Intracellular and Extracellular Carbonic Anhydrases Cooperate Non-enzymatically to Enhance Activity of Monocarboxylate Transporters

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Background: Monocarboxylate transporters (MCTs) mediate the shuttling of high-energy metabolites between different cells.

Results: Transport activity of MCTs is augmented by intracellular and extracellular carbonic anhydrases (CAs).

Conclusion: Intracellular and extracellular CAs can work in concert to ensure rapid shuttling of metabolites across the cell membrane.

Significance: CAs play a pivotal role as regulators of metabolite transport in different tissues.

Proton-coupled monocarboxylate transporters (MCTs) are carriers of high-energy metabolites such as lactate, pyruvate, and ketone bodies and are expressed in most tissues. It has previously been shown that transport activity of MCT1 and MCT4 is enhanced by the cytosolic carbonic anhydrase II (CAII) independent of its catalytic activity. We have now studied the influence of the extracellular, membrane-bound CAIV on transport activity of MCT1/4, heterologously expressed in Xenopus oocytes. Coexpression of CAIV with MCT1 and MCT4 resulted in a significant increase in MCT transport activity, even in the nominal absence of CO₂/HCO₃⁻. CAIV-mediated augmentation of MCT activity was independent of the CAIV catalytic function, since application of the CA-inhibitor ethoxyzolamide or coexpression of the catalytically inactive mutant CAIV-V165Y did not suppress CAIV-mediated augmentation of MCT transport activity. The interaction required CAIV at the extracellular surface, since injection of CAIV protein into the oocyte cytosol did not augment MCT transport function. The effects of cytosolic CAII (injected as protein) and extracellular CAIV (expressed) on MCT transport activity, were additive. Our results suggest that intra- and extracellular carbonic anhydrases can work in concert to ensure rapid shuttling of metabolites across the cell membrane.

Monocarboxylate transporters (MCT, SLC16)² are carriers of high-energy metabolites, such as lactate, pyruvate, and ketone bodies. The SLC16 gene family comprises 14 isoforms, the first four of which (MCT isoforms 1–4) have been reported to transport monocarboxylates in an electroneutral transport mode of 1 H⁺ : 1 monocarboxylate with different substrate affinities (1). MCT1, which is found in nearly all tissues studied so far, has a $K_m$ value of 3–5 mM for L-lactate (2, 3). MCT4 is a low-affinity, high-capacity carrier with a $K_m$ value for lactate of 17–35 mM (4), and is found prominently in glycolytic tissues such as e.g. white skeletal muscle fibers and astrocytes (1, 5, 6). 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, while MCT1 can both serve as a lactate importer and exporter. MCT1 and MCT4 require the ancillary protein CD147 (basigin, EMMPRIN), an analog of which is intrinsically expressed in Xenopus oocytes, for proper expression in the plasma membrane and hence transport activity (7–9).

Mammalian carbonic anhydrases (CA) included in the α-class of CAs, of which 16 isoforms are identified, catalyze the reversible hydration of CO₂ to HCO₃⁻ and H⁺ (10, 11). CAII is found in the cytosol, while CAIV is linked to the extracellular surface of the cell membrane via a glycosyl-phosphatidyl-inositol (GPI) anchor (12, 13). Both the intracellular isoform CAII and the extracellular isoform CAIV have been found to interact with different acid/base transporting proteins: In vitro studies and experiments using heterologous protein expression revealed that CAII binds to, and enhances the activity of, the chloride/bicarbonate exchanger AE1 (14, 15), the sodium-bicarbonate cotransporter NBCe1 (16, 17), the sodium/hydrogen exchanger NHE1 (18, 19) and the monocarboxylate transporters MCT1 and MCT4 (20–24). Extracellular CAIV has been shown to interact with NBCe1 (15, 25), AE1 (15, 26) and MCT2 (27). (For review of the various types of transport metabolons see Refs. 28–30.)

