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Transport Activity of MCT1 Expressed in *Xenopus* Oocytes Is Increased by Interaction with Carbonic Anhydrase*

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Injection of carbonic anhydrase isoform II (CA) into *Xenopus* frog oocytes increased the rate of H^+ flux via the rat monocarboxylate transporter isoform 1 (MCT1) expressed in the oocytes. MCT1 activity was assessed by changes of intracellular H^+ concentration measured by pH-selective microelectrodes during application of lactate. CA-induced augmentation of the rate of H^+ flux mediated by MCT1 was not inhibited by ethoxyzolamide (10 μM) and did not depend on the presence of added CO_2/HCO_3^- but was suppressed by injection of an antibody against CA. Deleting the C terminus of the MCT1 greatly reduced its transport rate and removed transport facilitation by CA. Injected CA accelerated the CO_2/HCO_3^--induced acidification severalfold, which was blocked by ethoxyzolamide and was independent of MCT1 expression. Mass spectrometry confirmed activity of CA as injected into the frog oocytes. With pulldown assays we demonstrated a specific binding of CA to MCT1 that was not attributed to the C terminus of MCT1. Our results suggest that CA enhances MCT1 transport activity, independent of its enzymatic reaction center, presumably by binding to MCT1.

Acid/base transport across cell membranes includes a variety of carriers, some of which are instrumental for cellular pH regulation or cellular import and export of metabolites. The monocarboxylate transporters (MCT)[2] belong to the *SLC16* human gene family, and 14 isoforms have been described so far for this lactate/pyruvate-proton cotransporter (1). MCT isoforms have been reported for most tissues, where the removal or supply of L-lactate (pyruvate or other monocarboxylates) is related to different metabolic conditions. The MCT isoform 1 (MCT1) has been found in most tissues; in the brain, MCT1 appears to be located mainly in glial cells (2). MCT1 has been expressed in oocytes of the frog *Xenopus laevis*, where it has been characterized molecularly and functionally (2–4). MCT1 has a K_m value for L-lactate of 3–5 mM and is believed to be associated in the glia-neuron lactate shuttle (5, 6). Lactate is exported from astrocytes via MCT1 and is then taken up by neurons via the high-affinity MCT2 (K_m~0.5 mM). The significance of this lactate shuttle has been demonstrated by showing that synaptic activity recovers and is maintained under glucose-deprived conditions after addition of lactate (7–9).

In the present study we have looked for a possible role for carbonic anhydrase (CA) in H^+ fluxes via MCT1. Extracellular CA isoform IV has been shown to facilitate lactate transport in astrocytes and neurons (10). Furthermore, CA has been reported to bind to various acid/base transporters, such as the chloride/bicarbonate anion exchanger (11), the sodium bicarbonate cotransporter (12, 13), and the sodium/hydrogen exchanger (14). We have expressed MCT1 in *Xenopus* oocytes and injected isoform II of CA, which has been shown to be present also in astrocytes (15). Our results suggest that CA binds to the MCT1 in the cell membrane of the oocyte and greatly enhances the H^+ flux via the MCT1. Surprisingly, the MCT1-CA interaction was independent of added CO_2/HCO_3^- and not sensitive to the CA inhibitor ethoxyzolamide. A preliminary account of some of the results has been communicated in abstract form (16).

