The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells

Kai-Stefan DIMMER*, Björn FRIEDRICH*, Florian LANG*, Joachim W. DEITMER† and Stefan BRÖER*‡[1]

*Physiologisches Institut der Universität, Gmelinstr. 5, D-72076 Tübingen, Germany, †Abteilung für Allgemeine Zoologie, FB Biologie, Universität Kaiserslautern, Postfach 3049, D-67653 Kaiserslautern, Germany, and ‡Division of Biochemistry and Molecular Biology, Faculty of Science, Australian National University, Canberra ACT0200, Australia

Transport of lactate and other monocarboxylates in mammalian cells is mediated by a family of transporters, designated monocarboxylate transporters (MCTs). The MCT4 member of this family has recently been identified as the major isoform of white muscle cells, mediating lactate efflux out of glycolytically active myocytes [Wilson, Jackson, Heddle, Price, Pilegaard, Juel, Bonen, Montgomery, Hutter and Halestrap (1998) J. Biol. Chem. **273**, 15920–15926]. To analyse the functional properties of this transporter, rat MCT4 was expressed in *Xenopus laevis* oocytes and transport activity was monitored by flux measurements with radioactive tracers and by changes of the cytosolic pH using pH-sensitive microelectrodes. Similar to other members of this family, monocarboxylate transport via MCT4 is accompanied by the transport of H^+ across the plasma membrane. Uptake of lactate strongly increased with decreasing extracellular pH, which resulted from a concomitant drop in the K_m value. MCT4 could be distinguished from the other isoforms mainly in two respects. First, MCT4 is a low-affinity MCT: for L-lactate K_m values of 17 ± 3 mM (pH-electrode) and 34 ± 5 mM (flux measurements with L-[U-^{14}C]lactate) were determined. Secondly, lactate is the preferred substrate of MCT4. K_m values of other monocarboxylates were either similar to the K_m value for lactate (pyruvate, 2-oxoisohexanoate, 2-oxoisopentanoate, acetoacetate) or displayed much lower affinity for the transporter (β-hydroxybutyrate and short-chain fatty acids). Under physiological conditions, rat MCT will therefore preferentially transport lactate. Monocarboxylate transport via MCT4 could be competitively inhibited by α-cyano-4-hydroxycinnamate, phloretin and partly by 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid. Similar to MCT1, monocarboxylate transport via MCT4 was sensitive to inhibition by the thiol reagent *p*-chloromercuribenzoesulphonic acid.

Key words: astrocytes, proton transport, pH regulation, white muscle, *Xenopus laevis* oocytes.

INTRODUCTION

Transport of lactate, pyruvate and the ketone bodies, acetoacetate and β-hydroxybutyrate, is of major physiological importance in almost all cells [1]. Transport of this class of substrates is mediated by a family of monocarboxylate transporters (MCTs), which can be found from bacteria to man [2–5]. Monocarboxylate transport into mammalian cells has been intensively studied in a number of cell preparations, including erythrocytes [6,7], hepatocytes [8], tumour cells [9], cardiac myocytes [10,11], skeletal muscle cells [12,13], kidney tubule cells [14] and brain astrocytes [15]. Comparison of the data from these investigations indicate that cells express different MCT isoforms adapted to their physiological role. Most cell types express the erythrocyte-type MCT1, which has K_m values for pyruvate, lactate and ketone bodies ranging from 1–10 mM [15,16]. This transporter is adapted to supply cells with ketone bodies and lactate for energy production, and to release lactate under hypoxic conditions [9,15,16]. High-affinity lactate transport has been reported in cardiac myocytes [10,11] and neurons [17]. In the latter cell type, at least, lactate transport is mediated by MCT2 [15]. The substrate specificity of this transporter is virtually identical with that of MCT1 [18]. Its prime physiological role is to supply cells with lactate and ketone bodies for the needs of energy metabolism. It is expressed in cells which rarely release lactate. MCT2 expression increases stongly in endothelial cells of the blood/brain barrier during suckling [19,20,21], when the brain consumes significant amounts of ketone bodies. In the adult brain, MCT2 expression is found in neurons [19,20,21], which again are thought to consume lactate derived from astroglial cells. The isoform MCT4 has recently been identified as the major lactate transporter of white glycolytic muscle cells [13,22], whereas oxidative-muscle fibre cells mostly express MCT1 [13,22]. The kinetic data derived from studies on giant sarcolemmal vesicles and cultured myotubes [12,23,24], however, are at variance with the kinetic properties of MCT4 as analysed in COS, ETL and NBL-1 cells, which express this isoform endogenously [13]. All those cell types showed K_m values for lactate which were similar to MCT1. In contrast, data from giant sarcolemmal vesicles indicate the presence of an MCT with low affinity [12]. The recent introduction of the *Xenopus laevis* oocyte expression system for the investigation of MCTs [15,16] now allows characterization of MCT isoforms in an expression system with low background activity.

In this study we have analysed the kinetic properties of the cloned rat MCT4 following expression in *X. laevis* oocytes to clarifiy the differences reported between transport studies in vesicles, myotubes and mammalian cells. Our results show that MCT4 is a low-affinity, lactate-preferring transporter, which is adapted to the release of lactate from glycolytic cells.

EXPERIMENTAL

Materials

L-[U-^{14}C]lactate (5.62 Gbq/mmol) was purchased from Amersham Buchler (Braunschweig, Germany). [1-^{14}C]2-Oxoiso-

Abbreviations used: DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid; pCMBS, *p*-chloromercuribenzoesulphonic acid; MCT, monocarboxylate transporter; rMCT, rat MCT; cRNA, complementary RNA; RT, reverse transcriptase.

[1] To whom correspondence should be addressed at the Physiologisches Institut der Universität (e-mail stefan.broeer@uni-tuebingen.de).

hexanoate was a gift from Katarzyna Nalecz, Nencki Institute, Warsaw, Poland. Sodium L-lactate, 2-oxoisohexanoate, DL-β-hydroxybutyrate, α-cyano-4-hydroxycinnamate, 2-oxoisopentanoate and acetoacetate were ordered from Fluka/Sigma (Deisenhofen, Germany). The cap-analogue P1-5′-(7-methyl)-guanosine-P3-5′-GTP was obtained from New England Biolabs (Schwalbach, Germany) and Ultima Gold scintillation cocktail from Canberra Packard (Dreieich, Germany). Collagenase (EC 3.4.24.3; 0.6–0.8 unit/mg was from Boehringer (Ingelheim, Germany). All other chemicals were of analytical grade and were purchased from E. Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Roche (Mannheim, Germany).

