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Nonenzymatic Augmentation of Lactate Transport via Monocarboxylate Transporter Isoform 4 by Carbonic Anhydrase II

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Abstract Monocarboxylate transporters (MCTs) are carriers of high-energy metabolites like lactate and pyruvate, and different MCT isoforms are expressed in a wide range of cells and tissues. Transport activity of MCT isoform 1 (MCT1), heterologously expressed in *Xenopus* oocytes, has previously been shown to be supported by carbonic anhydrase II (CAII) in a noncatalytic manner. In the present study, we investigated possible interactions of CAII with MCT4, expressed in *Xenopus* oocytes. MCT4 transport activity is enhanced both by injected and by coexpressed CAII, similar to MCT1, with the highest augmentation at low extracellular pH and low lactate concentrations. CAII-induced augmentation in MCT4 transport activity is independent from the enzyme's catalytic function, as shown by application of the CA inhibitor ethoxyzolamide and by coexpression of MCT4 with the catalytically inactive mutant CAII-V143Y.

Keywords Membrane transport mechanism · Ion transport · Electrophysiology · *Xenopus* oocytes

Introduction

High-energy metabolites, such as lactate, pyruvate and ketone bodies, are transported into and out of cells via monocarboxylate transporters (MCT, SLC16), of which 14 isoforms have been described (Halestrap and Meredith 2004). MCT isoforms 1–4 have been reported to transport monocarboxylates in an electroneutral transport mode of 1 proton:1 monocarboxylate with different substrate affinities. MCT1 is found in nearly all tissues studied and has a K_m value of 3–5 mM for L-lactate (Broer et al. 1997, 1998). MCT4 is a low-affinity, high-capacity carrier with a K_m value of 20–35 mM (Dimmer et al. 2000) and is found prominently in glycolytic tissues such as white skeletal muscle fibers and astrocytes (Halestrap and Meredith 2004; Halestrap and Price 1999; Pellerin et al. 2005). This suggests that MCT4 is the main pathway to export lactate out of glycolytic cells, which may produce larger amounts of lactate during metabolic demand. In the brain, MCT4 is expressed in astrocytes, where it has been suggested, together with MCT1, to be responsible for the export of lactate to provide neurons with the high-energy metabolite during increased activity (Bergersen 2007; Deitmer 2002; Magistretti et al. 1999; Walz and Mukerji 1988).

The enzyme carbonic anhydrase II (CAII), catalyzing the reversible hydration of CO_2, has been shown to bind to and enhance the activity of various acid/base-transporting proteins like the chloride/bicarbonate exchanger AE1 (Alvarez et al. 2005; McMurtrie et al. 2004; Vince and Reithmeier 1998, 2000), the sodium/hydrogen exchanger NHE1 (Li et al. 2002, 2006) and the sodium bicarbonate cotransporter NBC (Becker and Deitmer 2007; Loiselle et al. 2004; Pushkin et al. 2004). Lactate transport via MCT1, heterologously expressed in *Xenopus* oocytes, has previously been shown to be enhanced by CAII (Becker and Deitmer 2008; Becker et al. 2005). Surprisingly, this supporting effect of CAII does not depend on the enzyme's catalytic activity and appears to involve interaction between the C terminus of MCT1 and the N terminus of CAII.

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In the present work, MCT4, expressed in *Xenopus* oocytes, was studied to elucidate its relationship to injected and coexpressed CAII. The results indicate that lactate transport via MCT4 is enhanced by CAII, independent of the enzyme's catalytic activity, and that the interaction is highly sensitive to changes in extracellular lactate and proton concentrations.

Materials and Methods

Constructs, Oocytes and Injection of cRNA and CA

Human CAII cDNA (CAII-WT) was kindly provided by Dr. Reinhart Reithmeier (Toronto, Canada) (Vince et al. 2000). The catalytically inactive mutant CAII-V143Y was a gift from Dr. Carol Fierke (Ann Arbor, MI) (Alexander et al. 1991; Fierke et al. 1991). The two constructs were subcloned into the oocyte expression vector pGEM-He-Juel, which contains the 5′ and the 3′ untranscribed regions of the *Xenopus* β-globulin flanking the multiple cloning site. Rat MCT4 cDNA cloned into the oocyte expression vector pGEM-He-Juel was kindly provided by Dr. Stefan Bröer (Canberra, Australia) (Dimmer et al. 2000). Plasmid DNA was transcribed in vitro with T7 RNA-Polymerase (mMessage mMachine; Ambion, Austin, TX) as described earlier (Becker et al. 2004). *Xenopus laevis* females were purchased from Xenopus Express (Vernassal, France). Segments of ovarian lobules were surgically removed under sterile conditions from frogs anesthetized with 1 g/l of 3-aminobenzoic acid ethylester (MS-222; Sigma-Aldrich, Taufkirchen, Germany) and rendered hypothermic. The procedure was approved by the Landesuntersuchungsamt Rheinland-Pfalz (Koblenz, Germany; 23 177-07/A07-2-003 §6). As described earlier (Becker et al. 2004), oocytes were singularized by collagenase (Collagenase A; Roche, Mannheim, Germany) treatment in Ca^{2+}-free oocyte saline (pH 7.8) at 28°C for 2 h. Singularized oocytes were left overnight in an incubator at 18°C in Ca^{2+}-containing oocyte saline (pH 7.8) to recover. Oocytes of stages V and VI were injected with 5 ng of cRNA coding for MCT4, dissolved in DEPC-H_2O. Measurements were carried out 3–6 days after injection of cRNA. CAII was either injected as protein or coexpressed with MCT4. For injection of protein, 50 ng of CAII, isolated from bovine erythrocytes (C3934, Sigma-Aldrich), dissolved in 25 nl DEPC-H_2O, were injected 20–24 h before electrophysiological measurements. For coexpression of CAII, 12 ng cRNA coding for either CAII-WT or CAII-V143Y were injected together with MCT4 cRNA. The oocyte saline had the following composition (in mM): NaCl, 82.5; KCl, 2.5; $CaCl_2$, 1; $MgCl_2$, 1; Na_2HPO_4, 1; HEPES, 5—titrated with NaOH to the desired pH. In lactate-containing saline, NaCl was replaced by an equivalent amount of Na-L-lactate. In bicarbonate-containing saline, NaCl was replaced by an equivalent amount of $NaHCO_3$ and the solution was aerated with 5% CO_2/95% O_2. Lactate was added to HEPES-buffered solution at pH 7.0, unless otherwise stated, in the nominal absence of CO_2/HCO_3^-, containing around 0.008 mM of CO_2 from air and, hence, a HCO_3^- concentration of <0.2 mM.

