\ [butyrate] \quad (2)$$

Butyrate equilibrates rapidly across the membrane by free diffusion of the undissociated acid, and this was confirmed by demonstrating that addition of inhibitors to block the monocarboxylate transporter had no effect on the observed change in fluorescence signal.

The H^+ flux equals the lactate flux, since the lactate/proton transport ratio is 1:1 [19]. Once the fluorescence signal from the cells had reached a steady state, usually after 30 s, substrate (lactate or pyruvate) was added through a light-sealed port in the cuvette, and the fluorescence change was monitored. The change in pH_i, which represents carrier-mediated lactate or pyruvate transport, was determined after the total pH_i change was corrected for diffusion of the undissociated lactic or pyruvic acid. This free diffusion could be quantified from the pH_i change in the presence of suitable inhibitors of lactate/proton transport, i.e. 0.5 mM 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), 5 mM mersalyl and 10 mM α-cyano-4-hydroxycinnamate (CHC). Only a small fraction of lactate transport in muscles is mediated by a DIDS-sensitive lactate carrier or via the inorganic anion exchanger [30], e.g. less than 10% inhibition with DIDS was observed in mammalian skeletal muscle [20,36]. Mersalyl is a protein-modifying agent which at 5 mM caused a 70% inhibition of the lactate transport [36]. CHC is a competitive inhibitor causing an inhibition of 63% at a concentration at 10 mM [36]. Together DIDS, mersalyl and CHC are expected to block more than 90% of the

carrier mediated lactate transport at a concentration of 1 mM lactate [18] and it is assumed that the pyruvate transport is similarly inhibited. In the present study we found that 75% of the pyruvate transport was blocked with the same inhibitors as for lactate transport, and this fraction was found to be the maximal inhibition with any of the tested combinations of inhibitors in the present system. There was no interference of the inhibitors on the pH measurements. The pH_i was linear during the first 15 s at any substrate concentration used. Therefore, the initial rates of transport were estimated from the linear pH_i change in the first 10 s time period immediately after lactate or pyruvate had been applied. The K_m and V_{max} values were obtained by fitting the Michaelis-Menten equation to the flux data.

The possibility that the Na^+/H^+ antiport system had influenced the pH measurements where investigated using the amiloride derivative EIPA (5-(*N*-ethyl-*N*-isopropyl)amiloride). Two single experiments (run in 6-duplicate) with or without EIPA demonstrated no difference in the pH measurements associated with lactate incubation (concentrations from 5 to 30 mM lactate).

2.4.3. MCT determination

After washing the cells (13 and 16 days after plating) three times in an ice-cold phosphate buffer, the cells were harvested and dissolved in 200 μl glycyl-glycine buffer. Western blotting was carried out using polyclonal antibodies to MCT1 and MCT4 according to the method described by McCullagh et al. [27].

3. Results

3.1. Differentiation

3.1.1. Morphology and creatine kinase activity

Myogenic differentiation is associated with a number of changes such as alterations in total activity and isoenzyme distribution of several enzyme systems [16,39] and synthesis of contractile proteins and isoforms of myosin [31]. The rat primary myoblast reached confluence at day 2 after plating, the cells were aligned and a minor fraction of small myotubes was observed. When medium was changed to fusion medium, most of the myoblasts fused into multinucleated myotubes. At days 4–5, the first spontaneous contractions were observed, and 1 day after, cross-striations were visible by light microscopy. The nuclei remained in clusters until about day 9 and were then gradually laterally displaced in the myotubes, until day 12 (Fig. 1). The specific activity of creatine kinase, which may be taken as a quantitative marker of myogenic differentiation, increased when myoblasts fused into multinucleated myotubes (0.65 ± 0.10 U/mg protein, 48 h after seeding, just before fusion) and reached a maximum at day 10