Cloning

Two primers were used to amplify the coding sequence of rat MCT4 [13] from astroglial cDNA [25]. The sense primer (5′-TCG GAT CCT GGC CAT GGG AGG AGC T-3′) contained a *Bam*HI site additional to the sequence corresponding to the 5′ end of MCT4 (bases 82–101); the antisense primer (5′-GCT CTA GAC CGC TCA CAC GCT GGT TT-3′) contained an *Xba*I site additional to the sequence corresponding to the 3′ end of MCT4 (bases 1492–1509). The amplified MCT4 cDNA was cut with *Bam*HI and *Xba*I and cloned into the corresponding sites of vector pGEM-He [26] (kindly provided by Dr J. Ludwig, Tübingen). This vector contains the 5′ and 3′ untranslated regions of the *Xenopus* β-globin interrupted by a multiple cloning site.

Cell culture

Astroglia-rich primary cultures were prepared from newborn rat brains as described by Hamprecht and Löffler [27]. Total brains were passed successively through two nylon nets of 211 μm and 135 μm mesh width. The suspended cells of a complete offspring (10–15 animals) were collected by centrifugation and resuspended in 20 ml of Dulbecco's modified Eagle's medium/10 % fetal-calf serum. After determination of the cell number, cells were diluted in Dulbecco's modified Eagle's medium/10 % fetal-calf serum to a final density of 200000 cells/ml. Aliquots were dispensed in cell culture dishes and incubated in a cell incubator at 37 °C, air/CO_2 (9:1), for 14–21 days. Medium was renewed every week. For all experiments, 14–21-day-old confluent cultures were used. At least 90 % of these cells express glial acidic fibrillary protein. The cultures contain limited numbers of oligodendroglial, ependymal and microglial cells, but are devoid of neurons (results not shown).

Neuron-rich primary cultures were derived from embryonal rat brains (E16) as described by Löffler et al. [28]. Embryos were removed from the uteri of pregnant rats after 16 days gestation. Total brains were passed successively through two nylon nets of 135 μm and 20 μm mesh width. One million cells were seeded on to poly-D-lysine-coated 35 mm dishes. After 3 days in culture, cells were treated for 24 h with cytosine arabinoside at a final concentration of 0.5 μM to kill dividing cells. Subsequently, cells were incubated in glia-conditioned neuron culture medium [28]. The cultures were used at a culture age of 5–7 days. These cultures contain some astroglial cells but no oligodendroglial or ependymal cells, as determined by cell-specific markers [28].

Reverse transcriptase (RT)-PCR

Reverse transcriptase reaction

Total RNA was isolated from embryonal rat brain, adult rat brain, astroglia-rich rat primary cultures and different mouse tissues by the 'acid–guanidinium-thiocyanate–phenol–chloroform extraction' method [29]. For reverse transcription, 0.5 μg of oligo$(dT)_{15}$ was added to 1 μg of total RNA in a total volume of 12 μl. The mixture was incubated for 10 min at 65 °C then chilled on ice. Dithiothreitol, dNTPs, 5 × RT buffer and 30 units of RNasin (Promega) were added, and the whole mixture (20 μl) was further incubated at 37 °C for 2 min. For cDNA synthesis, 200 units of Superscript II-RT (Life Technologies) was added followed by incubation at 37 °C for 1 h. The cDNA was purified by using a PCR-product purification kit (Roche).

PCR

A standard PCR protocol with 100 pmol of each primer and a 10 μl aliquot of the purified cDNA was used for amplification of the fragments during 30 cycles (95 °C, 30 s; 50 °C, 1 min; 72 °C, 1 min) in a Thermocycler using *Taq* polymerase (Qiagen). After amplification the samples were extracted once with chloroform, and 20 μl were analysed by electrophoresis through a 1 % agarose gel.

For detection of the mouse or rat MCT4 mRNA, the sense primer (5′-TGC GGC CCT ACT CTG TCT AC-3′) and the antisense primer (5′-TCT TCC CGA TGC AGA AGA AG-3′) were used to amplify a specific fragment of 368 bp, corresponding to bases 969–988 and 1318–1337 of the rat MCT4 cDNA (accession number U87627), and to bases 45–64 and 394–413 of a published expressed sequence tag database sequence (accession number AI552036), which corresponds to the mouse MCT4 cDNA (which has not been cloned as yet).

Oocytes and injections

X. laevis females were obtained from the South African *Xenopus* facility (Knysna, Republic of South Africa). Oocytes (stages V and VI) were isolated by collagenase treatment as described before [30], and allowed to recover overnight.

Plasmid DNA was linearized with *Sal*I and transcribed *in vitro* with T7 RNA polymerase in the presence of the cap-analogue P1-5′-(7-methyl)-guanosine-P3-5′-GTP with the T7-mMessage mMachine Kit according to the protocol of the manufacturer (Ambion, Austin, Texas). Template plasmid was removed by digestion with RNase-free DNaseI. The complementary RNA (cRNA) was purified by phenol/chloroform extraction followed by precipitation with 0.5 vol. of 7.5 M ammonium acetate and 2.5 vol. of ethanol to remove unincorporated nucleotides. Integrity of the transcript was checked by denaturing agarose gel electrophoresis. Oocytes were microinjected with 15 nl of rat MCT (rMCT4) cRNA in water at a concentration of 1 μg/μl, by using a microinjection device (WPI Instruments, Sarasota, Florida, USA).

Immunohistochemistry

After testing for rMCT4 activity, oocytes were devitellinized with tweezers after incubation in hypertonic medium (200 mM potassium aspartate, 10 mM Hepes, pH 7.4), and fixed in Dent's solution (80 % methanol/20 % DMSO) overnight at −20 °C. The fixative was washed out and oocytes were incubated with the primary antibody (1:100) in the presence of 10 % goat serum at 4 °C for 12 h. The primary antibody was generously supplied by Andrew Halestrap (University of Bristol, Bristol, U.K.). The properties of the antibody were described by Wilson et al. [13]. After washing with PBS, incubation with secondary Alexa 488 goat anti-rabbit IgG antibody (dilution 1:200; Molecular Probes,

Eugene, OR, U.S.A.) was performed at room temperature for 1 h. After washing with PBS, stained oocytes were post-fixed with 3.7% paraformaldehyde for 30 min. The embedding procedure in acrylamide (Technovit 7100) was carried out according to manufacturer's instructions (Heraeus Kulzer, Wehrheim, Germany). Embedded oocytes were cut into 5-μm sections and analysed with a fluorescence microscope (Nikon Optiphot, Düsseldorf, Germany).