Western Blot Analysis

For comparison of protein levels of heterologously expressed MCT4 and expressed or injected CAII, Western blot analysis was performed. For each sample 20 oocytes were lysed by sonification in 2% sodium dodecyl sulfate solution with protease inhibitor (protease inhibitor cocktail tablets, Roche) 4 days after injection of cRNA or 24 h after injection of CAII protein. Total protein content was determined using the BCA protein assay kit (Pierce, Fisher Scientific, Schwerte, Germany). Extracts, containing 25 µg protein per sample, were separated by 4–12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Proteins of interest were detected by antibody staining: For detection of MCT4, rabbit anti-MCT4 polyclonal antibody (diluted 1:250; Millipore, Schwalbach, Germany); for detection of expressed CAII-WT and CAII-V143Y, rabbit anti-CAII (human erythrocytes) polyclonal antibody (diluted 1:500; Millipore); and for detection of injected CAII rabbit anti-CAII (bovine erythrocytes), polyclonal antibody (diluted 1:500; Millipore) were used. As a loading control, actin was labeled with mouse anti-actin monoclonal antibody (diluted 1:400; MP Biomedicals, Eschwege, Germany). Primary antibodies were labeled with goat anti-rabbit or anti-mouse IgG horseradish peroxidase–conjugated secondary antibody (diluted 1:4,000, Millipore). Membranes were analyzed after incubation with Lumi-light Western blotting substrate (Roche) with a Versa Doc imaging system (Bio-Rad, Munich, Germany). Proteins were quantified with the software Quanti One 4.5 (Bio-Rad). For each protein band the pixel density per square millimeter was calculated and the background pixel density subtracted. To allow comparison of different Western blots, all measured protein concentrations on one blot were normalized to the concentration of one protein on the same blot.

Intracellular pH Measurements

For measurement of intracellular pH (pH_i) and membrane potential, double-barreled microelectrodes were used; the manufacture and application have been described in detail previously (Becker and Deitmer 2004; Deitmer 1991).

Briefly, for double-barreled microelectrodes, two borosilicate glass capillaries of 1.0 and 1.5 mm diameter were twisted together and pulled to a micropipette. The ion-selective barrel was silanized with 5% tri-*N*-butylchlorsilane in 99.9% pure carbon tetrachloride, backfilled into the tip. H^+-sensitive cocktail (95291; Fluka, Buchs, Switzerland) was backfilled into the tip of the silanized ion-selective barrel and filled with 0.1 M Na-citrate (pH 6.0). The reference barrel was filled with 3 M KCl. To increase the opening of the electrode tip, it was beveled with a jet stream of aluminum powder suspended in H_2O. For calibration, electrodes were perfused with HEPES-buffered oocyte saline (pH 7.0). After a stable electrode potential was reached, oocyte saline (pH 6.4) was applied until the electrode again reached a stable potential.

As described previously (Bröer et al. 1998), optimal pH changes were detected when the electrode was located near the inner surface of the plasma membrane. This was achieved by carefully rotating the oocyte with the impaled electrode. All experiments were carried out at room temperature.

Calculation of $[H^+]_i$

The measurements of pH_i were stored digitally using homemade PC software based on the program LabView (National Instruments, Munich, Germany) and routinely converted into intracellular H^+ concentration, $[H^+]_i$. This should provide changes in the $[H^+]_i$, which take into account the different pH baseline, as measured in HEPES- and CO_2/HCO_3^--buffered salines (Becker et al. 2004). The rate of change of $[H^+]_i$ was analyzed by determining the slope of a linear regression fit using the spreadsheet program OriginPro 7 (OriginLab, Northampton, MA).

Buffer Capacity and Proton Fluxes

The intrinsic buffering power, β_i, was calculated from the maximal "instantaneous" pH_i changes recorded when changing from HEPES- to 5% CO_2/10 mM HCO_3^--buffered saline. The CO_2-dependent buffering power, β_{CO2}, was calculated from the intracellular bicarbonate concentration in the oocytes ($\beta_{CO2} = 2.3 * [HCO_3^-]$), and the bicarbonate concentration was obtained from the Henderson-Hasselbalch equation, assuming a $[CO_2]$ of 0.00835 mM in the HEPES-buffered, nominally CO_2-free solution. The total buffer capacity, β_t, was defined as the sum of β_i and β_{CO2}.

Net H^+ flux, J_H (mM/min), defined as the net transport of acid and/or base equivalents across the cell membrane, was calculated as the product of the rate of pH_i change, $\Delta pH_i/t$, and the total buffer capacity, β_t (Becker and Deitmer 2004).

Voltage-Clamp Recording

A borosilicate glass capillary, 1.5 mm in diameter, was pulled to a micropipette and backfilled with 3 M KCl. The resistance of the electrodes measured in oocyte saline was around 1 MΩ. For voltage clamping, the electrode was connected to the head stage of an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA). Oocytes were clamped to a holding potential of −40 mV.

Calculation and Statistics

Statistical values are presented as means ± standard error of the mean (SEM). For calculation of significance in differences, Student's *t*-test or, if possible, a paired *t*-test was used. In the figures, significance levels are indicated as follows: $*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.001$.

