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Relations Between the Stage of Cell Maturation and Lactate Transporter Activities in Rat Neonatal Muscle Cells in Culture

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Abstract. Lactate transport was investigated in newborn rat muscle cells in culture. The aim was to study the lactate transport function at two stages of cell differentiation in culture: (i) during the proliferative phase characterized by myoblasts and myotubes (MyB/MyT2) obtained after 2–3 seedings, (ii) when myotubes (MyT1) grow old in culture after 8–9 seedings. In both developmental stages MyB/MyT2, lactate was carried following a saturable and sigmoidal velocity curve: the Hill and the Scatchard plot analyses confirmed an allosteric or multisite mechanism of lactate transport with two classes of carriers: one of low and one of high affinity i.e., 8.6 and 0.95 mM, respectively, which are associated with high and low transport capacities (V_m) i.e., 9.1 and 0.67 nM/min/mg, respectively. With MyT1, the velocity curve of lactate transport presented a hyperbolic profile, and the Hill plot analysis gave a Hill number near one suggesting that for cell aging in culture the decrease in cooperativity shows that lactate transport essentially occurs through the low affinity transport system. Inhibitor effects also contributed to evidence for at least two systems of transport. Results obtained from primary cells give evidence for the early activity of lactate transport system at the Myb/MyT2 stage and its evolution during cell aging in culture (MyT1). Sarcolemmal lactate transport in primary cultures of myocytes is accomplished by multiple carriers, neither of which are MCT1 or MCT2 as confirmed by immunoblots.

Key words: Monocarboxylate carrier — Kinetic mechanism — Lactate transport — Muscle cells

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Introduction

The transport of lactate and monocarboxylic acids such as pyruvate is of major physiological importance in all mammalian cells [4, 22]. Cells and tissues that use glycolysis to produce ATP, such as erythrocytes and white muscles, or cells that are experiencing epinephrine stimulation export lactate. Once borne by blood, lactate is transported to gluconeogenic tissues (liver and kidney) for conversion into glucose or to heart and red muscles for consumption as a respiratory fuel [4, 5, 24, 26].

Whether lactate is imported or exported, membranes form barriers to its transport. Previously, diffusion of the nondissociated acid was thought to be the only mechanism of lactate transmembrane flux. Now it is well recognized that in most mammalian cells the transport of lactate across the plasma membrane occurs via facilitated diffusion involving a family of specific proton-linked lactate transport proteins [6, 8, 11, 13, 17, 24, 27, 29]. These transporters are stereospecific, vary in sensitivity to inhibitors (e.g., α-cyanohydroxycinnamates, disulfonates and organomercurial thiol reagents) [12, 14, 29] according to the tissue [14, 15, 20, 28] . In previous experiments, lactate transport was realized in L6 muscle cells. We demonstrated that lactate was transported by simple diffusion in myoblasts [1] and by a specific proteinic carrier in myotubes [7]. Kinetic analysis indicated that lactate was transported by a multicarrier system with onset related to myogenic differentiation [2, 3, 18].

Subsequently, kinetic analysis of lactate transport in sarcolemmal membranes confirmed the presence of a multicarrier system [3]. Moreover, the low sensitivity of lactate transport to DIDS provided evidence that muscle cells express distinct forms of the erythrocyte monocar-

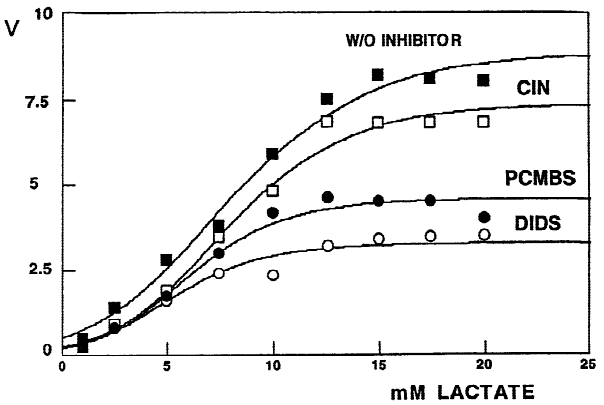


Fig. 1. Velocity curve of lactate transport in MyB/MyT2 with and without inhibitors. Values are means ± SE of measurements performed in triplicate from four separate experiments.

boxylate carrier. In view of previous results suggesting developmental influences on expression of sarcolemmal lactate transporters in myocytes, the aim of the present investigation was to confirm the original results concerning the lactate transport mechanism obtained with immortalized L6 muscle cells and to precisely analyze the kinetic parameters of each transport system and the effect of inhibitors. Kinetic analysis of the lactate transport system was performed in neonatal cultured satellite muscle cells. These experiments were undertaken with hindlimb muscle satellite cells from newborn rats. These cells that differentiate in culture provide a tool for studying lactate transport in a mature cell system.