Recording of intracellular pH (pH_i) values

Double-barrelled pH-sensitive microelectrodes, to measure pH_i and membrane potential, were prepared as previously described [31]. Briefly, the electrodes were pulled in two stages and silanized by filling a drop of 5% tri-*N*-butylchlorosilane in 99.9% pure CCl_4 into the prospective ion-selective barrel, and then baking the pipette on a hot plate at 475 °C for 4.5–5 min.

For pH-selective microelectrodes a small amount of H^+ cocktail (Fluka 95291) was backfilled into the tip of the silanized barrel and the remainder filled with 0.1 M sodium citrate, pH 6.0. The reference barrel was filled with 2 M potassium acetate. Electrodes were accepted for experiments if their response exceeded 50 mV per unit change in pH; on average they responded with a change of 54 mV to a change in pH by one unit.

The recording arrangement was the same as described previously [31,32]. The central and the reference barrel were connected by chlorided-silver wires to the headstages of an electrometer amplifier. The voltage signal of the reference barrel was also used for voltage-clamping and subtracted from the signal of the ion-selective barrel to obtain direct readings of pH_i.

Flux measurements

For each determination, groups of seven cRNA, water, or non-injected oocytes were washed twice with 4 ml of oocyte Ringer #2 buffer supplemented with $CaCl_2$ (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM Hepes, titrated with NaOH to pH 7.0) before incubation at room temperature in a 5 ml polypropylene tube containing 70 μl of the same buffer supplemented with 5 kBq of L-[U-^{14}C]lactate and different amounts of unlabelled substrate. Transport was stopped after different time intervals by washing oocytes three times with 4 ml of ice-cold oocyte Ringer buffer. Repeated washing steps did not result in leakage of labelled lactate [16]. Single oocytes were placed into scintillation vials and lysed by addition of 200 μl of 10% SDS. After lysis, 3 ml of scintillation fluid was added, and the radioactivity determined by liquid-scintillation counting.

Calculations

For the determination of kinetic parameters, non-linear regression algorithms of Microcal Origin software (Microcal Software, Northampton, MA, U.S.A.) were used. The initial slopes of pH transients from single oocytes were first fitted to a logistic equation, and then normalized by calculation of v/V_{max}. The final curve was then calculated from v/V_{max} values of the indicated number of experiments. K_m values were determined by non-linear regression analysis of values to the equation $v/V_{max} = [S]/(K_m + [S])$. Competition experiments were analysed by non-linear regression of data to the equation $v = V_{max}\ [S]/(K_m\ (1 + [I]/K_i) + [S])$; where [S] is the substrate concentration, [I] is the inhibitor concentration and K_i is the inhibition constant. The lactate concentration used in these experiments was well below the K_m value. The number of investigated oocytes (n) is given in the text. When using pH-sensitive microelectrodes, single oocytes could be superfused with complete sets of concentrations (e.g. pH dependence and concentration dependence). For radioactive flux measurements, each individual datapoint represents the difference between the mean ± S.D. uptake activity of 'n' MCT4 expressing and 'n' non-injected oocytes ($n = 7$). The S.D. of the difference was calculated by Gauss's law of error propagation.

RESULTS

Expression of rMCT4 in the plasma membrane of *X. laevis* oocytes

Oocytes expressing the rMCT4 isoform showed an increased uptake activity for L-[^{14}C]lactate. At a lactate concentration of 1 mM, rMCT4-injected oocytes took up 323 ± 25 pmol/10 min ($n = 7$), in contrast with non-injected oocytes, which took up 60 ± 3 pmol/10 min ($n = 7$). Uptake was linearly correlated to the incubation time for up to 20 min (results not shown). To compare rMCT4 with the other isoforms of the family, the basic kinetic constants of monocarboxylate transport were determined. Using L-[U-^{14}C]lactate, a K_m value of 34 ± 5 mM and a V_{max} value of 4200 ± 300 pmol/10 min was determined (Figure 1). This K_m value was considerably higher than the corresponding values of rMCT1 (3.5 mM; [16]) and rMCT2 (0.5 mM; [18]). Similar to the other MCT isoforms, a closer inspection of the concentration dependence revealed the presence of a high-affinity component, which was readily visible in the Eadie–Hofstee transformation of the data. The V_{max} value of rMCT4 was found to be similar to that of rMCT1, but was 18 times higher than the V_{max} value of rMCT2. To investigate whether the differences in the transport capacity reflected the protein content in the plasma membrane, or whether the transporter proteins had different turnover numbers, oocytes were analysed by immunocytochemistry

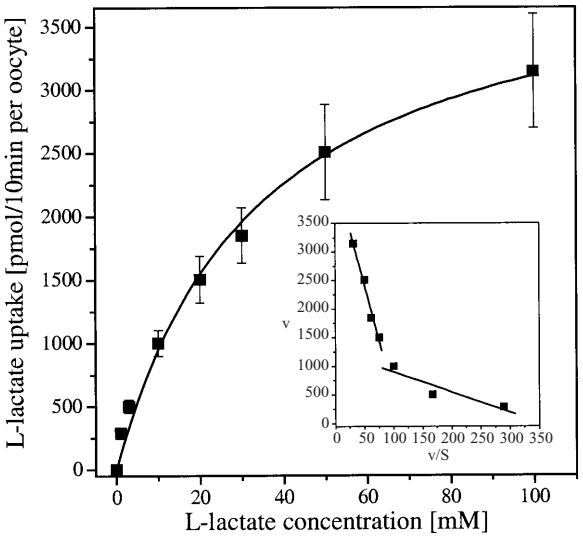

Figure 1 Determination of the kinetic constants for L-lactate transport via MCT4

Oocytes were injected with 15 ng of rMCT4 cRNA. After an expression period of 3 days, uptake of labelled lactate was determined at pH 7.0 over a time period of 10 min. For each concentration, the mean uptake activity of seven oocytes was determined. The transport activity of the non-injected oocytes has already been subtracted. Rat MCT4 showed a K_m value of 34 ± 5 mM and a V_{max} value of 4200 ± 300 pmol/10 min. The inset shows a transformation of the data according to Eadie and Hofstee, showing two kinetic components with affinities of approx. 38 mM and 4 mM.