Results

Injection of CAII into MCT4-Expressing Oocytes

Transport activity of MCT4 was determined by the rate of change in intracellular H^+ concentration ($\Delta[H^+]_i/t$) during application of 3 and 10 mM lactate at extracellular pH (pH_o) 7.0 in MCT4-expressing oocytes. Addition of lactate induced an intracellular acidification due to uptake of lactate and H^+ via MCT4, which was absent in native oocytes. Injection of 50 ng CAII, but not H_2O, into MCT4-expressing oocytes resulted in an increase in $\Delta[H^+]_i/t$ during application of 3 and 10 mM lactate, even in the nominal absence of CO_2/HCO_3^-, by a factor of 2, indicating a CAII-mediated increase in MCT4 transport activity (Fig. 1a, c). Withdrawal of extracellular lactate led to an intracellular alkalinization induced by the efflux of lactate and H^+ via MCT4 (Fig. 1d). Efflux of H^+ was also increased by a factor of 2 when CAII was injected into MCT4-expressing oocytes (Fig. 1d). Changes in $\Delta[H^+]_i/t$ indicated similar rates of influx and efflux of substrates via MCT4. Catalytic activity of CAII was checked by application of 5% CO_2/10 mM HCO_3^- (Fig. 1a, e). Injection of 50 ng CAII resulted in a ninefold increase in $\Delta[H^+]_i/t$ during application of CO_2/HCO_3^- compared to non-CAII-injected oocytes (Fig. 1e) and a fivefold increase in $\Delta[H^+]_i/t$ during withdrawal of CO_2/HCO_3^- (Fig. 1f). Interestingly, in CAII-injected oocytes, the rate of alkalinization during withdrawal of CO_2/HCO_3^- was only 70% of the rate of acidification measured during the application of CO_2/HCO_3^-, whereas in non-CAII-injected cells, acidification and alkalinization displayed the same time course (Fig. 1e, f). To check whether the CAII-induced change in transport activity was due to a change in the expression level of

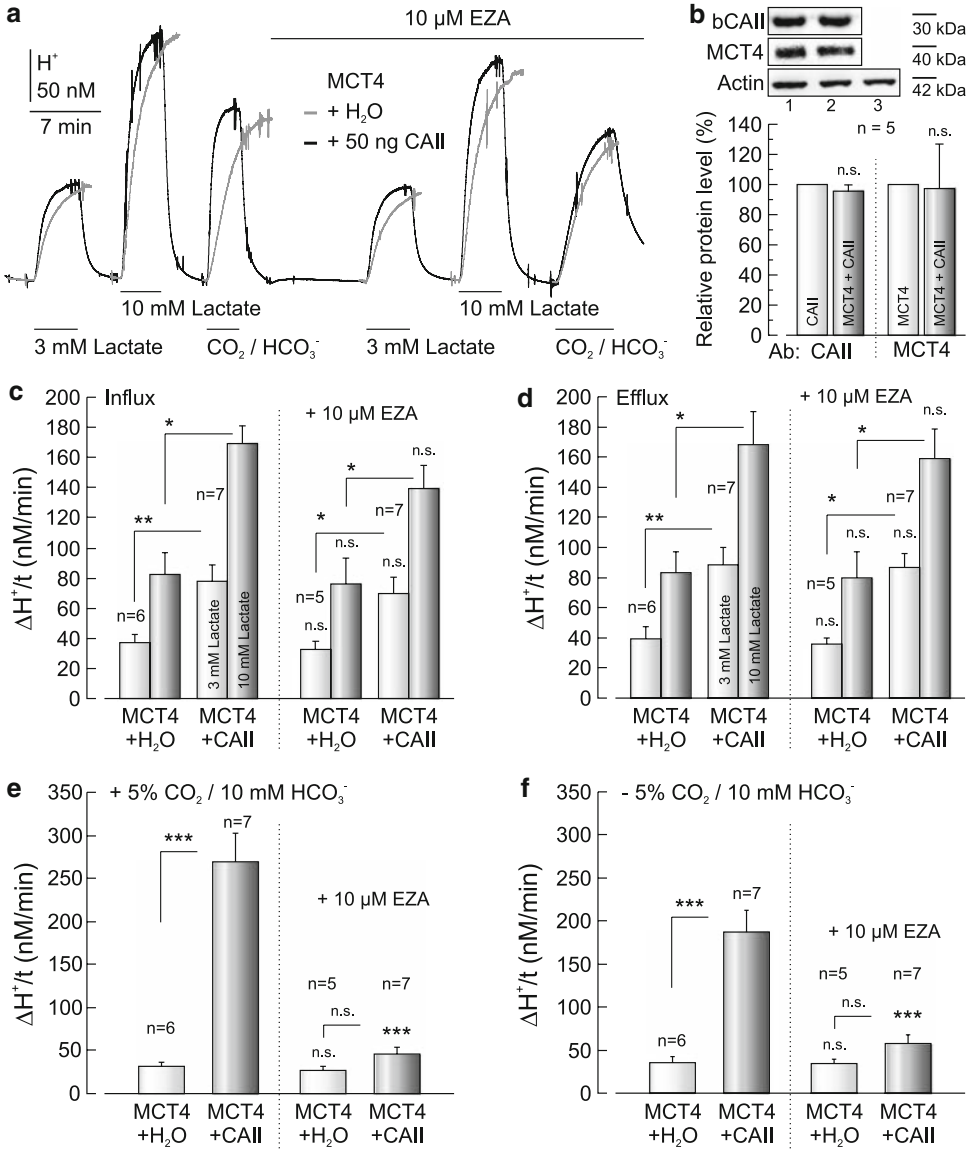

Fig. 1 Transport activity of MCT4, expressed in oocytes with and without injected CAII. (**a**) Original recordings of $[H^+]_i$ in MCT4-expressing oocytes injected with 50 ng of CAII protein (*black traces*) and H_2O (*gray traces*), respectively, during application of 3 and 10 mM lactate and 5% CO_2/10 mM HCO_3^- before and during application of 10 µM EZA. (**b**) Representative Western blot and analysis of relative protein levels of bovine CAII, MCT4 and actin, respectively. *Upper blot* shows antibody (Ab) staining against bovine CAII in lysates of native (*1*) and MCT4-expressing (*2*) oocytes injected with 50 ng of CAII. *Middle blot* shows antibody staining against MCT4 in lysates of MCT4-expressing oocytes either injected with (*1*) or without (*2*) 50 ng of CAII. As a loading control, the *lower blot* shows staining for actin in lysates of native oocytes injected with CAII (*1*) and MCT4-expressing oocytes either injected with (*2*) or without (*3*) CAII. Quantification of relative protein levels revealed no significant changes in the level of CAII (*left part of the diagram*) and MCT4 (*right part of the diagram*). (**c**) Rate of change of H^+ concentration in MCT4-expressing oocytes injected with 50 ng of CAII or H_2O, respectively, as induced by application and withdrawal (**d**) of 3 and 10 mM lactate before and after blocking CAII with 10 µM EZA. (**e**) Rate of change in H^+ concentration in MCT4-expressing oocytes injected with 50 ng of CAII or H_2O, respectively, as induced by application and withdrawal (**f**) of 5% CO_2/10 mM HCO_3^- before and after blocking CAII with 10 µM EZA. *Asterisks* at the bars for EZA refer to the values of the bars before application of EZA. Data are presented as means ± SEM. Significant relationships: * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$