Even if MCT1 is represented in muscle [9, 16, 30] and in primary muscle cells [25], these data confirm our previous results with L6 muscle cells, showing that in cultured skeletal cells, lactate is transported by a multicarrier system whose forms present specific sensitivities to inhibitors that are different from MCT1 and MCT2 [14, 15] as demonstrated by immunodetection in this work. The cell model used kinetically makes it possible to characterize each family of transporters.

Materials and Methods

Cell Culture

Satellite cells were obtained from leg muscles of newborn (5 days) white Wistar rats as previously described [7]. After 3–4 days of culture the proliferative phase is over and myoblasts begin to fuse and differentiate into myotubes. After 10 days of culture, myotubes can contract.

These cultures have a definite lifespan and cannot be seeded more than 10 times. From the first to the third subculture, cells actively proliferate and differentiate, from then until the 10th seeding, proliferation and differentiation slow down and eventually stop.

The transport experiments have been performed on myoblasts and myotubes. MyT2 myotubes were obtained after 7 days of culture and 3 seedings while MyTI myotubes were obtained after 16 days of culture and 8 seedings.

Transport Studies

Subconfluent monolayer cultures were harvested by trypsin treatment (trypsin 0.25%, 10 min, 37°C) and seeded in 2 cm^2 tissue culture multiwell plates until myoblasts, MyT2 and MyT1 myotubes stages were reached.

Transport experiments were performed as previously described [3]. Specific inhibitors of lactate transport such as α-cyano-4-hydroxycinnamicacid (CIN), parachloromercuribenzenesulfonic acid (pCMBS), 4,4′-diisothiocyanostilbenedisulfonic acid (DIDS) were used at 2.5 mM, 3 mM and 10 μM, respectively (maximal inhibiting concentrations previously determined [1, 7]) in PBS Dulbecco containing 15 mM HEPES pH 6.8. All chemicals were purchased from Sigma. Uptake was stopped after 10 sec of incubation as determined by time course experiments (*not shown*). It has been previously demonstrated that L-lactate is not metabolized during the first 10 sec [26] and in our laboratory by thin layer chromatography on cellulose in *n*-butanol:acetic acid:water (12:3:5).

Sarcolemmal Vesicles and Immunoblotting

Sarcolemmal vesicles from newborn rat cultured muscle cells were isolated according to [19]. The pellet was resuspended in 150 mM NaCl, 1% NP40, 1 mM $CaCl_2$, 0.1% SDS, 2 mM pMSF, 0.5 mg/ml leupeptin, 50 mM Tris-HCl, pH 7.4 for 15 min at 4°C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose (Amersham). The nitrocellulose was then blocked in 5% nonfat dry milk in TBST (TBS Tween 20 at 0.1%). Primary antibodies were used at a dilution of 1/1,000 for both antibodies against rat MCT1 and MCT2 (generous gift from Dr. A. Halestrap). Immunoreactivity of the antibodies with polypeptides in sarcolemmal vesicles was detected with horseradish-peroxidase goat anti-rabbit antibody conjugates (Biosys, France), followed by ECL (Amersham, UK) detection. Controls were performed on both rat erythrocytes and liver membrane extracts for antibodies against rat MCT1 and MCT2, respectively.

Abbreviations

The abbreviations used are: MyB: Myoblasts; MyT2: Myotubes (3rd seedings); MyT1: Myotubes (8th seedings); CIN: α-cyano-4-hydroxycinnamic acid; pCMBS: parachloromercuri-benzylsulfonic acid; DIDS: 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; MCT: Monocarboxylate transporter.

Results

Lactate Transport in Myoblasts and MyT2 Myotubes

The rate of lactate uptake varied sigmoidally with increasing extracellular lactate concentrations (Fig. 1). Initial transport velocities were unchanged at saturation between 15 and 25 mM suggesting the absence of significant lactate-free diffusion. The apparent V_m obtained by $V = f$(lactate) plot was 8.5 nmoles/mg prot/min.