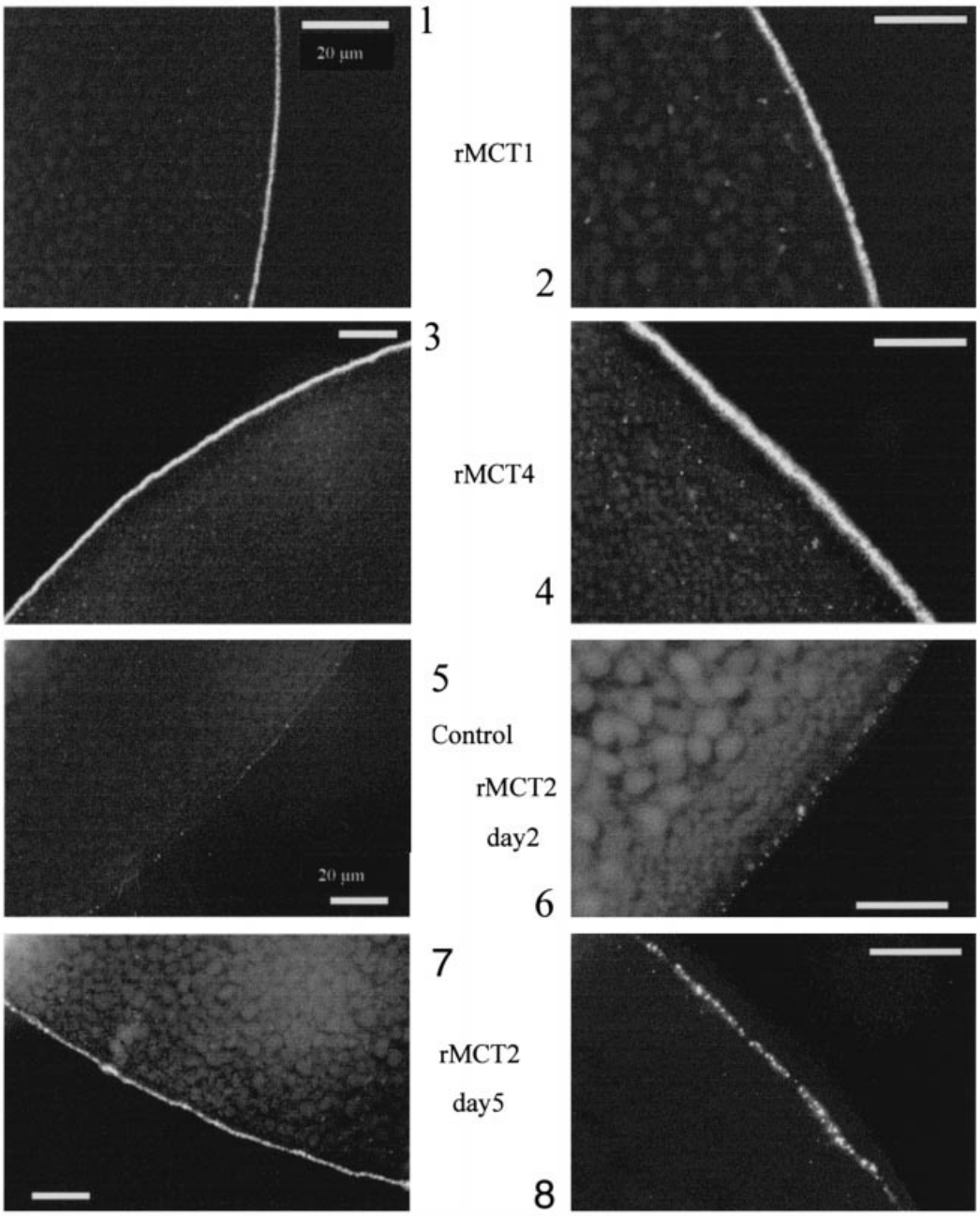

Figure 2 Immunohistochemistry of different MCTs expressed in *X. laevis* oocytes

Oocytes were injected with 15 ng of rMCT1, rMCT2, or rMCT4 cRNA, or remained non-injected. MCT1- and MCT4-expressing oocytes were used for immunohistochemistry after an expression time of 2 days (panels 1–4). Both MCTs are located in the plasma membrane. MCT2 oocytes were used for immunohistochemistry after 2 and 5 days (panels 6–8). After 2 days of expression only small amounts of MCT2 could be detected in the plasma membrane (panel 6). After 5 days MCT2 showed a spotted pattern in the plasma membrane, but still with a much smaller amount of transport protein than for MCT1 or MCT4 after day 2 (panels 7–8). Non-injected oocytes did not show significant immunoreactivity with any of the antibodies (MCT4 shown, panel 6). Panels are shown in two different magnifications. The white bar denotes 20 μm.

(Figure 2). When analysed 2 days after injection of the cRNA, only rMCT1- and rMCT4-like immunoreactivities were found in significant amounts (Figure 2). Both transporters were clearly localized in the plasma membrane, whereas no staining was observed in submembraneous compartments. In contrast, oocytes which had been injected with identical amounts of rMCT2 cRNA, showed only small numbers of scattered spots in the membrane 2 days after cRNA injection. After incubation for 5 days, significant amounts of rMCT2 immunorecativity could also be detected in the plasma membrane (Figure 2). Similar to the other isoforms, no staining in submembraneous compartments was detected; the immunoreactivity still showed a spotted appearance. Although different antisera do not allow a quantitative comparison of protein expression, due to differences in the affinity, the intensity of the staining showed similar grading to the V_{max} values of rMCT1, rMCT4 and rMCT2.