MCT4, the amount of expressed MCT4 was quantified by Western blots (Fig. 1b). Antibody staining of the blots showed a band at around 39 kDa, corresponding to the size of MCT4 (Manning Fox et al. 2000). Native oocytes, not expressing MCT4, produced no bands in the Western blots ($n = 5$, data not shown). Comparison between MCT4-expressing oocytes injected with 50 ng of CAII and MCT4-expressing oocytes without CAII revealed no significant

changes in the relative level of MCT4 protein. To test the accuracy of the method, the protein level of CAII injected in native and MCT4 expressing oocytes was determined the same way as for MCT4 (Fig. 1b). Antibody staining for CAII revealed a band at around 29 kDa, the size of CAII (Vince and Reithmeier 1998), while blotting of native oocytes showed no bands at all ($n = 5$, data not shown). Comparison of the CAII protein level showed no significant changes between MCT4-expressing and native oocytes injected with CAII. After determination of MCT4 and CAII protein level, blots were also labeled for actin to ensure consistent transfer of protein to the blotting membrane (Fig. 1b). Actin was quantified the same way as described for MCT4 and CAII. Comparison of the actin protein level showed no significant difference between MCT4-expressing and native oocytes with and without CAII, respectively ($n = 5$).

For different cell types it has been shown that CA can influence intracellular buffer capacity (Ro and Carson 2004; Vaughan-Jones et al. 2002). Therefore, the buffer capacity of the oocyte cytosol was determined by the CO_2-induced pH_i change (intrinsic buffer capacity β_i) and using the bicarbonate concentration calculated under steady-state conditions (CO_2-dependent buffer capacity, β_{CO2}), the sum of both giving the total buffer capacity, β_t. As seen in Fig. 2a, the buffer capacities remained unaffected by the presence of either CAII or ethoxyzolamide (EZA, 10 µM), indicating that CAII did not contribute to the *global* cytoplasmic buffering of oocytes. This does not exclude, however, as will be discussed later, that CAII might still contribute to the *local* buffer capacity near the cell membrane, where the CAII is located in frog oocytes.

With the buffer capacity and the rate of pH change known, the rate of H^+ flux (J_H) was calculated and plotted for MCT4-expressing oocytes with and without injected CA, in the absence and presence of EZA (Fig. 2b). Since the global oocyte buffer capacity was not affected (see above), the H^+ flux rate reflected a difference between the CAII-injected and the H_2O-injected oocytes, similar to the rate of change in $[H^+]_i$: A twofold increase in H^+ flux rate in CA-injected oocytes was obtained during activation of MCT4 by lactate, both in the presence and in the absence of EZA.

The Role of CAII Catalytic Function in the Support of MCT4 Activity

It has been shown for MCT1 that interaction with CAII does not depend on the catalytic activity of the enzyme (Becker and Deitmer 2008; Becker et al. 2005). To study the dependence of MCT4 transport activity on CAII catalytic function, we either blocked catalytic activity of injected CAII with 10 µM EZA or coexpressed MCT4 with wild-type CAII (CAII-WT) or with a catalytically inactive mutant of CAII (CAII-V143Y).

The CAII-induced augmentation of MCT4 transport activity persisted in the presence of EZA, for both influx and efflux of lactate and H^+ (Fig. 1a, c, d), while catalytic activity of CAII, determined by the rate of intracellular acidification and alkalinization during application and withdrawal of 5% CO_2/10 mM HCO_3^-, was reduced to values measured in oocytes injected with H_2O (Fig. 1e, f).

Coexpression of MCT4 with CAII-WT and CAII-V143Y, respectively, enhanced transport activity, as determined by the rate of rise in $[H^+]_i$ during lactate application (Fig. 3a, b), to a similar extend as observed for injection of 50 ng of CAII, indicating that CAII catalytic activity is not involved in augmentation of MCT4 transport activity. Application of 5% CO_2/10 mM HCO_3^- to oocytes coexpressing MCT4 and CAII-WT resulted in an intracellular acidification, the rate of which was again ninefold higher compared to MCT4-expressing and native oocytes

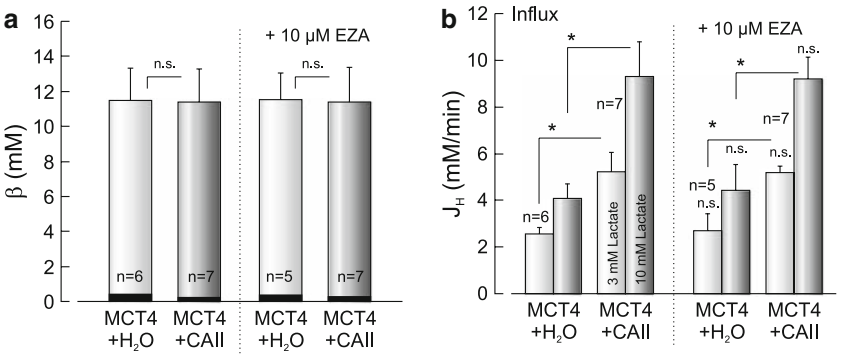

Fig. 2 Buffer capacity and proton flux in MCT4-expressing oocytes with and without CAII. **a** CO_2/HCO_3^--dependent buffer capacity (β_{CO2}, *dark part of the bar*) and intrinsic buffer capacity (β_i, *gray part of the bar*) of MCT4-expressing oocytes injected with 50 ng of CAII or H_2O before and during application of 10 µM EZA as determined by application of 5% CO_2/10 mM HCO_3^-. **b** Proton flux (J_H) in MCT4-expressing oocytes injected with 50 ng of CAII or H_2O, respectively, as induced by application of 3 and 10 mM lactate before and after blocking CAII with 10 µM EZA. Data are presented as means ± SEM. Significant relationships: * $P \leq 0.05$