We have recently shown that CAII can enhance transport activity of MCT1 and MCT4, when heterologously expressed, or injected as protein, in Xenopus oocytes, in a non-catalytic manner, and have proposed that CAII acts as a so called “proton-collecting antenna” for the transporter, presumably by dissipation intracellular proton microdomains via an intramolecu-
ular proton shuttle (20–24). Proton shuttling requires close proximity between transporter and enzyme, which is achieved for MCT1 and CAII by binding of CAII to the acidic cluster E489EE in the C-terminal tail of MCT1 (31). In contrast to MCT1 and MCT4, CAII failed to alter transport activity of the high-affinity monocarboxylate transporter MCT2 (27), possibly because it lacks the appropriate binding domain.

In the present study, we have tested whether extracellular CAII can increase transport activity of MCT1 and MCT4 when heterologously coexpressed in Xenopus oocytes, and whether intracellular CAII and extracellular CAIV act independently and can cooperate to drive MCT1 and 4 transport activity. Our results show that transport activity of MCT1 and MCT4 is enhanced by CAII in a non-catalytic manner, and that additional injection of CAII protein into MCT1/4 + CAIV-coexpressing oocytes increased MCT activity even further. This suggests that intra- and extracellular CA isoforms can functionally cooperate to enhance transport activity of the acid/base-coupled metabolite carriers MCT1 and MCT4.

**EXPERIMENTAL PROCEDURES**

**Constructs, Oocytes, and Injection of cRNA and Protein—**Human CAI-VWT and the CAIV mutant V165Y were provided by Dr. William S. Sly, St. Louis and subcloned into the oocyte expression vector pGEM-He-Juel, which contains the 5’ and the 3’ untranscribed regions of the Xenopus β-globin flanking the multiple cloning site. cDNA coding for rat MCT1 and rat MCT4, cloned into the oocyte expression vector pGEM-He-Juel, was kindly provided by Dr. Stefan Bröer, Canberra (3, 4). Plasmid DNA was transcribed in vitro with T7 RNA-Polymerase (mMessage mMACHINE, Ambion Inc., Austin) as described earlier (32). 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/liter of 3-amino-benzoic acid ethylester (MS-222, Sigma-Aldrich, Taufkirchen, Germany), and rendered hypothermic. The procedure was approved by the Landesuntersuchungsamt Rheinland-Pfalz, Koblenz (23 177- 07/A07-2-003 §6). As described earlier (32), oocytes were singularized by collagenase (Collagenase A, Roche, Mannheim, Germany) treatment in CaCl\textsuperscript{2+}-free oocyte saline (pH 7.8) at 28 °C for 2 h. The singularized oocytes were left overnight in an incubator at 18 °C in Ca\textsuperscript{2+}-containing oocyte saline (pH 7.8) to recover. Oocytes of the stages V and VI were injected with 5 ng of cRNA coding for MCT1 or MCT4, either together with 2 ng of cRNA coding for CAIV or alone. Measurements were carried out 3 to 6 days after injection of cRNA. CAII and CAIV were also directly injected as protein. For injection of protein, 50 ng of CAII, isolated from human erythrocytes, or 35 ng of CAIV, isolated from secretion medium of CHO cells expressing recombinant proteins (kindly provided by Dr. William S. Sly, St. Louis), dissolved in 27.6 nl of DEPC-H\textsubscript{2}O, were injected 12–24 h before electrophysiological measurements. Isolation of CAII and CAIV from human erythrocytes and from secretion medium of CAIV-expressing CHO cells, respectively, as well as purification of the proteins with CA inhibitor columns has been described in detail previously (33–35).

The oocyte saline had the following composition (in mM): NaCl, 82.5; KCl, 2.5; CaCl\textsubscript{2}, 1; MgCl\textsubscript{2}, 1; Na\textsubscript{2}HPO\textsubscript{4}, 1; HEPES, 5; titrated with NaOH to pH 7.0. In lactate-containing saline, NaCl was replaced by an equivalent amount of Na\textsubscript{1}-lactate. In the bicarbonate-containing saline, NaCl was replaced by an equivalent amount of NaHCO\textsubscript{3}, and the solution was aerated with 5% CO\textsubscript{2}/95% O\textsubscript{2}. Application of lactate was carried out in HEPES-buffered solution at pH 7.0 in the nominal absence of CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}, containing around 0.008 mM of CO\textsubscript{2} from air and hence a HCO\textsubscript{3}\textsuperscript{-} concentration of less than 0.2 mM and in 5% CO\textsubscript{2}/10 mM HCO\textsubscript{3}--buffered solution (pH 7.0), respectively.