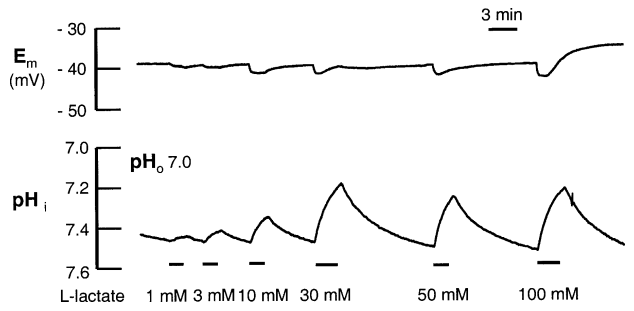

Figure 3 pH_i changes elicited in MCT4-expressing *X. laevis* oocytes by superfusion with L-lactate

Each oocyte was injected with 15 ng of MCT4 cRNA followed by an expression period of 3 days. pH_i and membrane potential were recorded with a double-barrelled pH-sensitive microelectrode. In the representative experiment depicted, an oocyte was superfused with lactate-containing solutions of different concentrations (marked by horizontal bars) and lactate-free solutions (intervals between bars). From measurements with $n = 6$ oocytes, a K_m value of 17 ± 3 mM was determined using the initial slopes of the acidification as transport velocity.

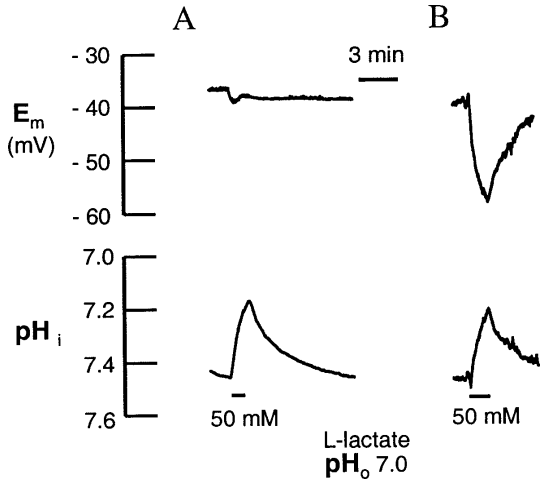

Figure 4 Changes of the membrane potential in MCT4-expressing *X. laevis* oocytes in response to superfusion with L-lactate

Each oocyte was injected with 15 ng of rMCT4 cRNA, measurements were done after 3 days of expression. (**A**) Acidification due to superfusion with lactate-containing solution (50 mM L-lactate) has no influence on the membrane potential within this batch of oocytes. (**B**) Oocyte of a different preparation, in which the membrane potential drops as a reaction to the acidification. The changes in the membrane potential in some oocytes had no influence on the kinetic data of rat MCT4 (results not shown).

Mechanism of lactate transport via rMCT4

Wilson et al. [13] previously showed that lactate transport in MCT4-expressing cells is accompanied by changes in cytosolic pH. These observations were confirmed by experiments using pH-sensitive microelectrodes. Superfusion of rMCT4-expressing oocytes with increasing concentrations of lactate generated an increasing acidification of the cytosolic pH, indicating concentration-dependent H^+ influx (Figure 3). Although lactate transport was accompanied by changes of the membrane potential in some experiments, these were not correlated with the influx velocity (Figures 4A and 4B). These changes most likely resulted from secondary effects of the cytosolic pH on endogenous oocyte ion channels and therefore depended on the oocyte preparation (Figures 4A and 4B). The transport mechanism of rMCT4 could

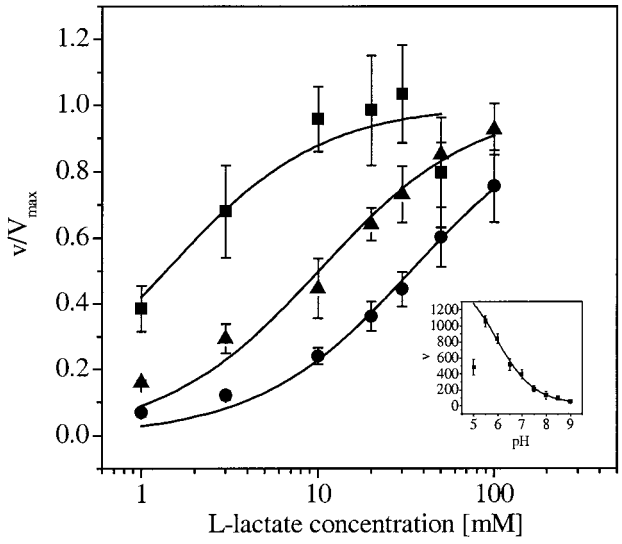

Figure 5 pH-dependence of L-lactate transport via MCT4

Oocytes were injected with 15 ng of rMCT4 cRNA and used for measurements after an expression period of 3–4 days. The uptake of labelled L-lactate was determined at different concentrations of L-lactate and different pH-values: pH 5 (■), pH 6 (▲) and pH 7 (●). Each data point represents the mean of seven injected oocytes, the value for non-injected oocytes are already subtracted. A drop in the K_m value from 34 ± 5 mM (pH 7) to 10 ± 2 mM (pH 6) to 1.4 ± 0.5 mM (pH 5) was observed by lowering the pH. The inset shows the transport activity of rMCT4 cRNA-injected oocytes ($n = 7$) in pmol L-lactate/10 min with variation of pH at a constant L-lactate concentration of 1 mM.

therefore, similar to the other members of the family, be described as an electroneutral $1H^+$/monocarboxylate$^-$ cotransport. In agreement with this mechanism, lactate transport was pH dependent, increasing with decreasing pH. The increase in transport velocity, however, was mainly caused by a decrease in the K_m value (Figure 5) and less by an increase in the V_{max}. The V_{max} remained constant between pH 7.0 (4200 ± 300 pmol/10 min) and pH 6.0 (4300 ± 200 pmol/10 min) and even decreased at pH 5.0 (2200 ± 100 pmol/10 min). This behaviour, which is similar to that observed in MCT1- and MCT2-expressing cells, has been interpreted as being a result of an ordered binding mechanism with H^+ binding before the monocarboxylate anion. Uptake of labelled lactate could be *trans*-stimulated by pre-loading the cells with substrates of the transporter. When oocytes were incubated with different substrates at a concentration of 100 mM (pH 7.0) for 20 min, lactate uptake (50 mM) was increased between 1.5- and 2-fold in comparison to uptake in control oocytes (Table 1). This result suggests that the conformational change of the unloaded carrier is the rate-limiting step in the transport mechanism.