MATERIALS AND METHODS

Constructs, Oocytes, and Injection of cRNA and Carbonic Anhydrase—For oocyte expression rat MCT1 cloned in the vector pGEMHeJuel, which contains the 5′- and the 3′-untranscribed regions of the *Xenopus* β-globulin flanking the multiple cloning site, was used (2). Plasmid DNA was linearized with NotI and transcribed *in vitro* with T7 RNA-polymerase in the presence of the cap analogon $m^7G(5')ppp(5')G$ (mMessage mMachine, Ambion Inc.) to produce a capped RNA transcript. The cRNA was purified using the Qiagen RNeasy MinElute cleanup kit and stored at −70 °C in DEPC-H_2O. Integrity of the cRNA was checked by formaldehyde-gel electrophoresis. *X. laevis* females were purchased from Horst Kähler, Hamburg, Germany. Oocytes were isolated and singularized by collagenase (collagenase A, Roche Applied Science) treatment in Ca^{2+}-free oocyte Ringer solution at room temperature for 2 h. The singularized oocytes were left overnight in Ca^{2+}-containing oocyte Ringer solution to recover. The oocyte saline solution (OR2+) 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 pH 7.0 or 7.4. The bicarbonate-containing saline solution contained (in mM): NaCl, 72.5; KCl, 2.5; $CaCl_2$, 1; $MgCl_2$, 1; Na_2HPO_4, 1; $NaHCO_3$, 10 (pH 7.0) or 24 (pH 7.4), gassed with 5% CO_2 and HEPES, 5, to stabilize the pH. Lactate (3 and 10 mM) was added as sodium lactate and exchanged for equimolar amounts of NaCl. Oocytes at stages V and VI were then selected and injected with 7 ng of MCT1-cRNA using glass micropipettes and a microinjection device (Nanoliter 2000, World Precision Instruments, Berlin, Germany). Control oocytes were injected with an equivalent volume of DEPC-H_2O.

CA was directly injected into the oocytes. Therefore 50 ng of CA, isolated from bovine erythrocytes (C3934, Sigma), dissolved in 25 nl of DEPC-H_2O, was injected 20–28 h before using oocytes for electrophysiological measurement. Control oocytes were injected with 25 nl of DEPC-H_2O alone. For co-injection of CA with anti-CA, a mixture of CA and a 1.5-fold amount of anti-CA (rabbit anti-carbonic anhydrase II (Bovine Erythrocytes) polyclonal antibody, AB1243, Chemicon Europe, Hofheim, Germany) was prepared 1–2 days before injection to allow

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[2] The abbreviations used are: MCT, monocarboxylate transporter; CA, carbonic anhydrase; EZA, ethoxyzolamide; BSA, bovine serum albumin; GST, glutathione *S*-transferase; NBC, sodium bicarbonate cotransporter; AE, chloride/bicarbonate exchanger; DEPC, diethylpyrocarbonate.

enough time for anti-CA to bind to the CA. Injection of the CA + anti-CA mixture was carried out as described for the CA.

C-terminal Deletion in the MCT1D56 Mutant—Deletion of the C-terminal tail of MCT was carried out by using site-directed mutagenesis with modified oligonucleotide primers, as first described by Weiner *et al.* (18). For the deletion, the base triplet, coding for the 56th amino acid from the C-terminal onward, was changed from CGA (arginine) to TGA (stop). For selection of the mutants, a second SpeI restriction side (ACTAGTG) was added by changing the base triplet coding for the 55th amino acid (leucine) from CTT to CTA. The mutation was carried out by using a PCR reaction with the whole pGHJMCT1 plasmid as template and the following sense and a complementary antisense primer (changed nucleotides are written in bold): 5′-GGC ATC AAT TAT **T**GA CT**A** GTG GCC AAA GAA-3′.

After DpnI digestion of the template DNA, the mutated construct was retransformed into the *Escherichia coli* strain XL1 blue. Mutations were verified by restriction digestion and cDNA sequencing. RNA was then produced as described above.

Intracellular pH Measurements—For measurement of intracellular pH and membrane potential, double-barreled pH-sensitive microelectrodes were used; the manufacture and application have been described in detail previously (19). Briefly, two borosilicate glass capillaries of 1.0 and 1.5 mm in diameter were twisted together and pulled to a micropipette. The ion-selective barrel was silanized with a drop of 5% tri-*N*-butylchlorsilane in 99.9% pure carbon tetrachloride backfilled into the tip. The micropipette was baked for 4.5 min at 450 °C on a hotplate. H^+-sensitive mixture (Fluka 95291, Fluka, Buchs, Switzerland) was backfilled into the tip of the silanized ion-selective barrel and filled up with 0.1 M sodium citrate, pH 6.0. The reference barrel was filled with 3 M KCl. To increase the opening of the electrode tip, it was bevelled with a jet stream of aluminum powder suspended in H_2O. Calibration of the electrodes was carried out in OR2+ with a pH of 7.0 and 6.4. The recording arrangement was the same as described previously (19, 20). The central and the reference barrel of the electrodes were connected by chlorided silver wires to the head stages of an electrometer amplifier. Electrodes were accepted for use in the experiments when their response exceeded 50 mV per unit change in pH; on average, they responded with 54 mV for unit change in pH. In the experimental chamber they responded faster to a change in saline pH than the fastest reaction expected to occur in the oocyte cytosol.