**Immunohistochemical Analysis of MCT1, MCT4, and CAIV—**Xenopus oocytes, expressing MCT1 and MCT4, respectively, either alone or together with CAIV, as well as native control oocytes were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; Roti-Histofix 4%, Roth, Karlsruhe, Germany) 4 days after cRNA injection. Oocytes were treated with 100% methanol and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich). Unspecific binding sites were blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich), 1% normal goat serum (NGS; Sigma-Aldrich) or 1% normal donkey serum (Sigma-Aldrich), depending on the origin of the secondary antibody. Intact oocytes were incubated in PBS containing the primary antibody against MCT1 (1:200; chicken anti-rat MCT1 polyclonal antibody (AB1286), Millipore, Temecula), MCT4 (1:300; rabbit anti-rat MCT4 polyclonal antibody (AB3314P), Millipore, Temecula) or CAIV (1:25 mouse anti-human CAIV monoclonal antibody (MAB2186), R&D Systems GmbH, Wiesbaden-Norderstedt, Germany) overnight at 4 °C. After washing, oocytes were incubated in PBS with the secondary antibody (Alexa Fluor 546 donkey anti-mouse IgG (A10036), Alexa Fluor 488 donkey anti-rabbit IgG (A-10040) or Alexa Fluor 488 goat anti-chicken IgG (A-11039), Invitrogen GmbH, Darmstadt) at a 1:100 dilution. Oocytes were then analyzed with a confocal laser-scanning microscope (LSM 700, Carl Zeiss GmbH, Oberkochen, Germany).

**Western Blot Analysis**—For comparison of protein levels of expressed MCT1, MCT4, and CAIV, Western blot analyses were performed. For each sample 20 oocytes were lysed by sonication in 2% sodium dodecylsulfate solution with protease inhibitor (Complete Mini EDTA-free, Roche, Mannheim, Germany) 4 days after injection of cRNA. Total protein content was determined using BCA protein assay kit (Pierce, Fisher Scientific GmbH, Schwerte, Germany). Extracts were separated by 4–12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Proteins of interest were detected by antibody staining, using the following antibodies: MCT1 (1:200; chicken anti-rat MCT1 polyclonal antibody (AB1286), Millipore, Temecula), MCT4 (1:250; rabbit anti-rat MCT4 polyclonal antibody (AB3314P), Millipore) or CAIV (1:300 mouse anti-human CAIV monoclonal antibody (MAB2186), R&D Systems GmbH, Wiesbaden-Norderstedt, Germany). As a loading control, β-tubulin was labeled with anti-β-tubulin mouse monoclonal antibody (diluted 1:4000; Sigma Aldrich). Primary antibodies were labeled with goat anti-mouse, goat anti-rabbit or rabbit anti-chicken IgG horseradish peroxidase-conjugated secondary antibody (diluted 1:4000; Santa Cruz Biotechnology Inc.). Membranes were analyzed
after incubation with Lumi-Light Western blotting substrate (Roche) with a Versa Doc imaging system (Bio-Rad). Quantification of MCT1 and MCT4 protein was carried out with the software Quantity One 4.5 (Bio-Rad). For each protein band the pixel density per square millimeter was calculated and 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.