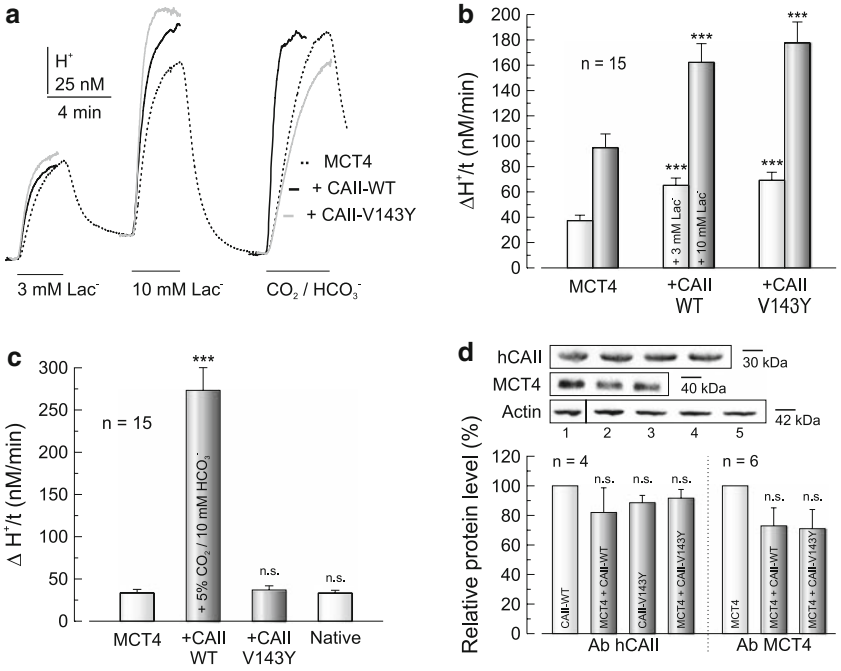

Fig. 3 Coexpression of MCT4 with CAII-WT and CAII-V143Y. **a** Original recordings of $[H^+]_i$ in oocytes expressing MCT4 alone (*dotted trace*), MCT4 + CAII-WT (*black traces*) and MCT4 + CAII-V143Y (*gray traces*) during application of 3 and 10 mM lactate and during application of 5% CO_2/10 mM HCO_3^-. **b** Rate of change in $[H^+]_i$ induced by application of 3 and 10 mM lactate to MCT4, MCT4 + CAII-WT and MCT4 + CAII-V143Y (co-) expressing oocytes. **c** Rate of change in $[H^+]_i$ induced by application of 5% CO_2/10 mM HCO_3^- to MCT4, MCT4 + CAII-WT and MCT4 + CAIIV143Y (co-) expressing and native oocytes. Asterisks on the bars for MCT4 + CAII-WT, MCT4 + CAII-V143Y and native cells refer to the values for oocytes expressing MCT4 alone. **d** Representative Western blot and analysis of relative protein level of expressed CAII, MCT4 and actin. *Upper blot* shows antibody (Ab) staining against human CAII in lysates of oocytes expressing CAII-WT (*2*) or CAII-V143Y (*4*) alone or coexpressing MCT4 and CAII-WT (*1*) or MCT4 and CAII-V143Y (*3*). *Middle blot* shows antibody staining against MCT4 in lysates of oocytes expressing MCT4 alone (*1*) or coexpressing MCT4 and CAII-WT (*2*) or MCT4 and CAII-V143Y (*3*). As a loading control, the *lower blot* shows staining for actin in lysates of oocytes expressing MCT4 alone (*1*) or coexpressing MCT4 and CAII-WT (*2*) or MCT4 and CAII-V143Y (*4*) and oocytes expressing CAII-WT (*3*) or CAII-V143Y (*5*) alone. Quantification of relative protein levels revealed no significant changes in the protein level between CAII-WT and CAII-V143Y either expressed alone or coexpressed with MCT4 (*left part of the diagram*) and of MCT4 either expressed alone or coexpressed with CAII-WT and CAII-V143 V (*right part of the diagram*). Data are presented as means ± SEM. Significant relationships: *** $P \leq 0.001$

(Fig. 3a, c). In contrast to CAII-WT, coexpression of MCT4 with the catalytically inactive CAII-V143Y resulted in no increase in the rate of acidification during application of CO_2/HCO_3^-. Potential differences in the expression level between CAII-WT and CAII-V143Y were determined by Western blots (Fig. 3d). Quantification of expressed CAII protein revealed no significant difference between CAII-WT and CAII-V143Y, expressed in oocytes. Furthermore, coexpression of MCT4 with CAII-WT and CAII-V143Y, respectively, resulted in no significant changes in protein level compared to MCT4 and CAII, respectively, expressed alone (Fig. 3d). In blots of native oocytes, no bands for MCT4 and CAII were observed ($n = 5$, data not shown). As a loading control, blots were also stained for actin (Fig. 3d). Quantification of actin protein levels showed no significant difference between oocytes expressing MCT4 and CAII-WT or CAII-V143Y either alone or together ($n = 4$–6).

As observed for injected CAII protein, coexpression of MCT4 with neither CAII-WT nor CAII-V143Y resulted in a significant change of the oocyte's apparent buffer capacity: MCT4-expressing oocytes had a β_t of 11.5 ± 0.7 mM ($n = 15$), oocytes that coexpressed MCT4 and CAII-WT had a β_t of 11.7 ± 0.5 mM ($n = 15$) and for cells coexpressing MCT4 with CAII-V143Y a β_t of 11.4 ± 0.7 mM ($n = 15$) was calculated.

The Role of the Substrate Gradients for the CAII-Mediated Support of MCT4 Activity

Transport activity of MCTs is determined by the gradients of their substrates, lactate and protons. Therefore, the question of what role these gradients play for the enhancing effect of CAII on transport activity of MCT4 is raised. The dependence of the CAII-induced increase in MCT4 activity on the extracellular H^+ concentration was studied by