The sigmoidal shape of the curve and saturation can indicate a protein transport system showing an allosteric or multisite carrier mechanism. The sigmoidal shape

Table 1. Kinetic parameters of lactate transport in MyB/MyT2

	Inhibitor	K_m*	V_m*
SITE 1	w/o	0.95 ± 0.08	0.67 ± 0.05
	CIN	1.50 ± 0.12	0.62 ± 0.05
	DIDS	1.34 ± 0.15	0.51 ± 0.04
	pCMBS	1.48 ± 0.11	0.70 ± 0.07
SITE 2	w/o	8.60 ± 0.7	9.10 ± 0.8
	CIN	11.30 ± 0.9	8.85 ± 0.7
	DIDS	8.50 ± 0.7	3.85 ± 0.3
	pCMBS	10.20 ± 0.6	5.53 ± 0.4

$K_{m(app)}$ (mM) represents the apparent dissociation constant of lactate transport. V_m (nmol/min/mg Prot) is the maximal velocity of lactate transport.

* Statistical values are the means ± SE of measurements performed in triplicate from four separate experiments.

was confirmed by the Hill plot (*not shown*) with a mean value of nH = 2 (Hill number), showing a nonlinear regression indicating a negative cooperativity of lactate uptake.

The Scatchard plot *V/L vs. V* was represented to determine the kinetic parameters of lactate transport in MyB/MyT2. A parabolic curve was obtained for the representation of these data.[1]

The mathematical regression analysis of the biphasic lactate uptake curves gave the asymptotes of each component and their kinetic parameters were reported in Table 1. This result indicated at least two systems implicated in lactate transport: one with high affinity (K_m) and low transport capacity (V_m) and another with low affinity and high transport capacity, subsequently referred to as sites 1 and 2.

Lactate transport in myoblasts and MyT2 myotubes was investigated using specific inhibitors: CIN (2.5 mM), pCMBS (3 mM) and DIDS (10 μM). These inhibitors did not modify the sigmoidal shape of the velocity curves and the inhibition was effective both at low and high extracellular lactate concentrations (Fig. 1).

In MyB/MyT2 18, 51, and 59% of lactate transport velocities were respectively inhibited at high lactate concentration by CIN, pCMBS and DIDS (Fig. 1).

The Hill plots (*not shown*) obtained with inhibitors were nonlinear and similar to those obtained without inhibitors, giving a Hill number of 2 and showing that the negative cooperativity of lactate transport was not modified by inhibitors. Scatchard plots of lactate transport showed two families of lactate carriers with high and low affinities for extracellular lactate (Fig. 2*a–c*).

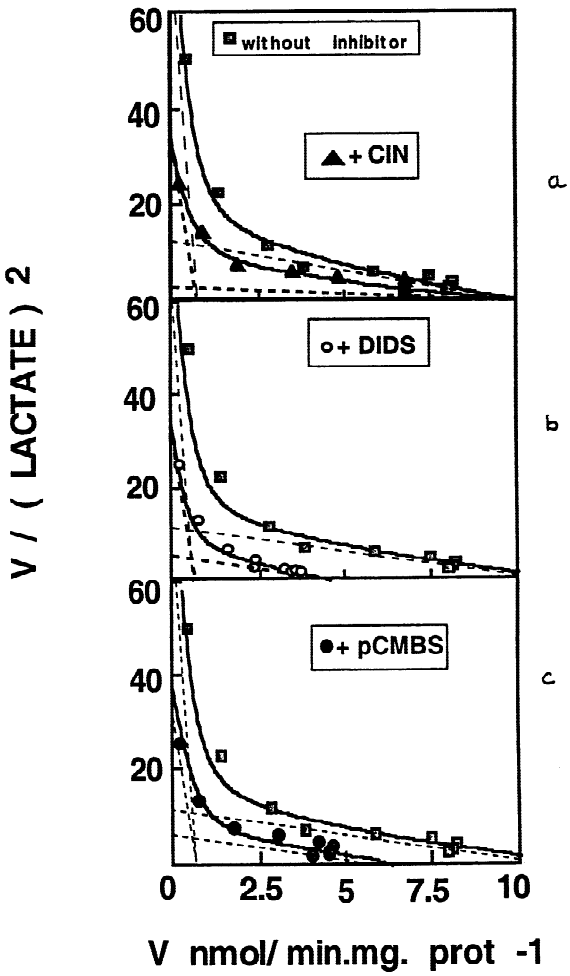


Fig. 2*a–c*. Scatchard Plot *V*/(Lactate)^2. 10^{-2} *vs. V* in MyB/MyT2 obtained from the data of Fig. 1 without and with inhibitor.