**Determination of Intra- and Extracellular CA Activity by Mass Spectrometry**—Intra- and extracellular activity of CA was determined by monitoring the $^{18}$O depletion of doubly labeled $^{13}$C$^{18}$O$^{16}$O through several hydration and dehydration steps of CO$_2$ and HCO$_3^-$ at 25 °C in intact and lysed oocytes, respectively (36–38). The reaction sequence of $^{18}$O loss from $^{13}$C$^{18}$O$^{15}$O ($m/z = 49$) over the intermediate product $^{13}$C$^{18}$O$^{16}$O ($m/z = 47$) and the end product $^{13}$C$^{16}$O$^{16}$O ($m/z = 45$) was monitored with a quadrupole mass spectrometer (OmniStar GSD 320; Pfeiffer Vacumm, Asslar, Germany). The relative $^{18}$O enrichment was calculated from the measured 45, 47, and 49 abundance as a function of time according to: log enrichment $= \log [49 \times 100/(49 + 47 + 45)]$. For the calculation of CA activity, the rate of $^{18}$O degradations was obtained from the linear slope of the log enrichment over the time, using the spreadsheet analyzing software OriginPro 8.6 (OriginLab Corporation, Northampton). For the experiments, the cuvette was filled with 6 ml of oocyte saline, pH 7.4. After addition of $^{13}$C$^{18}$O$_2$ the spontaneous degradation was measured for 5 min. For determination of extracellular CA activity batches of 100 CAIV-WT-expressing oocytes were added to the cuvette and the catalyzed degradation was determined for 10 min. Batches of 100 native oocytes were used as control. To determine total CA activity batches of 20 oocytes expressing CAIV and native oocytes, respectively, were lysed in 80 µl of oocyte saline and pipetted into the cuvette. To obtain the catalytic activity mediated by CA, the values of native oocytes were subtracted from the total catalytic activity of both intact and lysed oocytes expressing CAIV. The system was calibrated by determining the catalytic activity of 50, 100, 200, and 300 ng of isolated CAIV protein directly added to the cuvette. Based on this calibration total extra- and intracellular CAIV concentrations were calculated from the catalytic activity measured in intact and lysed CAIV-expressing oocytes.

**Intracellular pH Measurements**—Changes in intracellular proton concentration in oocytes were determined with ion-sensitive microelectrodes under voltage-clamp conditions. For measurement of intracellular pH and membrane potential, double-barreled microelectrodes were used; the manufacture and application have been described in detail previously (32, 39). 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. For two electrode voltage clamp a borosilicate glass capillary, 1.5 mm in diameter, was pulled to a micropipette and backfilled with 3 M KCl. This electrode was used for current injection and was connected to the head-stage of an Axoclamp 2A amplifier (Axon Instruments). The actual membrane voltage was recorded by the reference barrel of the double-barreled pH-sensitive microelectrode. Oocytes were clamped to a holding potential of $-40$ mV.

As described previously (3), optimal intracellular pH changes were detected when the ion-selective electrode was located near the inner surface of the plasma membrane. 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 Germany GmbH, München, Germany) and were 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 e.g. measured in HEPES- and CO$_2$/HCO$_3^-$-buffered salines (32). The rate of change of the measured [H$^+$]$_i$, was analyzed by determining the slope of a linear regression fit using the spreadsheet program OriginPro 8.6.

**Calculation of Buffer Capacity (β) and Proton Fluxes (J_H)**—Intrinsic buffer capacity $\beta_i$, was calculated from the change in pH$_i$ (amplitude) when changing from HEPES- to 5% CO$_2$/10 mm HCO$_3^-$-buffered saline. The CO$_2$-dependent buffer capacity $\beta_{CO2}$, was calculated from the intracellular bicarbonate concentration ($\beta_{CO2} = 2.3 \times [HCO_3^-]$), which was obtained from the Henderson-Hasselbalch equation, assuming a [CO$_2$]$_i$ of 1.33 mm in a solution aerated with 5% CO$_2$. The total buffer capacity, $\beta_i$, was defined as the sum of $\beta_i$ and $\beta_{CO2}$.

Net H$^+$ fluxes $J_H$ (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 change in intracellular pH and the total buffer capacity $\beta_i$ for lactate application in the presence of CO$_2$/HCO$_3^-$. For application of lactate in the nominal absence of CO$_2$/HCO$_3^-$, $J_H$ was calculated using the intrinsic buffer capacity $\beta_i$ (32).

**Statistics**—Statistical values are presented as means ± S.E. of the mean. For calculation of significance in differences, Student’s $t$ test or, if possible, a paired $t$ test was used. In the figures shown, a significance level of $p \leq 0.05$ is marked with *, $p \leq 0.01$ with ** and $p \leq 0.001$ with ***.