As described previously (3) 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 (22–25 °C). Only oocytes with a membrane potential negative to −30 mV were used for experiments.

Buffering Power and Proton Fluxes—The measurements of pH_i were stored digitally using homemade PC software based on the program LabView and could be converted into intracellular H^+ concentration, $[H^+]_i$. This should provide changes in the $[H^+]_i$, which take into account the different pH base lines, *e.g.* as measured in HEPES- and CO_2/HCO_3^--buffered saline solutions (20).

Amplitude and rate of change of the measured pH_i or of the $[H^+]_i$ were continuously recorded. 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 solution. The CO_2-dependent buffering power, β_{CO2}, was calculated from the intracellular bicarbonate concentration in the oocytes, $\beta_{CO2} = 2.3 \cdot [HCO_3^-]$, and the bicarbonate concentration was obtained from the Henderson-Hasselbalch equation. 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 buffering power β. For calculation of the flux rates in HEPES-buffered saline solution, $\Delta pH_i/t$ was multiplied with the intrinsic buffer capacity β_i of the oocyte. For calculation of the flux rate in CO_2/HCO_3^--buffered saline solution, $\Delta pH_i/t$ was multiplied with the total buffering capacity β_t (20).

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 electrode measured in oocyte saline solution was around 1 megohm. For voltage clamp recording, both electrodes were connected to the head stages of an Axoclamp 2B amplifier (Axon Instruments). The experimental bath was grounded with a chlorided silver wire coated by agar dissolved in OR2+.

Determination of CA Activity—Activity of CA was determined by monitoring the ^{18}O depletion of doubly labeled $^{13}C^{18}O_2$ through several hydration and dehydration steps of CO_2 and HCO_3^- at 25 °C (21, 22). The reaction sequence of ^{18}O loss from $^{13}C^{18}O^{18}O$ ($m/z = 49$) 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 (MSD 5970; Hewlett Packard, Waldbronn, Germany) by coupling the machine to a cuvette via an aqueous membrane inlet system. 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 × 100/(49 + 47 + 45)]. For the calculation of the CA activity of the sample, the rate of ^{18}O degradation was obtained from the linear slope of the log(enrichment) over the time, using the spreadsheet-analyzing software Origin 6.0 (Microcal Software Inc.). The rate was compared with the corresponding rate of the non-catalyzed reaction. For the experiments, the cuvette was filled with 10 ml of OR2+ with a pH of 7.35 according to the mean intracellular pH of CA-injected oocytes and tempered to 25 °C. 1 μg of CA was added directly as protein, dissolved in water in a concentration of 0.2 μg/μl. Oocytes, injected with 50 ng of CA each, were pipetted into the cuvette in a batch of 20, making a total amount of 1 μg of CA.

Covalent Coupling of Carbonic Anhydrase and Bovine Serum Albumin (BSA) to Sepharose and Non-covalent Binding of Glutathione S-transferase to Glutathione-Sepharose—For pulldown assays CA and, as control, BSA, were linked covalently to *N*-hydroxysuccinimide-activated Sepharose 4 Fast Flow (Amersham Biosciences). Briefly CA and BSA were dissolved in phosphate-buffered saline solution and incubated with activated *N*-hydroxysuccinimide-Sepharose beads (5 mg of protein/ml medium) overnight at 4 °C. The remaining active groups were blocked for 3 h at room temperature with 0.5 M ethanolamine/0.5 M NaCl, pH 8.0. To remove uncoupled protein, the beads were washed alternately with 0.2 M $NaHCO_3$/0.5 M NaCl, pH 8.0, and 0.2 M NaOAc/0.5 M NaCl, pH 4.0. The efficiency of protein coupling was assessed by comparing protein content before and after coupling on SDS-PAGE/Coomassie stain. GST expressed in *E. coli* was bound to glutathione-Sepharose 4 Fast Flow (Amersham Biosciences). BL21 cells then were transformed with the vector pGEX 4T-2 (Amersham Biosciences) and cultured, and at $A_{578\,nm}$ of 0.7, expression of the GST protein was induced by the addition of 0.1 mM isopropyl 1-thio-β-D-galactopyranoside. After incubation for 3 h at 29 °C, cells were harvested by centrifugation (15 min, 5000 × *g*) and lysed by sonification, and proteins were extracted with 1% (v/v) Triton X-100 in 150 mM NaCl/50 mM Tris (pH 7.4) (Tris-buffered saline) for 1 h at 4 °C. After centrifugation (50,000 × *g*, 4 °C, 20 min) the supernatant was incubated with glutathione-Sepha-