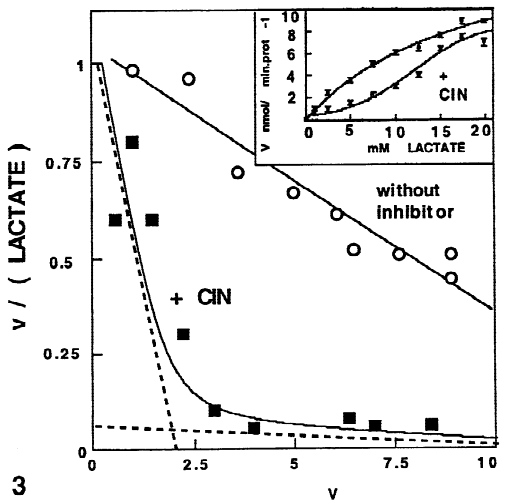
It was observed (Table 1) that the three inhibitors applied did not have the same effect on the two families of transport sites. The apparent affinity of lactate for the first site was decreased by the three inhibitors without modification of the apparent maximal capacity of transport $V_{m(app)}$ as for a competitive inhibition, whereas for the second site, the inhibitors showed a weak effect on the apparent lactate affinity but DIDS and pCMBS namely modified the apparent V_m of lactate transport as for noncompetitive inhibitors.

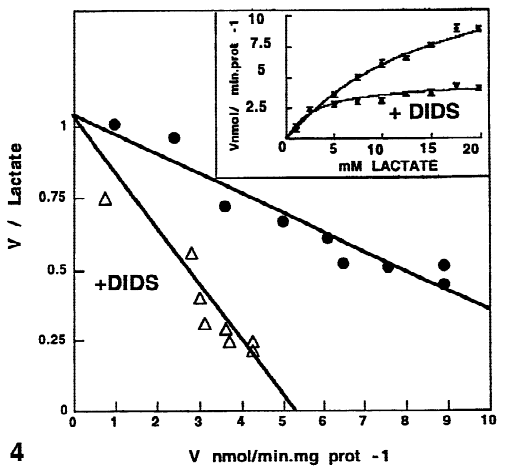
The kinetic parameters are reported in Table 1.

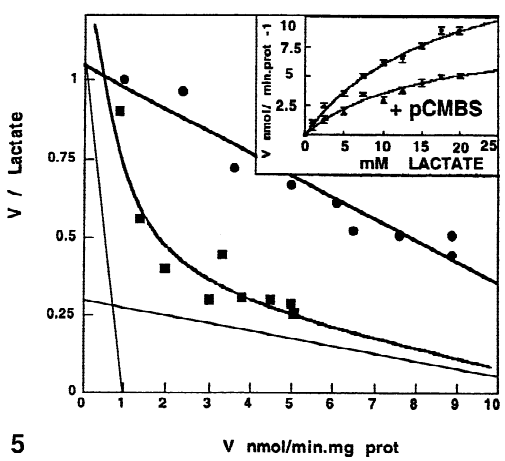
Lactate Transport in MyT1 Myotubes

The hyperbolic shape of the velocity curve in MyT1 myotubes (Fig. 3 inset) indicated that saturable lactate transport occurred macroscopically by mostly one transport system. This was confirmed by the linear regression of the Hill plot (*not shown*) with a nH nearly 1 and the

[1] The Scatchard plot of *V vs.* [Lactate]^2 *vs. V*, should give a linear curve for a cooperative or allosteric system and a Hill number of 2, however, in each case, a biphasic curve was observed to fit these data (Fig. 2). This representation confirmed the negative cooperativity observed on the Hill plot [22].







Figs. 3, 4, 5. Scatchard Plot *V*/(Lactate) *vs. V* in MTy1 obtained from the data obtained from the respective insets without and with inhibitor. In the insets the velocity curves of lactate transport in MyT1 were represented with and without inhibitors. Values are means ± SE of measurements performed in triplicate from four separate experiments.