**RESULTS**

**Expression of Extracellular CAIV Alters Transport Activity of MCT1 and MCT4 in Xenopus Oocytes in a Non-catalytic Manner**

We have previously shown that expression of extracellular, membrane-bound CAIV can enhance transport activity of the high-affinity monocarboxylate transporter MCT2 by a non-catalytic interaction. To investigate whether CAIV does augment transport activity of the monocarboxylate transporters MCT1 and MCT4, we coexpressed CAIV together with MCT1 and MCT4, respectively. Transport activity of MCT was determined by application of 3 and 10 mM lactate in the nominal absence and in the presence of 5% CO$_2$/10 mM HCO$_3^-$ (pH 7.0) before and during application of 10 µM of the CA inhibitor 6-ethoxy-2-benzothiazolesulfonamide (EZA), as shown for MCT1 in Fig. 1A. Both in the absence and in the presence of CO$_2$/HCO$_3^-$, coexpression of MCT1 with CAIV led to a ~2-fold increase in MCT1 activity as determined from the rate
of lactate-induced acidification ($\Delta[H^+] / \Delta t$) (Fig. 1, B and C). In both cases, the CAIV-induced increase in MCT1 activity was insensitive to the inhibition of CAIV catalytic activity by EZA (Fig. 1, A–C). Inhibition of CAIV catalytic activity by EZA was confirmed by application of CO$_2$/HCO$_3$ (Fig. 1A): The rate of CO$_2$-induced acidification was increased by a factor of 7 with
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257.0 ± 19.7 nm/min in MCT1+CAIV-expressing oocytes as compared with 36.8 ± 3.5 nm/min in non-CA-expressing cells (p < 0.001). In the presence of 10 μM EZA, the rate of acidification significantly decreased to 64.35 ± 4.5 nm/min in MCT1+CAIV-expressing oocytes (p < 0.001), while no difference was observed in non-CA-expressing cells (35.5 ± 4.1 nm/min; data not shown). Expression of CAIV not only increased the rate of lactate-induced acidification, but also doubled the rate of change in intracellular alkalinization during removal of lactate in the absence and presence of CO2/HCO3 and EZA (Fig. 1, D and E), which indicates that CAIV enhances MCT activity in both directions, influx and efflux of lactate.

To confirm that CAIV-induced augmentation in MCT transport activity is independent from CAIV catalytic activity, we coexpressed MCT1 with the catalytically inactive CAIV mutant CAIV-V165Y. Coexpression of MCT1 with CAIV-V165Y resulted in virtually the same rate of lactate-induced acidification as did coexpression of MCT1 with CAIV-WT (Fig. 1F); however, the rate of acidification induced by application of 5% CO2/10 mM HCO3 confirmed the lack of catalytic activity of CAIV-V165Y in contrast to CAIV-WT (Fig. 1G).

It has been shown for different cells that catalytic activity of carbonic anhydrase can influence intracellular buffer capacity (40, 41). Therefore we determined the intrinsic (β) and CO2/HCO3-dependent buffer capacity (βCO2) of the oocyte cytosol with and without CAIV. The total buffer capacity (β) was calculated from the sum of βi and βCO2 (see “Experimental Procedures”). As seen in Fig. 2A, the buffer capacity remained unaffected by CAIV both in the absence and presence of EZA, indicating that CAIV does not contribute to global cytoplasmic proton buffering of oocytes. However, this does not exclude that CAIV might still contribute to the local buffer capacity at the extracellular surface of the cell membrane, where CAIV is colocalized with MCT1 and MCT4, respectively (see Fig. 4, A and D).

With the buffer capacity known, the rate of H+ flux (JH) can be calculated from the rate of change in intracellular pH. Since the global oocyte buffer capacity was not affected (Fig. 2A), the rate of H+ flux reflected a difference between MCT1 and MCT1+CAIV-expressing oocytes that was similar to the rate of change in intracellular H+ concentration (Fig. 2, B and C): Both in the nominal absence (Fig. 2B) and in the presence of CO2/HCO3 (Fig. 2C), CAIV led to ~2-fold increase in JH. Under both conditions this augmentation in transport activity was not altered by the application of EZA, indicating that CAIV catalytic activity is dispensable for MCT transport function. Interestingly, only minor differences in JH could be observed between lactate application in CO2/HCO3-buffered solution and in the nominal absence of CO2/HCO3, although intracellular buffering power doubled in the presence of CO2/HCO3 (βi versus β). A possible explanation for this effect is that the increase in intracellular buffering power makes up for the decrease in the proton gradient due to the CO2-induced decrease in intracellular pH from 7.3 in HEPES-buffered to 6.8 in CO2/HCO3-buffered solution.