Substrate recognition by rMCT4

The most characteristic feature of rMCT4 was its low apparent substrate affinity as compared with that of MCT1 and MCT2. As pointed out above, a K_m value of 34 ± 5 mM was determined using labelled lactate. Owing to the extremely high concentrations which had to be used to achieve saturation of the transporter, K_m values between 25 and 35 mM were determined in our experiments. When pH-sensitive microelectrodes were used to analyse the kinetics of rMCT4, a somewhat lower value of 17 ± 3 mM was determined. The isoforms MCT1 and MCT2 have been shown to be non-specific transporters, accepting a wide variety of

Table 1 *Trans*-stimulation of L-lactate uptake by increased cytosolic substrate concentration

Rat MCT4-expressing oocytes (15 ng of cRNA, 2 days after injection) were first incubated for 20 min with a solution containing 100 mM of the indicated monocarboxylate. The transport activity of MCT4 (measured at an extracellular L-lactate concentration of 50 mM) was stimulated by increased cytosolic monocarboxylate concentration. Data points show the means ± S.D. of $n = 7$ injected oocytes (non-injected oocytes already subtracted).

Preincubation substrate	L-Lactate uptake (pmol/10 min per oocyte)	Transport activity (%)
Without substrate	1450 ± 200	100
L-Lactate	2860 ± 650	197
Acetoacetate	2160 ± 290	148
Pyruvate	2550 ± 540	176
2-Oxoisohexanoate	1970 ± 660	136
2-Oxopentanoate	2670 ± 410	184
β-Hydroxybutyrate	2380 ± 250	164
α-Hydroxyisobutyrate	2280 ± 260	157

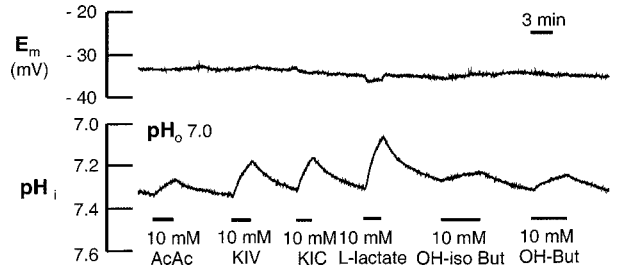

Figure 6 Substrate specifity of MCT4

Cytosolic pH was recorded in a representative oocyte that was injected with 15 ng of rMCT4 cRNA followed by an expression time of 2–4 days during superfusion with solutions containing different monocarboxylic acids [AcAc, acetoacetate; OH-But, β-hydroxybutyrate; OH-iso But, α-hydroxy-isobutyrate; KIC, α-ketoisocaproate (2-oxoisohexanoate); KIV, α-ketoisovalerate (2-oxoisopentanoate)].

Table 2 Kinetic constants for transport via MCT4 for different monocarboxylic acids

Oocytes were injected with 15 ng of rat MCT4 cRNA and used 2–4 days after injection. For flux measurements $n = 7$ injected oocytes were used (data from non-injected oocytes were already subtracted). K_i values were determined by measuring the uptake of L-lactate (1 mM) in the presence of increasing amounts of other monocarboxylates (1–100 mM) and analysing the curves as competitive inhibition when the monocarboxylate was accepted as a transported substrate. The results of measurements with pH-sensitive microelectrodes represent at least $n = 5$ oocytes, each being superfused with at least five different substrate concentrations. All measurements were performed in transport buffer at pH 7.0, unless indicated otherwise.

Substrate	K_m flux measurements (mM)	K_i flux measurements (mM)	K_m pH electrode (mM)
L-Lactate			
pH 7	33.7 ± 4.9		16.9 ± 2.7
	30.9 ± 7.5		15.6 ± 0.2
pH 6	10.6 ± 5.4		
	10.1 ± 1.9		
pH 5	1.4 ± 0.5		
	1.4 ± 0.8		
D-Lactate		55.4 ± 9.7	55.1 ± 13.9
		40.0 ± 5.0	
Pyruvate			
pH 7.0		18.9 ± 6.1	16.2 ± 1.5
		25.4 ± 8.9	
pH 7.4			36.3 ± 2.0
pH 7.5		22.3 ± 12.1	
pH 8.0		21.9 ± 12.7	
2-Oxoisohexanoate	12.8 ± 10.1	11.4 ± 0.8	
	5.9 ± 2.2	9.0 ± 2.6	
2-Oxopentanoate		27.5 ± 7.3	
		22.5 ± 7.8	
Acetoacetate		22.6 ± 6.7	31.1 ± 6.2
		23.3 ± 3.9	
D/L-β-Hydroxybutyrate		45.2 ± 19.4	64.7 ± 8.6
		35.5 ± 7.8	
α-Hydroxyisobutyrate		29.9 ± 7.9	
		30.1 ± 4.3	
Formate		> 100	
Acetate		> 100	

monocarboxylates. Superfusion of rMCT4-expressing oocytes with different monocarboxylates similarly resulted in the acidification of the cytosol (Figure 6). However, in contrast with MCT1 and MCT2, lactate seemed to be the preferred substrate of rMCT4, eliciting much faster responses than the other monocarboxylates. To decide whether this discrimination resulted from differences in the apparent affinity, or from different maximum velocities, the kinetic constants for a number of monocarboxylates were determined (Table 2). In general, apparent substrate affinities for hydroxy and oxo acids were all found to be in the range of 10–50 mM. In contrast, short-chain fatty acids displayed much higher K_m values of > 100 mM. The K_m values of physiologically important substrates were mostly similar to the K_m value for lactate. Only 2-oxoisohexanoate, a substrate with increased hydrophobicity, displayed a significantly lower K_m value for transport via rMCT4. Although rMCT4 did not discriminate strongly between hydroxy and oxo acids, transport showed stereospecificity of L-lactate over D-lactate (Table 2).