Scatchard plot $V/L = f(V)$ (Fig. 3) showing a linear regression for one lactate transport system with low affinity for external lactate i.e., $K_{m(app)}$ = 1.4 mM and $V_{m(app)}$ = 17.5 nmol/min/mg protein.

To characterize this mechanism further, CIN (2.5 mM), pCMBS (3 mM) and DIDS (10 μM) were tested on lactate uptake.

On Fig. 3 inset with CIN, the hyperbolic shape of the velocity curve is transformed into a sigmoidal one as observed in MyB and MyT2 (with or without inhibitor). The sigmoidal shape of the velocity curve seemed to show that lactate transport is mainly inhibited in a low range of lactate concentrations i.e., 0–15 mM. This was confirmed by the Hill plot which presented a nonlinear regression and a medium value of nH > 1 (*not shown*). The Scatchard Plot (Fig. 3) confirmed these results and presented a biphasic curve whose asymptotes (obtained by mathematical regression) could be related to two transport systems. These parameters reported in Table 2 showed that a high affinity and low transport capacity family of sites are implied in a low range of lactate concentrations and another one with a very low affinity ($K_{m(app)}$ = 230 mM) and high lactate transport capacity.

DIDS inhibited lactate transport at high external lactate concentrations (Fig. 4 inset). Thus, it appeared that DIDS had mostly an inhibitor effect on lactate transport sites with low affinity for lactate. In the presence of DIDS, lactate transport presented an apparent hyperbolic velocity curve. This was confirmed by the Scatchard plot (Fig. 4) which presented a linear regression related to one type of transport site. The kinetic parameters of lactate transport in the presence of DIDS were reported in Table 2. The remaining transport was attributed to the first family of sites, since the affinity ($K_{m(app)}$ 5.2 mM) was in a three times higher range of values and *Vm* ($V_{m(app)}$ 5.3 nmol/min/mg prot) was lower, compared to the range of values expressed for the second family of sites in absence of inhibitors.

In the presence of pCMBS, the lactate velocity curve was hyperbolic for all the external lactate concentrations applied (Fig. 5 inset). The biphasic Scatchard plot analysis gave evidence for a multiple lactate transport system (Fig. 5). Both asymptotes of these biphasic curves indicated the apparent kinetic parameters of two transport sites and are reported in Table 2.

Immunodetection of MCT1 and MCT2 in Neonatal Sarcolemmal Vesicles

The above results suggest that there are at least two families of lactate carriers. To test if these carriers present any analogy with MCT1 and MCT2, immunodetection was carried out using specific antibodies against these transporters. The presence of MCT1 and MCT2 corresponding polypeptides under reducing and nonre-

ducing conditions, was not detectable in blotting experiments performed with lysates of sarcolemmal vesicles obtained from neonatal cultured muscle, while the antibodies recognized polypeptides of 40 kDa in rat erythrocytes and liver membrane extracts (*data not shown*).

Discussion

Kinetic analysis of lactate transport in myoblasts and myotubes obtained in primary culture from newborn rat hindlimb muscles have shown that the mechanism of transport is carried out by several pathways.

Lactate Transport in Myoblasts and MyT2 Myotubes

It has been observed that the lactate transport presents similar kinetic characteristics in myoblasts and MyT2 myotubes i.e., time course, sigmoidal shape of the velocity curve, kinetic parameters. Thus, in contrast to the case of L6 myoblasts [3], it appears that primary myoblasts have the advantage of presenting lactate-specific carriers on their plasma membranes. Myogenic differentiation from myoblasts to myotubes does not occur with modifications of kinetic characteristics of transport, already obtained at the myoblastic stage.

The extensive analysis of lactate transport in myoblasts and myotubes have shown a multicarrier system, as previously described for L6 cells [3]. The Hill plot (*not shown*) obtained from the velocity curve gives evidence for at least two independent lactate transport systems. This is confirmed by the Scatchard plot: V/L^2 *vs.* V which presents a concave curve. Two major components seem to be involved in lactate transport: two transport sites, one with high affinity and low capacity and another one with low affinity and high capacity. The kinetic parameters are reported in Table 1.