Determination of CAIV Expression and Activity—We have shown previously that CAIV can display not only extracellular, but also robust intracellular catalytic activity in CAIV-expressing...
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FIGURE 3. Transport activity of MCT1 and MCT4 is enhanced by coexpression of CAIV, but not by injection of CAIV protein. A, original recordings of [H\(^+\)], in Xenopus oocytes either expressing MCT4 (black trace) or coexpressing MCT4 + CAIV (blue trace) and MCT4-expressing oocytes, additionally injected with 35 ng of mature CAIV protein (green trace) during application of 3 and 10 mM lactate and 5% CO\(_2\)/10 mM HCO\(_3\)\(^-\) in the absence and presence of 30 \(\mu\)M EZA. B, rate of change in [H\(^+\)], induced by application of lactate in oocytes expressing MCT4 or coexpressing MCT4 + CAIV and MCT4-expressing oocytes, additionally injected with CAIV protein in the absence and presence of EZA. C, rate of change in [H\(^+\)], induced by application of 5% CO\(_2\)/10 mM HCO\(_3\)\(^-\) in oocytes expressing MCT4 or coexpressing MCT4 + CAIV and MCT4-expressing oocytes, additionally injected with CAIV protein in the absence and presence of EZA. D, rate of change in [H\(^+\)], induced by application of lactate in oocytes expressing MCT1 or coexpressing MCT1 + CAIV and MCT1-expressing oocytes, additionally injected with CAIV. Asterisks at the bars for MCT1 + CAIV-coexpressing oocytes refer to the values of MCT1-expressing cells. E, amount of extracellular (blue bars) and intracellular (orange bars) localized CAIV in oocytes injected with 0.5, 1, 3, and 6 ng of CAIV-coding cRNA, as determined by mass-spectrometric analysis. The percent values indicate the relative amount of extracellular CAIV.

ing Xenopus oocytes and in mouse neurons in situ (38). Therefore we determined the expression level of intra- and extracellular CAIV in Xenopus oocytes at different amounts of injected cRNA. Although the level of expressed CAIV increased with increasing amounts of injected cRNA, the relative distribution of CAIV consistently showed ~25% extracellular and ~75% intracellular CAIV (Fig. 3E). To investigate whether the CAIV-mediated augmentation in MCT transport activity is mediated by intra- or extracellular CAIV, we either coexpressed MCT4 with CAIV, yielding both intra- and extracellular CAIV, or directly injected 35 ng of purified CAIV protein 1 day before the measurements. Since the mature protein lacks the GPI anchor and presumably cannot be incorporated into the cell membrane, injection of CAIV protein will only result in intracellular, but no extracellular CAIV.

Catalytic activity of cytosolic CAIV was monitored by the rate of intracellular acidification induced by 5% CO\(_2\)/10 mM HCO\(_3\)\(^-\) (Fig. 3, A and C). Since CA increases the rate of conversion of CO\(_2\) entering the oocyte, to HCO\(_3\)\(^-\) and H\(^+\), the rate of the CO\(_2\)-induced acidification can be taken as a direct measure for intracellular CA activity. Both injection of cRNA coding for CAIV and injection of mature CAIV protein lead to a significant increase in intracellular CA activity, which can be inhibited by EZA (Fig. 3C).
Transport activity of MCT4 was determined by application of 3 and 10 mM lactate in the nominal absence of CO$_2$/HCO$_3^-$, before and during application of 30 μM EZA (Fig. 3A). Like transport activity of MCT1, activity of MCT4 was significantly increased by coexpression of CAIV, both in the absence and presence of EZA. However, no increase in MCT4 transport activity could be observed when purified CAIV protein was injected into MCT4-expressing oocytes instead of coexpressing CAIV by injection of cRNA (Fig. 3B). The same observation could be made when CAIV protein was injected into oocytes expressing MCT1 (Fig. 3D). Therefore, it can be concluded that CAIV-mediated augmentation of MCT transport activity requires the enzyme to be localized at the extracellular surface of the plasma membrane.