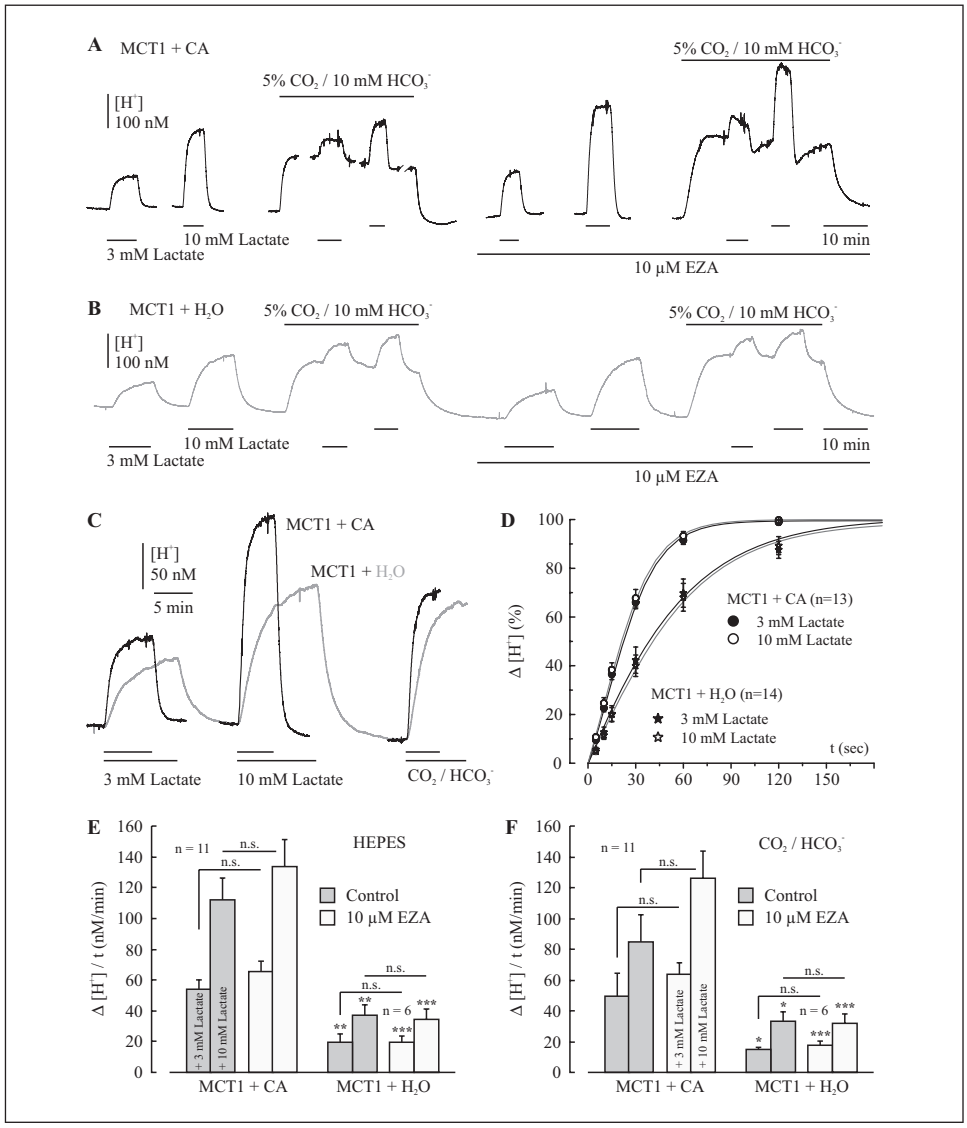

FIGURE 1. **Increase of the rate of $[H^+]$ change via MCT1 by carbonic anhydrase II.** *A* and *B*, intracellular pH recordings, converted to $[H^+]$, during repeated application of 3 and 10 mM L-lactate in oocytes expressing MCT1 injected with 50 ng of CA (*A*) or with H_2O (*B*) in HEPES-buffered and in CO_2/HCO_3^--buffered saline solutions at pH 7.0 in the absence and in the presence of the CA inhibitor EZA (10 μM). *C–F*, superimposed traces (*C*), the mean relative changes of $[H^+]$ (*D*), and rates of H^+ changes (*E* and *F*) as induced by lactate in MCT1-expressing oocytes with or without (H_2O) injected CA in the presence and absence of CO_2/HCO_3^- and the CA inhibitor ethoxyzolamide (*E* and *F*). Significance levels: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

rose beads. Beads were washed with Tris-buffered saline, and the amount of purified GST bound to the Sepharose was determined by SDS-PAGE and Coomassie staining.

In Vitro Translation of MCT1 and MCT1D56 and Pulldown Assays on Immobilized CA-Sepharose—In vitro translation of MCT1 and MCT156 in pGEMHeJuel was performed under the control of the T7 promoter using the TNT7® Quick translation kit (Promega). Therefore 1 μg of cDNA and 10 μCi of L-[^{35}S]methionine were incubated with rabbit reticulocyte lysate in a final volume of 30 μl. After incubation for 90 min at 30 °C, the probes were frozen at −20 °C. The *in vitro* translated products were checked by SDS-PAGE and analyzed by a phosphorimaging device before they were used for the pulldown assays. 