Inhibitors of rMCT4

Similar to the other members of the MCT-family, α-cyano-4-hydroxycinnamate was found to be an inhibitor of monocarboxylate uptake with an IC_{50} of 350 ± 150 μM. 4,4′-Diisothiocyanostilbene-2,2′-disulphonic acid (DIDS), at a concentration of 500 μM, reduced lactate transport by 60 %; the remaining transport activity was insensitive even to millimolar (up to 2 mM) concentrations of DIDS (Figure 7). Lactate uptake via MCT1, in contrast, is completely blocked by DIDS, with an IC_{50} of 1.1 mM. Phloretin blocked lactate transport completely with high affinity ($IC_{50} = 40 \pm 20$ μM). MCT4, unlike MCT2 but similar to MCT1, was found to be sensitive to inhibition by *p*-chloromercuribenzoesulphonic acid (pCMBS). After 5 min preincubation with 1 mM pCMBS in oocyte Ringer buffer, lactate uptake decreased by 91 %.

Tissue distribution of MCT4

To analyse the expression pattern of MCT4, mRNA was isolated from different tissues and the MCT4 mRNA was detected by RT-PCR. Significant amounts of MCT4 mRNA were detected in testis, small intestine, parotid gland, lung and brain. Small amounts were detected in heart, kidney and spleen, and no significant transcript level was detected in liver (Figure 8). Significant species differences have been observed in the tissue distribution of MCT1 and MCT2 [5]. In contrast with these isoforms, we found a good match between expression of MCT4 in

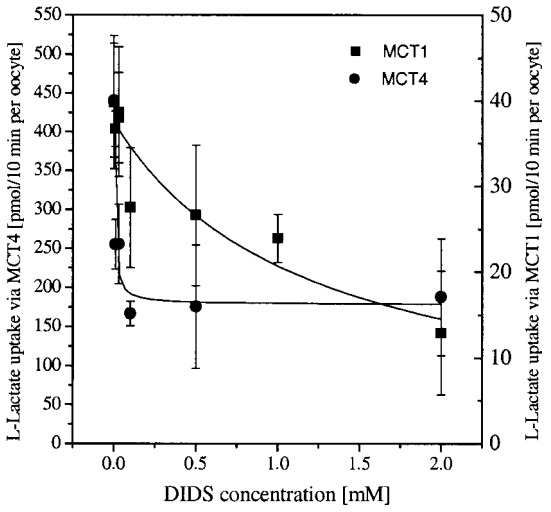

Figure 7 Inhibition of L-lactate transport via MCT1 and MCT4 by DIDS

Oocytes were injected with 15 ng of rMCT1 or rMCT4 cRNA. After an expression period of 3 days, uptake of labelled lactate (extracellular concentration 100 μM for rMCT1 and 1 mM for rMCT4) was determined at pH 7.0 over a time period of 10 min in the presence of increasing DIDS concentrations. For each concentration, the mean uptake activity of seven oocytes was determined. The transport activity of the non-injected oocytes has already been subtracted. Note the two different ordinates.

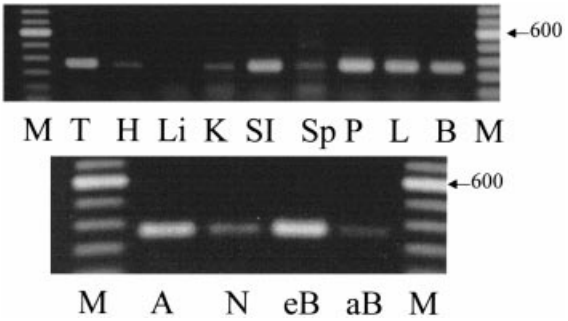

Figure 8 Expression pattern of MCT4 in different mouse tissues and different cultured rat brain cells

Total RNA was isolated from different mouse tissues (upper panel) and from cultured rat astrocytes, neurons, embryonic and adult brain (lower panel). A 1 μg aliquot of RNA was reverse transcribed into cDNA, and an aliquot was used for RT-PCR using MCT4-specific primers. The MCT4-specific fragment (368 bp) was amplified for 30 cycles. M, marker; T, testis; P, parotid gland; B, brain; SI, small intestine; Sp, spleen; H, heart; L, lung; Li, liver; K, kidney; A, cultured rat astrocytes; N, cultured rat neurons; eB, embryonic brain; aB, adult brain. A 100 bp marker was used as a standard, the 600 bp band is marked.

rat and mouse tissues (Figure 8) with the reported distribution in human tissues [5]. However, in addition to these data we detected significant expression of MCT4 in rat brain and cultured rat brain cell types. High expression was detected in cultured astrocytes, whereas MCT4 mRNA was barely detectable in cultured neurons. Embryonic brain contained more corresponding mRNA than adult brain (Figure 8).

DISCUSSION

Methodological aspects

One of the problems we encountered during the characterization of MCT4 was that different techniques generated different kinetic constants. When using labelled lactate, the complete amount of accumulated lactate is determined. This uptake was linear for about 20 min. In contrast, the pH-sensitive microelectrodes determine the local intracellular pH close to the plasma membrane. This technique generates pH transients which are linear for shorter time courses. The local pH shifts are net changes and presumably the sum of at least three different movements: (1) the influx of protons through the MCT4 (accompanied by lactate); (2) the efflux of protons through the transporter (accompanied by lactate); and (3) the diffusion of protons into the cytosol of the oocyte (accompanied by any anion). Therefore it can be expected that a steady state is reached earlier when the pH is determined locally, in comparison with the determination of net movement of labelled lactate. The measurement of proton movements detects only net transport of lactate, because efflux of lactate in the equilibrium situation is also accompanied by protons. Using labelled lactate, uptake occurs, even in exchange, until the specific activity in the cytosol and the extracellular fluid is in equilibrium.

Another factor which might contribute to disturbances is osmolarity. Owing to the extremely high K_m value of the transporter, saturation was generally reached at concentrations > 100 mM. Since oocyte Ringer contains 85 mM NaCl, a complete exchange of NaCl into sodium lactate is necessary. However, the K_m value for lactate in osmotically balanced (K_m approx. 30 mM) or unbalanced solution (K_m approx. 20 mM) did not differ strongly.

In most oocyte batches we observed a hyperpolarization of the membrane potential during superfusion with monocarboxylate-containing solutions. These changes in the membrane potential were unlikely to reflect an electrogenic monocarboxylate-transport mechanism for two reasons: the changes in membrane potential varied significantly between different oocyte preparations; and kinetic constants were not influenced by changes in the membrane potential (results not shown). A possible explanation for this phenomenon might be an effect of the intracellular pH on endogenous oocyte ion channels. The influx of lactate together with protons, resulting in local acidification of submembraneous compartments, might, in turn, modify the gating behaviour of ion channels, as has been shown for example for potassium channels [33].