**Determination of Expression Levels in Oocytes**—Localization of MCT1, MCT4, and CAIV at the oocyte plasma membrane was shown by antibody staining of intact oocytes (Fig. 4, A and D). To make sure that the observed CAIV-mediated increase in MCT transport activity is not due to an increase in the expression level of the transporters, we performed quantitative Western blot analysis of MCT1, MCT4, and CAIV protein in oocytes: Coexpression of CAIV did not induce an up-regulation of MCT expression, but led to a significant decrease in the expression level of MCT1 by 26% (Fig. 4, B and C) and MCT4 by 22% (Fig. 4, E and F). Therefore, the observed increase in the rate of proton/lactate cotransport is attributable to a CAIV-mediated increase in MCT transport function, which seems to be underestimated by up to 25%, rather than an increase in the expression level of transport protein when coexpressed with CAIV.

**Cytosolic CAII and Membrane-bound CAIV Can Functionally Cooperate**—Since both CAII and CAIV enhance transport activity of MCT1 and MCT4, respectively, we asked, whether CAII and CAIV can cooperate to further enhance MCT transport function. Therefore we expressed MCT1 in oocytes and either injected 50 ng of CAII protein, coexpressed MCT1 with CAIV, or injected 50 ng of CAII into oocytes coexpressing MCT1 with CAIV. Transport activity of MCT1 was again determined by application of 3 and 10 mM lactate in the nominal absence of CO$_2$/HCO$_3^-$ (Fig. 5A), while catalytic activity of CA was monitored by a pulse of 5% CO$_2$/10 mM HCO$_3^-$ (Fig. 5, B, C, E). Combination of CAII and CAIV led to a significant increase in MCT1-mediated transport activity by a factor of 3.5 as compared with MCT1 alone, while injection/expression of either CAII or CAIV only induced an increase in transport activity between 1.5 and 2.7 (Fig. 5B). The rate of alkalinization following removal of lactate was augmented by CAII and CAIV in a similar fashion as was the lactate-induced acidification. CAII and CAIV together increased MCT1 transport activity by a factor between 2.5 and 3, while each CA isoform alone increased transport activity from 1.4–2.2-fold (Fig. 5D). These results indicate that intracellular CAII and extracellular CAIV can functionally cooperate to enhance acid/base-coupled metabolite transport via MCTs, presumably independent of each other, on both sides of the cell membrane.

**DISCUSSION**

The present study shows that extracellular CAIV enhances transport activity of MCT1 and MCT4, and that this enhance-
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Transport activity can be augmented by additional injection of CAII protein into the cytosol. The actions of these CA isoforms on MCT activity persisted even after inhibition of the CA catalytic activity. These results suggest that extracellular and intracellular CA isoforms can cooperate functionally and appear to modulate MCT transport independently.

We have previously shown that transport activity of MCT1 and MCT4 is increased by CAII when the proteins are heterologously expressed in Xenopus oocytes (20–24). The mechanism of this augmentation has been found to be independent of the enzyme’s catalytic activity, since both in the absence and in the presence of CO₂/HCO₃⁻, inhibition of CAII catalytic activity with EZA, or mutation of the enzyme’s catalytic center (CAII-V143Y), had no effect on the CAII-induced increase in MCT1 and MCT4 transport activity (20–22). In contrast, removing the intramolecular H⁺/HCO₃⁻ shuttle of CAII (CAII-H64A) led to a loss of interaction between CAII and MCT1/MCT4 (24). From this it was concluded that CAII, directly bound to the transporter, can act as a “H⁺/HCO₃⁻-collecting antenna”, which directly supplies substrate to MCT1 and MCT4, to support proton/lactate cotransport. The necessity of such an antenna might derive from the low apparent diffusion rate of protons in a strongly buffered solution like the cytosol: It has been calculated that H⁺/HCO₃⁻-coupled transport proteins like MCTs, whose substrate is only available at very low concentrations, transport and thus remove or deliver H⁺ faster from/to the immediate vicinity around the membrane carrier than H⁺ can diffuse into or from this microdomain. This would lead to depletion or