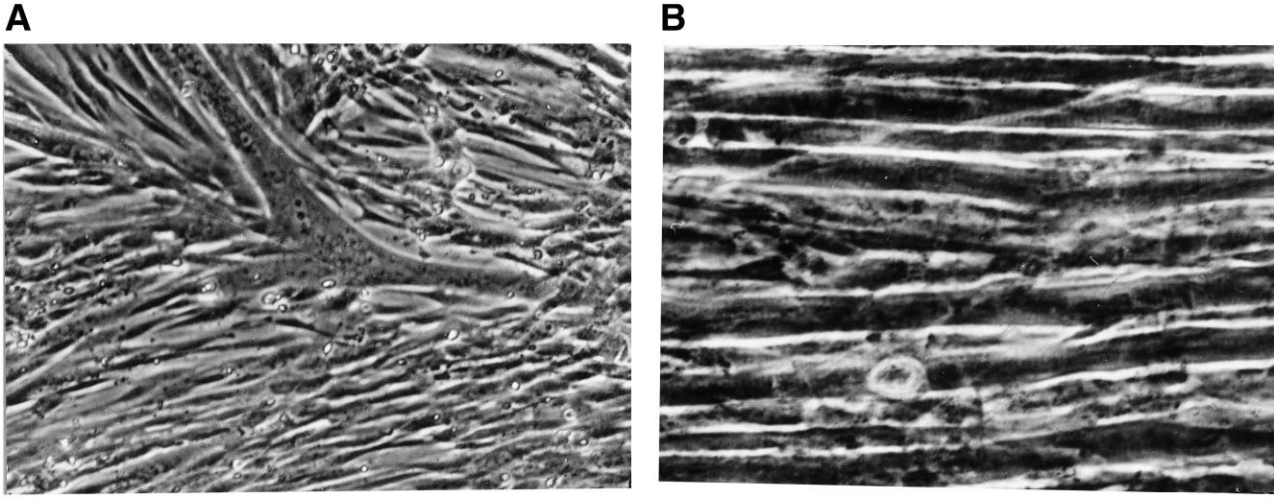

Fig. 1. Cultured rat myoblasts and myotubes photographed in a phase contrast microscope. (A) 2 day old myoblasts and small myotubes (×125). (B) 13 day old contracting myotubes (×400).

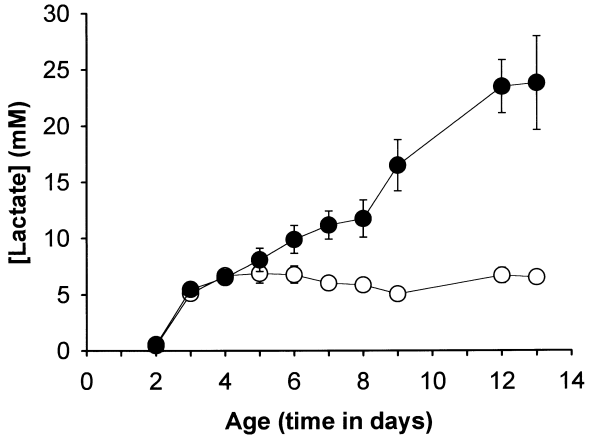

Fig. 2. Medium lactate concentration during differentiation at low (○) and high (●) medium glucose concentration. Values are means ± S.E.M. ($n = 5$), each run in duplicate. Error bars not shown fall within respective symbols.

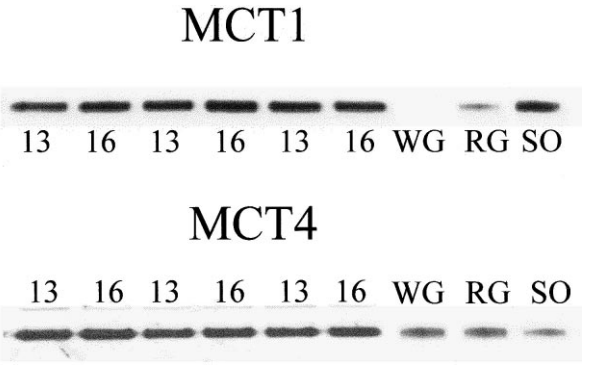

Fig. 3. Western blotting analysis of cultured rat myotubes. The myotubes were analyzed for the monocarboxylate isoforms MCT1 and MCT4 at days 13 and 16. The control groups are made from rat muscle homogenates with the same amount of protein per lane. WG, RG and SO indicate white gastrocnemius, red gastrocnemius and soleus muscles, respectively. The molecular mass of the bands detected is approx. 43 kDa.

after seeding. When seeded in high glucose medium (22.7 mM), the creatine kinase activity progressively decreased from day 10 to 16 ($P < 0.05$) from 3.51 ± 0.10 to 2.29 ± 0.05 U/mg protein (mean ± S.E.M. ($n = 4$)). When seeded in low glucose medium (5.05 mM), the creatine kinase activity remained constant: 3.70 ± 0.23 to 3.64 ± 0.06 U/mg protein. The cultured myotubes were used for experiments at days 12–14.