7 μl of the protein probes were diluted in 1 ml of RIPA-CaMg buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1% BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$) and incubated for 3 h at room temperature with 50 μl of CA-Sepharose (~3.75 mg/ml), GST-Sepharose (~0.1 mg/ml), and BSA-Sepharose (~3.75 mg/ml) equilibrated in RIPA-CaMg buffer. Protein columns were washed five times for 5 min with 1 ml of RIPA-CaMg buffer at room temperature. Columns and bound proteins were eluted with SDS-PAGE sample buffer (8% (w/v) SDS, 120 mM Tris, pH 6.8, 20% (v/v) glycerine, 0.01% (w/v) bromphenol blue, 10% (v/v) β-mercaptoethanol) and transferred to SDS-PAGE. After drying, gels were exposed to phosphorimaging screens.

Calculation and Statistics—Statistical values are presented as means ± S.E. For the calculation of significance in differences, Student's *t* test or, if possible, a paired *t* test was used.

RESULTS

A typical experiment, which we used for analysis of the effect of CA on the activity of MCT1, is shown in Fig. 1. Oocytes were injected with 14 ng of MCT1-cRNA and either 25 μl of CA (50 μg) or 25 μl of H_2O 20–28 h before the experiment. The oocytes were voltage clamped at −40 mV, and intracellular pH changes, converted to changes in $[H^+]_i$, were measured with ion-selective microelectrodes. Lactate (3 and 10 mM) was applied for 5–10 min to activate the MCT1, both in HEPES-buffered, nominally CO_2/HCO_3^--free saline solution (which contains less than 70 μM HCO_3^- at pH 7.0) and, for comparison, in saline solution buffered with 5% CO_2/10 mM HCO_3^- (pH 7.0). The $[H^+]_i$ changes in oocytes injected with CA (Fig. 1*A*) occurred much faster during the application of lactate and also upon the addition of CO_2/HCO_3^-, as compared with the $[H^+]_i$ changes measured in oocytes injected with H_2O instead of CA (Fig. 1*B*). Ethoxyzolamide (EZA, 10 μM), an inhibitor of CA, slowed the CO_2-dependent $[H^+]_i$ changes but not the lactate-