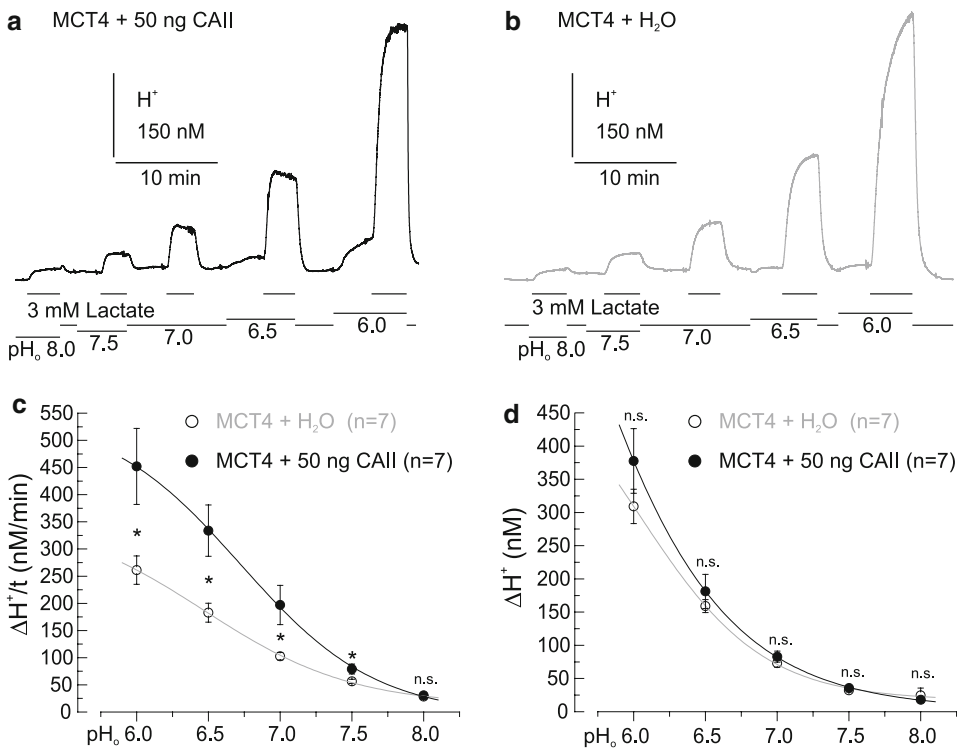

Fig. 4 Dependence of MCT4-mediated transport activity on the proton gradient. Original recordings of $[H^+]_i$ in MCT4-expressing oocytes injected with 50 ng of CAII (**a**) or H_2O (**b**) during application of 3 mM lactate at pH_o 6.0, 6.5, 7.0, 7.5 and 8.0. (**c**) Rate of rise in H^+ concentration in MCT4-expressing oocytes injected with 50 ng of CAII or H_2O during application 3 mM lactate, plotted against pH_o. (**d**) Amplitude of the change in H^+ concentration in MCT4-expressing oocytes injected with 50 ng of CAII or H_2O during application of 3 mM lactate, plotted against pH_o. Data are presented as means ± SEM. Significant relationships: * $P \leq 0.05$

application of 3 mM lactate to MCT4-expressing oocytes injected with either 50 ng of CAII (Fig. 4a) or H_2O (Fig. 4b) at five different pH_o values (6.0, 6.5, 7.0, 7.5 and 8.0). Both the rate of change ($\Delta[H^+]_i/t$) and the absolute change in lactate-induced acidification ($\Delta[H^+]_i$) increased with decreasing pH_o (Fig. 4c, d). $\Delta[H^+]_i$ remained unchanged following injection of CAII, but $\Delta[H^+]_i/t$, indicative of transport activity, increased significantly in MCT4-expressing oocytes injected with CAII, dependent on pH_o, compared to MCT4-expressing oocytes which were not injected with CAII. The data suggest that CAII supports the transport rate of H^+ and, hence, lactate flux but does not affect the total amount of H^+ and lactate transported into the oocytes via MCT4 until equilibrium is reached. The difference in $\Delta[H^+]_i/t$ as induced by the injection of CAII increased with decreasing pH value and was largest at pH_o 6.0. At pH_o 8.0, when the H^+ gradient would not support lactate uptake via MCT4, the effect of CAII on transport activity became negligible. Thus, injection of CAII augments the increase of MCT4 activity at a high proton gradient but has no or little effect on MCT4 activity when there is no gradient for protons across the cell membrane.

The impact of the lactate gradient on the CAII-induced increase in MCT4 activity was investigated by application of 0.1, 0.3, 1, 3, 10 and 30 mM lactate in MCT4-expressing oocytes, injected with either 50 ng of CAII or H_2O at pH_o 7.0 (Fig. 5a). Plotting of $\Delta[H^+]_i/t$ shows a dependence of MCT4 activity on the lactate concentration (Fig. 5b). For all lactate concentrations used, injection of 50 ng of CAII resulted in a significant increase in the rate of H^+ rise; however, this difference between oocytes with and without CAII decreased with increasing lactate concentration, as evident from the relative change in $\Delta[H^+]_i/t$ (Fig. 5c). Using an Eadie-Hofstee plot, a K_m value of 17.4 ± 1.3 mM lactate could be determined for MCT4-expressing oocytes without CAII. Injection of CAII caused a loss of the linear dependence between substrate and substrate affinity. Instead, an increase in substrate affinity could be observed at decreasing substrate concentration. For higher lactate concentrations (3–30 mM), a K_m value of 17.7 ± 1.2 mM, close to the K_m for MCT4-expressing oocytes, could be observed, while application of lactate concentrations lower than 3 mM appeared to result in a substantial increase in substrate affinity (Fig. 5d). Thus, CAII enhanced MCT4 activity more effectively at low lactate concentrations.

Discussion

It has recently been shown that both injection and coexpression of CAII can enhance transport activity of MCT1 expressed in *Xenopus* oocytes (Becker and Deitmer 2008; Becker et al. 2005). This effect is independent of the catalytic activity of the enzyme but appears to require direct binding of CAII to MCT1. In the present study we demonstrate that both injection of CAII and coexpression of wild-type CAII and the catalytically inactive mutant CAII-V143Y increase transport activity of the low-affinity,