3.1.2. Medium lactate concentration

The lactate concentrations measured immediately before the daily shift of the culture medium were dependent on the medium glucose concentration and reached 6–7 mM with low glucose, but 20–25 mM when high glucose was present in the culture medium (Fig. 2). For cells seeded with high (22.7 mM) and low (5.05 mM) glucose medium, the initial rate of medium lactate accumulation was constant at about 14.5 nmol (mg protein)$^{-1}$ min^{-1}, after which it decreased. Due to the large formation of lactate with the high glucose concentration, which could have an influence on the lactate transporter expression, medium containing 5.05 mM glucose was used to culture cells for the transport experiments.

3.1.3. Buffer capacity

Since the calculation of the lactate and pyruvate transport depends upon the buffer capacity, β, of the cells, it was necessary to measure this parameter for the different age of the cells used for the transport experiments. Table 1 shows that the buffer capacity increased by approx. 25% from day 12 to 16. Consequently, the transport data were corrected for the buffer capacity change with age.

3.1.4. MCT1 and MCT4 determination

The cultured myotubes were investigated for the presence of the monocarboxylate transporter isoforms, MCT1 and MCT4. The myotubes in culture were found to express both MCT1 and MCT4 in approximately the same amount per mg of total muscle protein as was observed in fully differentiated muscles, i.e. mature rat muscles (Fig. 3).

Table 1
Buffer capacity in myotubes as function of days in culture

12 days	13 days	14 days	16 days
13.3 ± 0.2 mM/pH unit	13.7 ± 1.5 mM/pH unit	16.2 ± 1.0 mM/pH unit	17.9 mM/pH unit

Values are means ± S.E.M. ($n = 6$). The medium glucose concentration was 5.05 mM.