FIGURE 5. Extracellular CAIV and intracellular CAII work together to enhance transport activity of MCT1. A, original recordings of [H⁺]i in Xenopus oocytes expressing MCT1 (black), expressing MCT1 + injected with 50 ng of CAII protein (green), coexpressing MCT1 + CAIV (blue) and co-expressing MCT1 + CAIV + injected with CAII protein (orange), respectively, during application of 3 and 10 mM lactate and 5% CO₂/10 mM HCO₃⁻. Rate of rise in [H⁺], induced by application of lactate (B) and 5% CO₂/10 mM HCO₃⁻ (C), respectively, in oocytes expressing MCT1, expressing MCT1 + injected with 50 ng of CAII protein, coexpressing MCT1 + CAIV and co-expressing MCT1 + CAIV + injected with CAII protein, respectively. Rate of intracellular alkalinization induced by removal of lactate (D) and 5% CO₂/10 mM HCO₃⁻ (E), respectively, in oocytes expressing MCT1, expressing MCT1 + injected with 50 ng of CAII protein, coexpressing MCT1 + CAIV and co-expressing MCT1 + CAIV + injected with CAII protein, respectively. Asterisks at the bars for MCT1 + CA refer to the values of MCT1-expressing cells.
Indeed we could show in this study that the presence of both intracellular CAII and extracellular CAIV enhance transport activity of MCT1 and MCT4 in both directions, i.e. influx and efflux of H<sup>+</sup>/lactate to a higher extent than did either isoform alone. While coexpression of either CAII or CAIV enhanced MCT1/4 transport activity by a factor of 1.5 to 2.7, the presence of both CA isoforms augmented the transport activity by a factor of up to 3.5, which was significantly greater than with either CA isoform alone.

A H<sup>+</sup>-collecting antenna on only one side of the membrane might lead to an increased formation of a proton microdomain on the other side of the membrane, since supply or removal of H<sup>+</sup> is enhanced on the cis-side, which leads to an increase in MCT transport activity (as seen by single expression of either CAII or CAIV), which then would result in an increased accumulation or depletion of H<sup>+</sup> at the trans-side, depending on transport direction. This formation of a H<sup>+</sup> micro-domain would then again decrease MCT transport activity. With a H<sup>+</sup>-collecting antenna on both sides of the membrane, formation of H<sup>+</sup> microdomains would be suppressed both at the cis- and at the trans-side. With respect to lactate/proton influx, H<sup>+</sup> would be delivered from the extracellular antenna, mediated by CAIV, while the intracellular antenna, mediated by CAII, would remove H<sup>+</sup> (Fig. 6). On the other hand, during lactate/proton efflux the two antennae would work in the opposite mode from that shown in Fig. 6, delivery of H<sup>+</sup> intracellularly by CAII and removal of H<sup>+</sup> by CAIV extracellularly. In such a scenario extracellular CAIV and intracellular CAII would cooperate by a “push and pull principle” pushing protons to the transporter on one side, and pulling them away on the other side of the cell membrane (Fig. 6). By this mechanism intracellular CAII and extracellular CAIV can cooperate to enhance metabolite transport into and out of cells by MCT1 and MCT4.

This mechanism might be of special importance for cells that transport high amounts of lactate like e.g. astrocytes in the mammalian brain. Astrocytes, which are highly glycolytic, have been shown to export lactate which is taken up by neurons for energy production, a mechanism coined “Astrocyte to Neuron Lactate Shuttle” (ANLS; 45–48). Moreover, it has been suggested, that astrocytes could also take up lactate, dependent on the concentration gradient (49). As reported before, uptake and/or release of lactate in astrocytes are mediated via MCT1 and MCT4 (50–52). Furthermore, astrocytes play a pivotal role in acid/base regulation in the brain, and thus highly express intracellular CAII and extracellular CAIV (31, 53, 54). Therefore, MCT1, MCT4, CAII, and CAIV might cooperate in astrocytes to ensure both rapid shuttling of lactate to neurons and efficient uptake of lactate from the extracellular space under different physiological conditions. However, it should be pointed out that a functional interaction between MCTs and CAIV has so far only been demonstrated in the overexpression system of the *Xenopus* oocyte, but has not yet been investigated in astrocytes or other tissue cells.

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