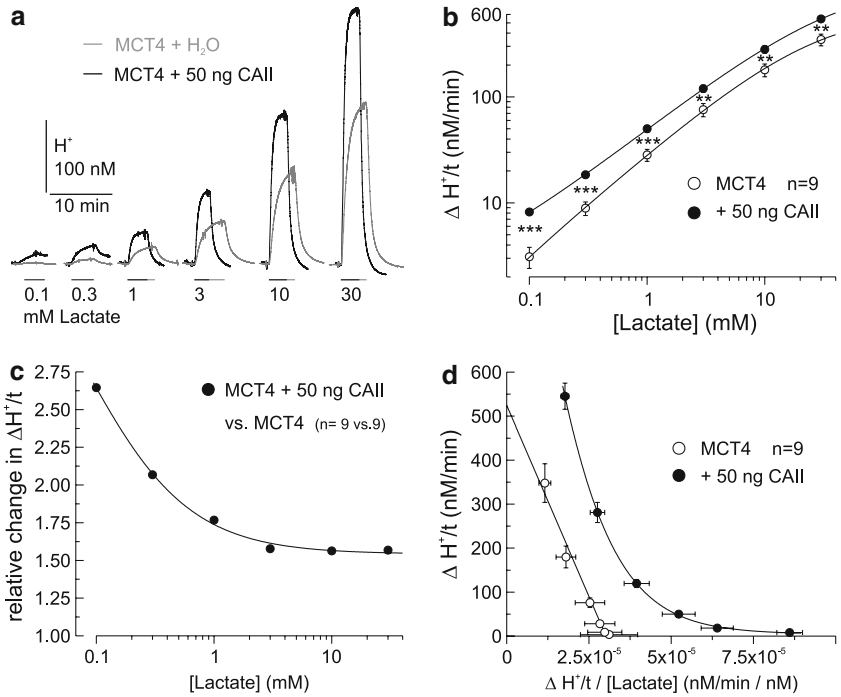

Fig. 5 Dependence of MCT4-mediated transport activity on lactate concentration. **a** Original recordings of $[H^+]_i$ in MCT4-expressing oocytes injected with 50 ng of CAII (*black trace*) or H_2O (*gray trace*) during application of 0.1, 0.3, 1, 3, 10 and 30 mM lactate. **b** Double-logarithmic plot of the rate of rise in H^+ concentration induced by application of lactate in MCT4-expressing oocytes injected with either 50 ng of CAII or H_2O against lactate concentration. **c** CAII-induced increase in the rate of rise in H^+ concentration plotted as a quotient of the values for CAII-injected oocytes and the values for H_2O-injected cells against extracellular lactate concentration. **d** Eadie-Hofstee plot for determination of the MCT4 K_m value for lactate in oocytes injected with either 50 ng of CAII or H_2O. Data are means ± SEM. Significant relationships: ** $P \leq 0.01$ and *** $P \leq 0.001$

high-capacity lactate/pyruvate transporter MCT4, even in the nominal absence of CO_2/HCO_3^- and in the presence of the CA inhibitor EZA. From these results it can be concluded that CAII supports MCT4 transport activity, presumably by removing H^+ from the transporter mouth in a noncatalytic manner, as suggested for MCT1.

CAII-induced augmentation of MCT4 activity displays similar magnitude and kinetics as CAII-induced increase in MCT1 activity: (1) transport activity is increased twofold in the presence of either injected or coexpressed CAII; (2) an increase in extracellular H^+ concentration—leading to an increase in the inwardly directed H^+ gradient—amplifies the effect of CAII; (3) an increase in extracellular lactate concentration reduces CAII-induced augmentation in MCT4 activity. During transport activity of MCT4, protons are expected to accumulate or deplete at the mouth of the transporter, depending on the direction of lactate movement. This would create a local microdomain with either much higher or lower H^+ concentration at the cell membrane, which would reduce the effective H^+ gradient and, hence, reduce MCT4 transport activity in the inward and outward modes, respectively. In a hypothetical model presented recently (Becker and Deitmer 2008), CAII would allocate H^+ along the plasma membrane and thereby increase the area in which H^+ can be released from the membrane into the bulk during lactate uptake or moved from the bulk to the membrane during lactate release. CAII would hence suppress the buildup or depletion of protons within local microdomains at the pore of the transporter. This would then lead to an increased transport activity of MCT4. The increase in MCT4 activity might lead to the depletion of H^+ at the outer mouth of the transporter. In this case, an increase in extracellular H^+ concentration would further increase transport activity and thereby increase the effect of CAII. An increase in extracellular lactate concentration at constant extracellular H^+ concentration, however, would increase overall flux but would also exacerbate the extracellular depletion of H^+ and thereby counteract the effect of intracellular CAII. Thus, the enhancing effect of CAII would be increased at high extracellular H^+ concentrations but would decrease at higher extracellular lactate concentration when depletion of extracellular H^+ limits transport activity.

It has been shown previously that MCTs are bidirectional transporters and responsive to trans-stimulation (Brown and Brooks 1994; Juel and Halestrap 1999); in a recent report we demonstrated that CAII enhances transport activity of MCT1 for both influx and efflux of H^+/

lactate (Becker and Deitmer 2008). Likewise, in the present study a CAII-induced enhancement of H^+ efflux via MCT4 could be observed (data not shown). This suggests that CAII could also attract H^+ and funnel it to MCT4, which would prevent depletion of protons at the side of the transporter and facilitate efflux of H^+ and lactate when the gradients are reversed.

It is noteworthy that the CO_2-dependent buffer capacity was not affected by injection of CA, although H^+ shuttling was greatly accelerated. It has been shown in other cells, e.g., cardiac myocytes (Vaughan-Jones et al. 2002) and cultured oligodendrocytes (Ro and Carson 2004), that membrane-attached CA can create pH microdomains and increase local buffering. In smaller cells (<20 μM), these CA-mediated pH microdomains may significantly affect the global cytosolic buffer capacity. In larger cells, such as *Xenopus* oocytes, however, pH microdomains near the cell membrane created by CA-catalyzed conversion of CO_2 to and from H^+ and HCO_3^- may have little effect on the global cytosolic H^+ buffer capacity, unless soluble, freely diffusing CA increases the diffusion of acid/base equivalents. CA-coupled metabolons of acid/base transporters may increase pH differentials at the cell membrane, which likely creates gradients of H^+ and HCO_3^- concentrations and, hence, changes buffer capacity. As discussed by Ro and Carson (2004), these gradients would be influenced by the partition coefficient between freely diffusing and metabolon-associated CA.

Another explanation for a CAII-induced increase in MCT4 activity might be that CAII acts as a chaperone for MCT4, which increases the overall amount of MCT4 in the oocyte membrane. Nevertheless, as MCT4 protein level does not change due to injection or coexpression of CAII, this possibility seems unlikely.

Potential Implications for the Cell-to-Cell Lactate Shuttle

In some tissues, like muscle and brain, different cell types express different MCT isoforms, indicating an intercellular distribution and compartmentalization of lactate/pyruvate metabolism. For these tissues it has been proposed that lactate could be transferred from glycolytic, lactate-producing cell types to oxidative, lactate-consuming cells, a mechanism referred to as the "cell-to-cell lactate shuttle" (Brooks 1985; Gladden 2004).