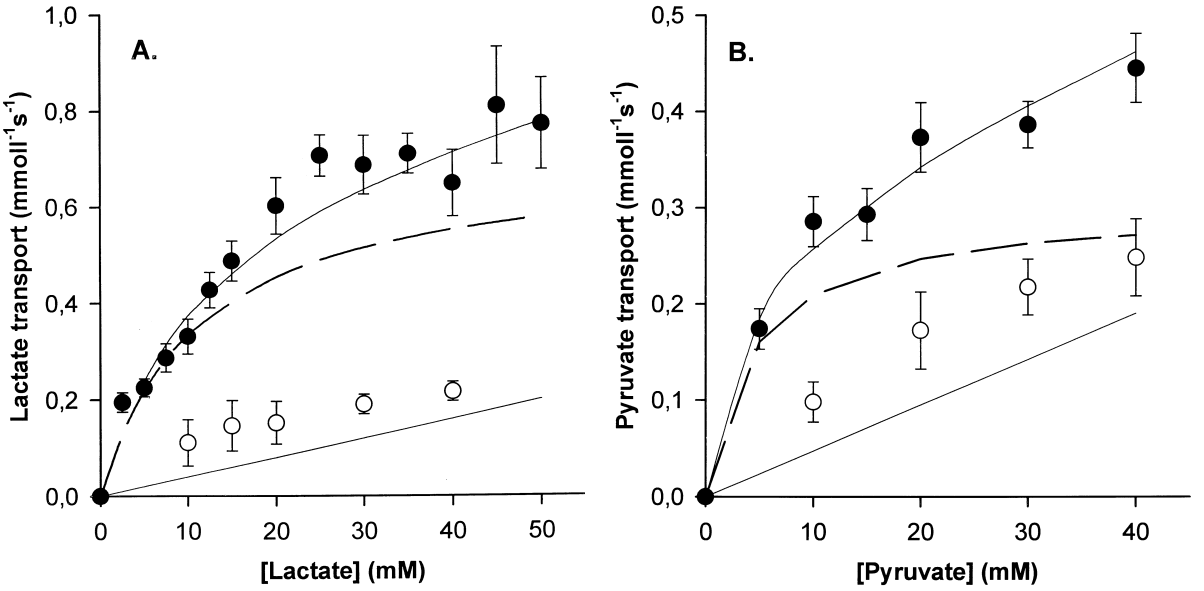

Fig. 4. (A) Lactate uptake into myotubes. Values are means ± S.E.M. ($n = 9$). (B) Pyruvate uptake into myotubes. Values are means ± S.E.M. ($n = 10$). Uptake was measured in the presence (○) and absence (●) of 0.5 mM DIDS, 5 mM mersalyl and 10 mM CHC. The values obtained in the presence of inhibitors were assumed to represent simple diffusion. The simple diffusion, α[S], was determined as the mean of three separate experiments. The straight lines, in both A and B, are the best linear regression fit to the data. The filled circles in both A and B represent the total transport $v_0 = \frac{V_{max}[S]}{K_m + [S]} + \alpha[S]$. The open circles represent the non-inhibitory component in both A and B, whereas the dashed lines represent carrier-mediated transport (total transport subtracted simple diffusion of undissociated acid). The curves depicted are the best Michaelis-Menten fit to the data.

3.1.5. Lactate and pyruvate transport

In order to quantify simple diffusion of the lactate and pyruvate transport, experiments were carried out in the presence of three lactate transport inhibitors, DIDS, mersalyl and CHC. However, as it was impossible to inhibit the transport entirely, the measured non-inhibitory component was split into two components. The diffusion component of the total transport (determined as described in Section 2) must therefore be approximated by a straight line, with a slope of 0.0040 ± 0.0005 ($n = 4$) for lactate and 0.0048 ± 0.0003 ($n = 4$) for pyruvate (Fig. 4A, B), whereas the remaining part could be non-inhibited transport. The diffusion components were subtracted from the total transport, in order to quantify the carrier-mediated transport. The carrier-mediated transport appeared to follow Michaelis-Menten kinetics with $K_{m(lactate)} = 9.9 \pm 1.1$ mM, $V_{max(lactate)} = 0.69 \pm 0.02$ mmol l^{-1} s^{-1} (Fig. 4A) and $K_{m(pyruvate)} = 4.4 \pm 1.3$ mM, $V_{max(pyruvate)} = 0.30 \pm 0.05$ mmol l^{-1} s^{-1} (Fig. 4B).

4. Discussion

4.1. Lactate and pyruvate transport

In the past it was assumed that lactic acid moves across skeletal muscle sarcolemma solely by simple diffusion [14]. However, the finding that mercury compounds inhibited the uptake of lactate suggested the presence of a facilitated diffusion mechanism for lactate [8]. Since then the kinetics of lactate transport have been studied in many model systems. In the present study primary cultures of myotubes were used as a model system and were found to have a K_m value of 9.9 mM which is within the range of values obtained in other studies on lactate transport in skeletal muscles. Previous reports from tracer studies have shown a range for K_m from 13 to 40 mM in intact skeletal muscle and vesicles isolated from rat skeletal [18,28,29,36], and 12.5 mM for the myotubes of the L6 cell line [7]. Furthermore, the K_m for lactate transport in giant vesicles prepared from

human skeletal muscle is within the same range as the values obtained in sarcolemmal giant vesicles from rats [22]. In contrast, the K_m value for pyruvate in rat primary skeletal muscle was in the present study found to be 4.4 mM, which was markedly lower than values previously reported for other skeletal muscle model systems (>50 mM [20]).

The maximum velocity (V_{max}) of the transport process is determined by the number of carrier molecules in the membrane, as well as the turnover number of the individual transporters. The present study demonstrates that rat muscle cells in culture display a maximal velocity for lactate and pyruvate transport of 0.69 mmol l^{-1} s^{-1} and 0.30 mmol l^{-1} s^{-1}, respectively. In addition, the diffusion component at 20 mM of lactate was approx. 20% of the total transport, a value slightly lower than that of human skeletal muscle (30% [22]) and similar to that of rat muscle sarcolemmal vesicles (20% [18]) at the same concentration. The V_{max} value for lactate is of the same order of magnitude as in human skeletal muscle in vivo where a maximal release of lactate after exhaustive exercise was found in the range 0.03–0.15 mmol l^{-1} s^{-1} with a gradient of 20–40 mM lactate [2]. As the K_m value lies within the range of 20–40 mM, V_{max} can, however, be estimated to be twofold higher than the observed lactate release. The V_{max} values reported for other skeletal muscle model systems are difficult to compare due to the different units used. Furthermore, the present analysis of MCT indicates that there are a large amount of transport molecules in rat myotubes which result in a high V_{max} value.