Mechanistic aspects

Similar to the other members of the MCT family [15,16,18] we detected two kinetic components in the Eadie–Hofstee analysis of the concentration dependence of lactate uptake (Figure 1). Presently it is not clear whether the appearance of the two components is a property of the oocyte expression system or whether it is an intrinsic feature of MCT-type transporters. Another not fully resolved discrepancy was observed during the evaluation of V_{max} and K_m values. The *trans*-stimulation experiments suggested that the maximum velocity of the transporter is limited by the conformational transition of the unloaded carrier. To explain the differences of uptake rates for the various substrates of the transporter at identical concentrations (Figure 6) divergent K_m values have to be expected. However, most of the substrate K_m values ranged between 20 and 30 mM, whereas the estimated V_{max} values varied between 30% and 100% as compared with L-lactate (results not shown). Additionally, we found similar values by using flux studies as well as by monitoring changes in the cytosolic pH. Some of the K_m values may nevertheless be slightly underestimated, at the most by a factor of 2. In agreement with this notion, higher K_m values are determined when the fluorescent pH indicator 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein is used to follow the changes in

the cytosolic pH in MCT4-expressing oocytes (A. P. Halestrap, personal communication).

Physiological aspects

Two features discriminate rMCT4 from the other, so far characterized, members of the monocarboxylate transporter family, MCT1 and MCT2. The first one is the extremely low substrate affinity, and the second one is the preference for lactate as a substrate under physiological conditions. MCT1, MCT2 and MCT4 span a wide range of substrate affinities. For L-lactate, K_m values of approx. 500 μM, approx. 4 mM and approx. 30 mM have been determined for the isoforms MCT2, MCT1 and MCT4 respectively ([16,18], and the present study). All isoforms mediate transport of lactate in cotransport with a proton, thereby allowing the cell to release the acid equivalents which have been produced during glycolysis together with lactate. When the substrate specificity of the transporters is analysed, MCT1 and MCT2 can be characterized as 'real' MCTs, i.e. they display a similar capacity for the transport of a number of monocarboxylates. α-Oxo acids, for example, generally have lower K_m values than the corresponding hydroxy acids. The V_{max} value for L-lactate is slightly higher than the V_{max} of pyruvate, but this is more than compensated for by a 5-fold lower K_m value in the case of both MCT1 and MCT2. Therefore transport of pyruvate is preferred over transport of lactate, when both are offered at similar concentrations. Similarly, the ketone bodies β-hydroxybutyrate and acetoacetate have similar K_m values and V_{max} values as lactate. Even branched-chain oxo acids are accepted as a substrate of MCT1 and MCT2. This ensures an inter-organ transfer of monocarboxylates, i.e. from liver to brain, or from liver to the muscle. MCT1 particularly seems to be expressed in cells which use ketone bodies for energy production, but which also release lactate under certain conditions. The high-affinity isoform MCT2, in contrast, is mostly expressed in cell types which use lactate, like liver cells, or neurons of the brain. MCT4, on the other hand, seems to be adapted for highly glycolytic cells for two reasons: (1) it has a high capacity and is almost non-saturable, and (2) pyruvate and lactate have similar K_m values. In most cells the equilibrium between lactate and pyruvate is governed by the redox potential of the cells, which ensures that lactate is present at much higher concentrations than pyruvate. Cells with a low number of mitochondria might be unable to keep the redox potential constant when both lactate and pyruvate are released at similar rates, because NADH produced during glycolysis could not be regenerated. The MCT4 isoform is therefore adapted for the release of lactate from cells with few mitochondria. Only when pyruvate is accumulated would it be able to compete with lactate for release. Cells with low numbers of mitochondria are not able to use lactate as an energy source. MCT4 can therefore be characterized as a transporter which is adapted for lactate release. The kinetic characteristics of this transporter fit well with its expression in glycolytic myotubes of white muscle cells [13]. Another candidate of cell type for expression of MCT4 are Müller cells of the retina. They also do not contain mitochondria and are known to release lactate as an energy metabolite for photoreceptor cells [34]. It has, however, been reported that Müller cells mostly express MCT1 [35]. For the first time, we showed significant expression of MCT4 mRNA in rat and mouse brain, indicating that certain cell types in the brain may also contain fewer mitochondria, and release lactate as a possible energy metabolite for neighbouring cells. The distribution of MCT4 mRNA in mouse peripheral tissues was in good agreement with the Northern-blot studies of Price et. al. [5] using human tissues.

Evolutionary aspects

We have recently started to analyse structure–function relationships in MCT1 [36], which suggested that substrate binding and recognition occur in helix 8 and helix 10. Mutations in helix 10 are known to affect the substrate specificty of MCT1. The residues of helix 10 are not well conserved between the different family members. The exchange of phenylalanine-360 to cysteine in MCT1, for example, results in a gain of substrate recognition, allowing mevalonate to be transported in addition to the other monocarboxylates [2,33]. This residue is conserved in MCT2, the substrate specificity of which is almost identical with MCT1 [18]. In rMCT4, however, this residue is converted into a tyrosine. This exchange might influence the substrate specificity with respect to preferential recognition of lactate. One discriminating feature in the MCT family is the sensitivity to pCMBS. Whereas both MCT1 and rMCT4 are sensitive to inhibition by pCMBS, MCT2 is resistant to modification of cysteine residues by this compound. A sequence comparison shows that only two cysteines [residue 106 (rat MCT1) at the end of helix 4 and residue 188 (rat MCT1) at the end of helix 6 respectively] are present in MCT1 and rMCT4 but are missing in MCT2. These cysteine residues are obvious candidates for lining the transport pore in MCT1, the modification of which blocks translocation of substrates.

As judged from the sequence similarity, rMCT4 is more closely related to MCT3 (68 % similar residues) than to the other isoforms. MCT3 is predominantly expressed in the retina pigment epithelium [4]. Although a kinetic characterization of MCT3 is still lacking it is likely that the features of this transporter are similar to rMCT4. It has been reported that the retina produces more L-lactate than any other tissue [35]. Therefore an MCT isoform similar to rMCT4 with low affinity, high capacity and preference for lactate, as reported here, would enable the retina epithelium to release large amounts of L-lactate.

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