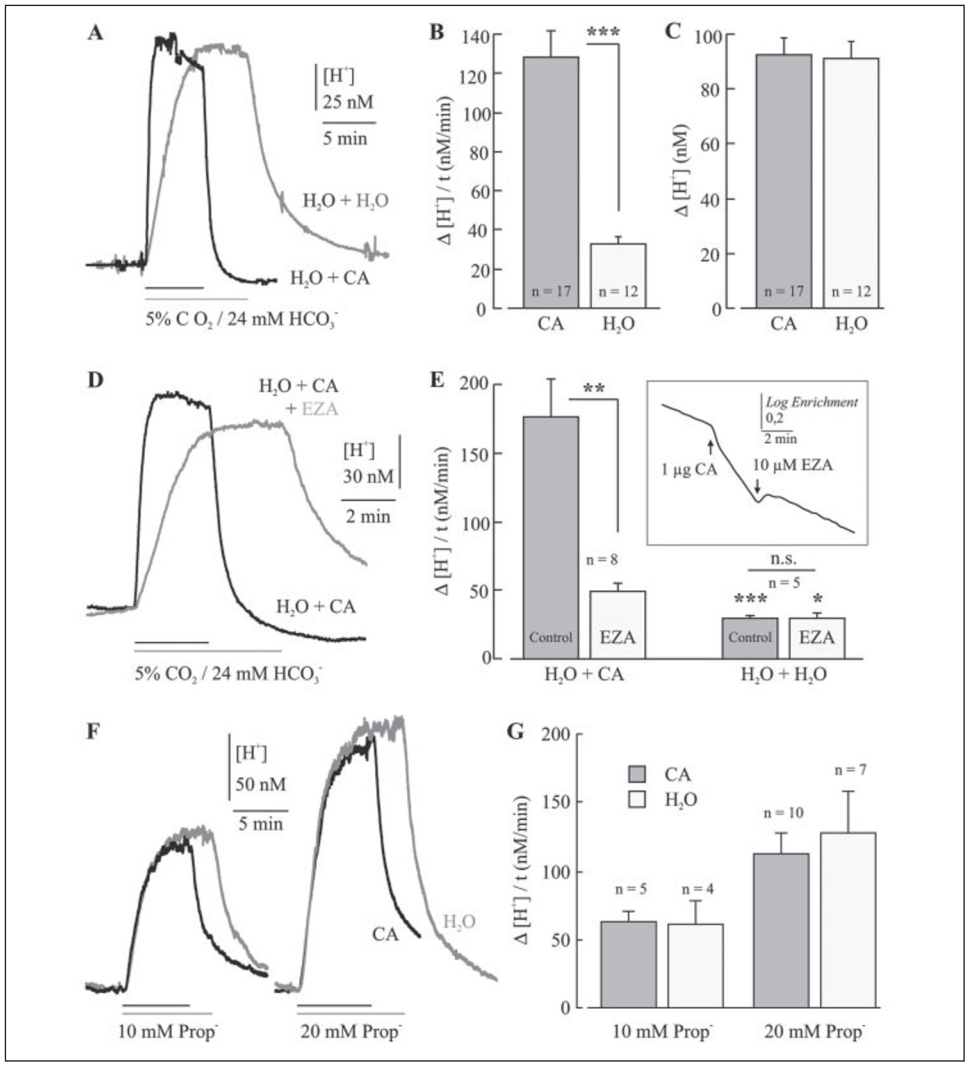

FIGURE 2. **The effect of carbonic anhydrase on acid fluxes in native oocytes.** *A–C*, CO_2/HCO_3^--induced change of $[H^+]$ and rates of $[H^+]$ changes in native oocytes (injected with H_2O instead of cRNA) that were injected with CA or again with H_2O. In *B*, ***, $p < 0.001$. Rate of $[H^+]$ change in native oocytes with and without CA in the absence and presence of the CA inhibitor *EZA* (10 μM) (*D* and *E*). In *E*, *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. The *inset* in *E* shows the CA activity measurement in mass spectrometry before and after the addition of 10 μM EZA. Rate of $[H^+]$ change as induced by propionic (*Prop*) acid in native oocytes with and without CA (*F* and *G*).

induced acidification in CA-injected oocytes and was without effect in the H_2O-injected oocytes (Fig. 1, *A* and *B*). We have analyzed the change of $[H^+]_i$ and the rate of $[H^+]_i$ change in both types of oocytes, as shown for the superimposed traces of $[H^+]_i$ changes evoked by lactate in HEPES-buffered saline solution (Fig. 1*C*). The change of $[H^+]_i$ plotted against time indicated for both lactate concentrations that transport is considerably faster in oocytes injected with CA (Fig. 1*D*). The half-time of the $[H^+]$ change was 22 s in CA-injected oocytes and 43 s in H_2O-injected oocytes. Surprisingly, this augmentation of transport activity via the MCT1 was achieved in HEPES-buffered, nominally CO_2/HCO_3^--free saline solution and was not affected by EZA. In both cases the enhancement of the rate of $[H^+]_i$ change by CA was 3–5-fold (Fig. 1, *E* and *F*). The membrane current measured at a holding potential of −40 mV remained virtually constant (not shown here), as the MCT1, cotransporting one lactate anion with one H^+, is electroneutral, as demonstrated previously (3, 23).