Such a diversity of parameters related to cloned MCT, has already been observed in the case of MCT1 and MCT2 [10, 14]; although MCT1 and MCT2 proteins were not detected on these newborn cultured rat muscle cells using anti-MCT1 and MCT2 antibodies, this does not mean that MCT1 and MCT2 do not exist but are only slightly expressed in this system.

Effect of Inhibitors on Lactate Transport in Myoblasts and MyT2 Myotubes

The three inhibitors applied do not modify the sigmoidal shape of the velocity curve. That means these inhibitors have macroscopically the same effect for the different lactate concentrations applied. Nevertheless, the analysis of the kinetic parameters (Table 1) shows a variation in the sensitivity of each family of transporters.

Table 2. Kinetic parameters of lactate transport in MyT1

	Inhibitor	K_m*	V_m*
SITE 1	w/o	0	0
	CIN	0.98 ± 0.09	1.76 ± 0.2
	DIDS	5.2 ± 0.4	5.3 ± 0.4
	pCMBS	0.97 ± 0.08	0.91 ± 0.07
SITE 2	w/o	14 ± 1.2	17.5 ± 1.5
	CIN	230 ± 27	13 ± 1.1
	DIDS	0	0
	pCMBS	37.1 ± 4	11.7 ± 1

$K_{m(app)}$ (mM) represents the apparent dissociation constant of lactate transport. V_m (nmol/min/mg Prot) is the maximal velocity of lactate transport.

* Statistical values are the means ± SE of measurements performed in triplicate from four separate experiments.

CIN works as a competitive inhibitor for both families of transporters. The modification of the affinity constant is more important for site 1 (high affinity) than for site II (low affinity) (inhibition of 50 and 30%, respectively).

PCMBS is competitive when applied to site 1 and noncompetitive for site 2. Thus, our results are inconsistent with those previously established for MCT1 or MCT2. Monocarboxylate transport by MCT1 is completely inhibited for 0.3 mM PCMBS and no inhibition is observed in the case of MCT2. These results are in agreement with the conclusion of Jackson [9] considering that the monocarboxylate isoforms of lactate transport in muscle could be different from MCT1 and MCT2.

DIDS modifies the affinity of lactate for the site 1 and V_m is weakly and significantly lowered (mixed type inhibition). On the contrary, site 2 affinity constant is not sensitive to DIDS, but V_m is lowered (noncompetitive type inhibition) showing that the interaction between DIDS and the two kinds of transport sites are different.

The catalytic efficiency (V_m/K_m) was determined in order to analyze activity of these two sites (Fig. 6). Firstly, we observe that the catalytic (or transport) efficiency is higher on the site 2 than on the site 1. Secondly, we observe that the inhibition rates for the site 1 with CIN and DIDS are the same (41.3 and 43% *vs.* control, respectively); with PCMBS the inhibition rate is 32.9%. The sensitivity of CIN on the site 2 is lower than on the site 1 (25% *vs.* control), with DIDS and PCMBS the inhibition rate is higher on the site 2 than on the site 1 (52.7 and 48.7% *vs.* control, respectively).

Lactate Transport in MyT1

The analysis of lactate transport in myotubes obtained after 8–10 subcultures has been performed to study the stability of the kinetic characteristics of lactate transport. At the MyT1 stage of slow proliferation of the cells, the velocity curve of lactate transport has lost its sigmoidal