In skeletal muscle, lactate is continuously produced even under aerobic conditions (Dubouchaud et al. 2000; Halestrap and Price 1999; Juel 2001; Richardson et al. 1998). Although glycolysis and glycogenolysis also appear in oxidative type I fibers (Halestrap and Meredith 2004), lactate production takes place mainly in glycolytic white muscle fibers, containing few mitochondria (Halestrap and Price 1999). Lactate is exported from white muscle fibers primarily via the high-capacity isoform MCT4 and is taken up and oxidized by red muscle fibers, which express large amounts of MCT1 but also MCT4 and, exclusively, CAII (Dubouchaud et al. 2000; Hashimoto et al. 2005; Jeffery et al. 1986; Juel 2001; Juel and Halestrap 1999). The expression patterns of MCT1, MCT4 and CAII suggest that during moderate activity CAII might increase lactate uptake into oxidative red muscle fibers via MCT1 and MCT4, while lactate efflux from glycolytic fibers via MCT4 remains unaffected (Fig. 6a).

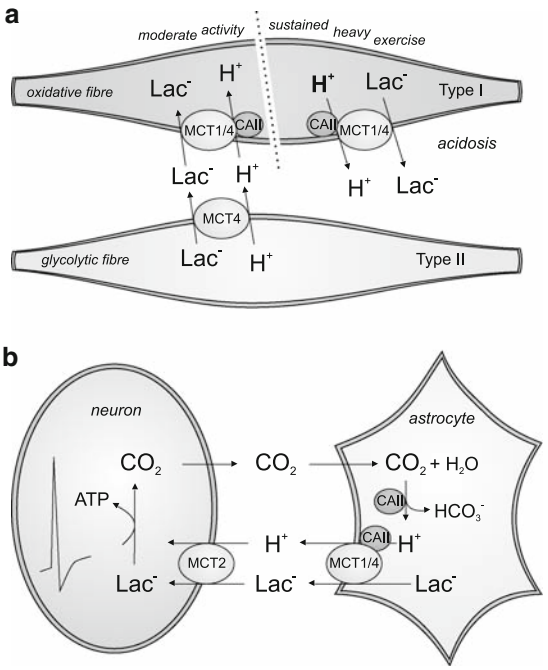

Fig. 6 Hypothetical model of lactate fluxes in muscle and brain as supported by carbonic anhydrase. **a** In skeletal muscle, lactate is primarily formed in white, glycolytic muscle fibers, from where it is exported via MCT4 and taken up by red oxidative fibers via MCT1 and MCT4. During sustained heavy exercise, lactate and protons also build up in red muscle fibers. From there they are removed via MCT1 and MCT4, supported by the enzyme CAII. **b** In the brain, active neurons release CO_2, which diffuses into astrocytes, where it is hydrolyzed, catalyzed by CAII. The proton, produced during hydrolysis, is then shuttled to glial MCT1 and MCT4 via CAII and transported out of the astrocytes, together with lactate, into the extracellular space, where it is taken up by the neurons via MCT2

Intense muscle activity is associated with a rapid drop in muscular pH (Bishop et al. 2007; Messonnier et al. 2007). Recent studies reported a pH change from 7.1 at rest to 6.8 immediately following a 45-s cycle test in humans (Bishop et al. 2007). Thus, during intense physical exercise, there is a large need for removal of H^+ from muscle, which is largely mediated by MCTs but is also attributed to the activity of cytosolic and membrane-bound CA (Juel et al. 2003; Westerblad and Allen 1992). It has recently been shown that increased expression of CAII results in

decreased accumulation of muscle lactic acid in vivo during supramaximal exercise in humans and in an increase in work capacity (Messonnier et al. 2007). Together with our findings that CAII increases lactate transport rate via MCT1 and MCT4, these results suggest that coexpression of CAII with MCT1 and MCT4 would increase export of H^+ and lactate from red muscle fibers during intense muscular activity, thereby enhancing the efficacy of the muscle to counteract lactacidosis and to use the excess, energy-rich lactate by supporting the reshuttling of lactate between different types of muscle fibers (Fig. 6a).

In the brain, CAII is prominently expressed in glial cells, in particular in oligodendrocytes and astrocytes, which play a crucial role in the energy supply of neurons. Lactate is believed to be transported out of glial cells via MCT1 and MCT4 into the extracellular space, where it is taken up by neurons via MCT2 (Hashimoto et al. 2008; Pellerin et al. 2005, 2007; Rafiki et al. 2003). Furthermore, astrocytes influence the size and spread of extracellular pH shifts accompanying neuronal activity (Deitmer and Szatkowski 1990; Grichtchenko and Chesler 1994; Pellerin et al. 2007). Extracellular pH shifts are shaped by the activity of the glial Na^+/HCO_3^- cotransporter (NBC) and extracellular CAIV but also depend on the transport function of acid/base-coupled metabolite transporters like the MCTs (Bevensee et al. 1997; Erlichman et al. 2008; Tong et al. 2000). With its direct influence on MCT1 and MCT4 transport activity, CAII would link glial lactate secretion to changes in extracellular pH and thereby to changes of neuronal activity (Fig. 6b): CO_2, released by active neurons, enters astrocytes, where its hydration, catalyzed by carbonic anhydrases, results in the formation of bicarbonate and protons. HCO_3^- is transported out of the astrocytes via the NBC, providing additional extracellular buffer capacity, while the protons left in the cells induce an intracellular acidification (Deitmer 1991). The increase in intraglial proton concentration might then drive efflux of H^+ and lactate via MCT1 and MCT4. As CAII supports MCT transport activity, presumably by shuttling H^+ along the plasma membrane to the transporter pore (Becker and Deitmer 2008), it might be speculated that H^+, formed by the hydration of CO_2 in the vicinity of the plasma membrane, might be shuttled to the MCT via CAII, driving the export of H^+ and lactate. By this mechanism, CAII would directly couple CO_2 uptake by astrocytes to lactate release. Due to the cell-specific expression of CAII in the brain, we hypothesize that the expression of CAII may enhance those MCT isoforms which predominantly release lactate, as is believed for MCT1 and MCT4 in astrocytes, and less so, or not at all, for MCT isoforms which primarily take up lactate, such as MCT2 in neurons. Nevertheless, it should be noted that neurons not only take up glia-derived lactate but also use glucose and systemic lactate taken up from the blood, when arterial lactate concentration rises during insulin-induced hypoglycemia (Lubow et al. 2006) or high-intensity exercise (Ide et al. 2000; van Hall et al. 2009).

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