MCT1 and CA, stained with fluorescently labeled antibodies, were clearly visible in the cell membrane of the oocytes injected with cRNA of MCT1 or injected with CA (data for CA not shown here; for MCT1 data see Ref. 23). There was no staining of the oocyte cytosol, indicating that both proteins were incorporated into or attached to the cell membrane. Native oocytes, labeled with antibodies against CA or MCT1, showed no staining at all.

Native oocytes, which were injected with H_2O instead of MCT1-cRNA but also injected with CA, showed a fast acidification upon the addition of CO_2/HCO_3^-; the rate of $[H^+]_i$ change was about four times greater than in oocytes injected with H_2O instead of CA (Fig. 2, *A–C*). This increase in the rate of $[H^+]_i$ change induced by CA was fully reversed by the CA inhibitor EZA (Fig. 2, *D* and *E*). The rate of acidification induced by propionate (10 and 20 mM) was not affected by the injection of CA in native oocytes (Fig. 2, *F* and *G*), indicating that active CA does not *per se* stimulate the rate of $[H^+]_i$ change.

The buffer capacity of the oocyte cytosol was determined by the CO_2-induced intracellular pH change (intrinsic buffer capacity β_i) and by using the bicarbonate concentration calculated under steady-state conditions in CO_2/HCO_3^--buffered saline solution (CO_2-dependent buffer capacity β_{CO2}), the sum of both giving the total buffer capacity β_t (23). As seen in Fig. 3*A*, the buffer capacities remained unaffected by the presence of either CA or EZA, indicating that CA did not contribute to the *global* cytoplasmic buffering of oocytes. This does not exclude, however, that CA might still contribute to the *local* buffer capacity near the cell membrane, where the CA is located (as will be discussed later).

With the buffer capacity and the rate of pH change known, the rate of H^+ flux (J_H) was calculated and plotted for the MCT1-expressing oocytes with and without injected CA in the absence and presence of EZA (Fig. 3, *B* and *C*). Because the global oocyte buffer capacity was not affected (see above), the H^+ flux rate reflected a similar difference between the CA-injected and the H_2O-injected oocytes. A 3–5-fold