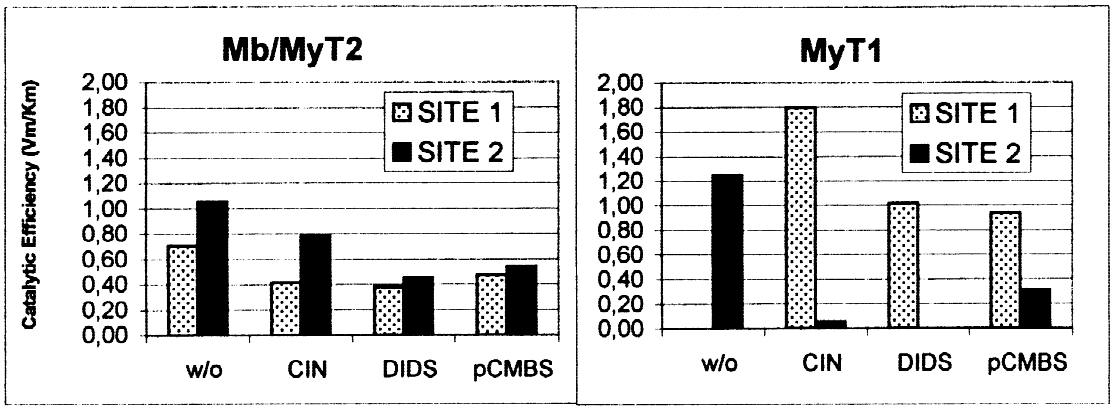


Fig. 6. Catalytic efficiency (V_m/K_m) is represented for MyB/MT2 and MyT1, with or without inhibitors. 100% of efficiency are obtained without inhibitors. Kinetic parameters (V_m and K_m) are reported in Tables 1 and 2.

shape to become hyperbolic. The Hill plot and the Scatchard plot *V/L vs. V* confirm this finding and show only one transport system with low affinity and high capacity of lactate transport.

But when CIN is applied, the sigmoidal shape of the velocity curve is restored and the inhibition of lactate transport is high for 0–12 mM lactate. The nonlinear shape of the Hill plot and the biphasic Scatchard plot V/L^2 *vs. V* obtained with CIN give evidence for two mechanisms of transport.

The results reported in Table 2 give evidence for a lactate transport system with high affinity whose parameters are similar to those observed in the case of MyB/MyT2 and another with low affinity ($K_{m(app)}$ = 14 mM, $V_{m(app)}$ = 17.5 mM). If compared to MyB/MyT2 system, CIN inhibits essentially the transporter with low affinity in MyT1, showing that the hyperbolic shape of the velocity curve hides the existence of two families of carriers. ($K_{m(app)}$ = 8.6 mM increases to $K_{m(app)}$ = 14 mM).

The action of pCMBS on myotubes MyT1 confirms the existence of two families of transport sites and its major effect occurs on transport site 2; moreover these results give evidence for the existence of a second family of carriers which appears only when this inhibitor is applied.

If we consider the ratio V_m/K_m (which characterizes the catalytic efficiency of the lactate carrier system) we observe (Fig. 6) that the site I presents similar sensitivities compared to those of myoblasts and MyT2 and a higher catalytic efficiency with inhibitors. But the second site appears to be very sensitive to CIN (ratio of 0.06) and to pCMBS. DIDS effect shows that only one family of the transport site is still functional and could be attributable to the first or to the second carrier site.

This kinetic analysis demonstrates that even if MyT1 presents a hyperbolic velocity curve, lactate is transported by a multicarrier system as confirmed by the inhibitor's effects. Moreover, these results validate the velocity curves obtained using myotubes from [3] and demonstrate that the sigmoidal shape of the velocity curves is not an experimental artefact.

This study gives evidence for several lactate carriers of which two families seem to have common kinetic parameters. The kinetics of lactate transport in cultured muscle cells that we observed are not comparable to those reported by Garcia [14, 15] for MCT1 or MCT2, McCullagh [16] and Von Grumbbowck [25]. So there appears to be a restricted functional analogy between the muscular lactate carriers and MCT1–MCT2. The sensitivity to pCMBS observed in cultured muscle cells does not correspond to those obtained for MCT1 and MCT2.

We make the hypothesis that MCT1 is undetectable in L6 muscle cell line as well as in satellite cells when they are subcultured.

Moreover, this study gives evidence for a multisite transport system in MyT2 that can shift into a one apparent transport site system in MyT1.

In conclusion, this model of cultured cells from neonatal rat muscle validates and completes our previous work using cells from the L6 line [3]. Each family is probably implied in a specific physiological role, concerning cell bioenergetics and regulation.

These results demonstrate firstly that kinetic transport analysis using specific inhibitors remain a good tool to characterize the efficiency and/or the recruitment of carrier isoforms. Secondly, this study using neonatal muscle subcultures at different stages of maturation completes our previous work using cells from the L6 line [3], and shows that we dispose of interesting tools at different stages of differentiation. These models are used to analyze the biogenesis of transporters and the changes of their functions. Myoblasts from the L6 line and from subcultures of satellite cells do not present the same level of function. Myotubes obtained after several seedings seem to lose the multisite carrier system. The regulation

of transporter synthesis will be studied using these models, nucleic probes and specific antibodies from the already characterized isoforms.

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