In the present study, the affinity of lactate transport was approx. 40% of that for pyruvate, which was markedly lower than the values previously reported by Roth and Brooks [36] and McDermott and Bonen [29] based on substrate inhibitor experiments for rat skeletal muscle sarcolemmal vesicles.

The present method used for affinity studies of lactate and pyruvate transport in single heart cells are similar to those determined with the radiotracer technique [33,40]. However, the V_{max} values in single heart cells measured with the BCECF technique at 23°C are similar to those measured with tracers at 11°C and 0°C for lactate and pyruvate, respectively. One reason for the discrepancy in V_{max} values could be that the tracer technique measures both net proton-linked uptake of lactate and pyruvate and their exchange with internal lactate and pyruvate, whereas the BCECF technique only measures the net proton-linked uptake. Another reason could be that with the tracer technique, the substrate will be metabolized before measurements of the transport rate have been made. Therefore, if kinetic properties of transport systems are to be investigated, it is essential that initial rates of uptake are measured before significant metabolism has occurred. The data presented in this paper demonstrate that the use of BCECF to measure pH_i allows estimates of accurate kinetic data for proton-linked transport of monocarboxylates into the muscle cell. The K_m values obtained in the present study, demonstrate that the carriers in primary rat muscles are similar to those found in other skeletal muscle cells. Indeed, since fluorescence can be measured continuously with very high time resolution, the technique provides accurate measurements of transport rates. Furthermore, the technique can be used for the study of proton-linked transport of any monocarboxylate, since it is dependent on the pH changes, and not on the existence of the substrate in a radioactivity labeled form.

In the range of physiological pH the buffer capacity is constant, but at a pH higher than 7.3 or lower than 6.1 the correlation is no longer linear [26]. In our experiments a buffer capacity of 13–17 mM/pH unit was found, which is of similar magnitude as that reported by Carpenter and Halestrap [4] for Ehrlich Lettre cells (13.7 mM/pH unit) and slightly lower than that reported by Wang et al. [41] for rat and guinea pig cardiac myocytes (23.0 and 24.3 mM/pH unit, respectively).

4.2. The cell model

The level of differentiation of the muscle cells used for transport studies is clearly of importance [7]. In this study the specific activity of creatine kinase was used as a quantitative marker of myogenic differentiation. The level of creatine kinase activity obtained in the present study is higher than previously reported activities [39] measured in muscle cells cultured on a collagen matrix, but similar to activities measured in cells cultured on Matrigel coated dishes [9]. The discrepancy may be explained by the finding that Matrigel enhances myogenic differentiation

[9,13]. Furthermore, the cell medium may affect the creatine kinase activity since it has been observed that the cells cultured in high glucose medium (22.7 mM) have a lower creatine kinase activity than cells cultured in low glucose medium (5.05 mM).

Another way to characterize the primary culture cells as a model system for the study of lactate transport is to compare the amount of lactate transporter isoforms with that obtained in muscle homogenates. Eight isoforms of the monocarboxylate transporters have been cloned [10,11,35], of which two, MCT1 and MCT4, are present in mammalian skeletal muscles.

Rat myotubes express mainly embryonic and neonatal fiber types [31] and have capacity for oxidative ATP production, but when glucose is abundant anaerobic glycolysis is preferred [15,38]. The MCT1 isoform exists mainly in oxidative fibers (SO and FOG) [27], whereas the MCT4 isoform seems to be present in all fiber types in mature skeletal muscle fibers [35]. Both MCT1 and MCT4 were expressed in the rat myotubes and based on the amount expressed per mg of total protein it was estimated that the transporter densities were higher than in mixed rat muscles. It could be speculated that those two isoforms, MCT1 and MCT4, in skeletal muscles have different kinetics. However, Juel and Pilegaard [23] found the same K_m for red and white muscles in studies with sarcolemma giant vesicles. Furthermore, in the present study DIDS incubation of myotubes from rat skeletal muscles revealed no sign of biphasic inhibition.

In conclusion, based on the finding of transport kinetic parameters similar to values in intact muscles and the finding of an expression of both MCT1 and MCT4 in rat primary skeletal muscle cells in culture, we propose that the model system is suitable for the study of muscle monocarboxylate transport.

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