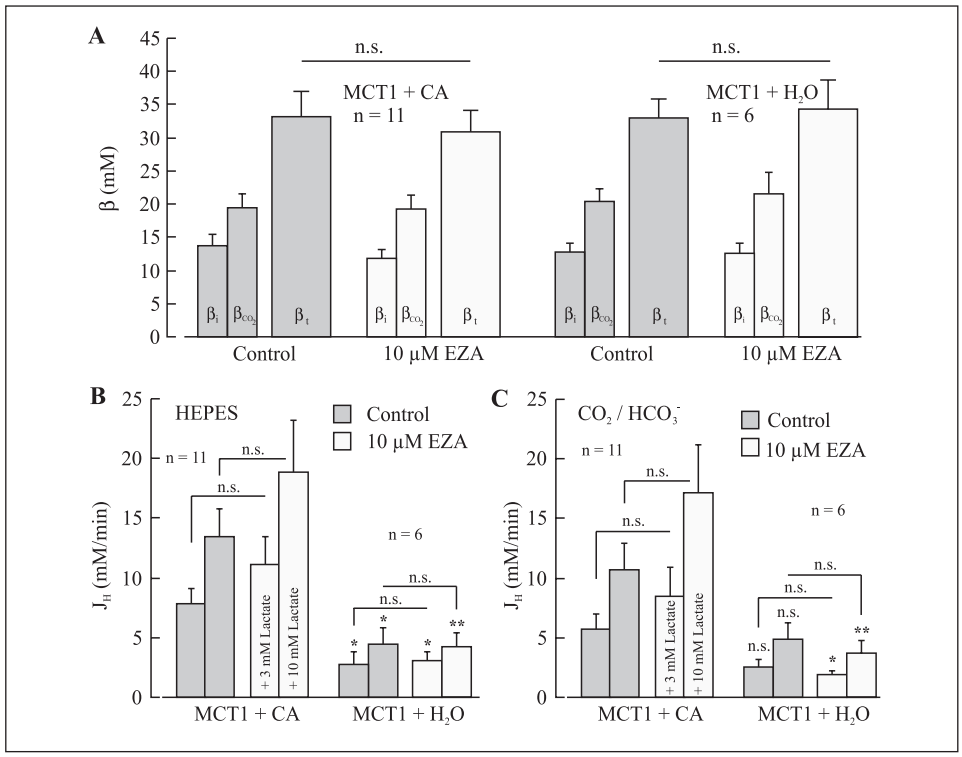

FIGURE 3. **H^+ buffer capacity and H^+ flux as affected by carbonic anhydrase.** *A*, the H^+ buffer capacity of *Xenopus* oocyte cytosol, indicated as intrinsic (β_i), CO_2-dependent (β_{CO2}), and total (β_t) buffer capacity in MCT1-expressing oocytes injected with CA or H_2O in the absence and presence of *EZA* (10 μM) and the rates of H^+ fluxes J_H, $J_H = \beta_t$, or $\beta_i \times dpH_i$, as induced by 3 and 10 mM L-lactate in HEPES (*B*)- and CO_2/HCO_3^- (*C*)-buffered saline solutions. In *B* and *C*, *, $p < 0.05$ and **, $p < 0.01$.

increase in H^+ flux rate in CA-injected oocytes was obtained during activation of MCT1 by lactate both in the presence and absence of EZA and in the absence and presence of CO_2/HCO_3^-. These results suggest that the presence of CA increases the transport activity of the MCT1 by a mechanism that is independent of CO_2/HCO_3^- and of the inhibition of the CO_2 reaction center of CA by EZA.

We tried to inhibit the effect of CA on MCT1 transport activity by injecting antibodies against CA (anti-CA) alone and together with CA into oocytes. In comparison with a MCT1-expressing oocyte with CA injected alone, the rate of $[H^+]_i$ change induced by lactate (in HEPES-buffered saline solution) was much slowed in oocytes into which CA and anti-CA or anti-CA alone was injected (Fig. 4, *A* and *B*). The lactate-induced rate of $[H^+]_i$ change in oocytes into which CA and anti-CA were injected was the same as in oocytes injected only with anti-CA but was 2–3 times smaller than in oocytes injected with CA. In contrast, the rate of CO_2-induced acidification in oocytes injected with CA and anti-CA was intermediate to that with oocytes injected with CA alone and to that in oocytes injected only with anti-CA (Fig. 4, *C* and *D*). In the presence of EZA (10 μM), no difference in the CO_2-induced rate of $[H^+]_i$ change was detected between all three types of oocytes. The injection of BSA, instead of anti-CA, in MCT1-expressing oocytes injected with CA had no effect on the CA-induced increase of the rate of the lactate-induced acidification and the amplitude of acidification caused by the application of CO_2. These results suggest that injection of CA together with its antibody results in complete inhibition of the CA-mediated enhancement of the lactate-induced rate of $[H^+]_i$ change via MCT1 activity but only in a partial inhibition of the CA-mediated facilitation of the CO_2-induced rate of $[H^+]_i$ change.

We measured the activity of the CA as injected into the oocytes 20–24 h after injection of 50 ng of CA into each oocyte using mass spectrometry. CA activity measured *in vitro* (addition of 1 μg of CA) and in 20 oocytes (corresponding to 1 μg of CA with 50 ng injected into each oocyte) showed an activity of around 25 units/ml, whereas no activity was measured in the absence of CA (Fig. 4, *E* and *F*). Thus, CA injected into oocytes had the same activity as CA *in vitro*, although the reaction of CA-injected oocytes was accompanied by a small delay, as expected. Addition of anti-CA antibody to CA, both *in vitro* and in oocytes, did not inhibit the measured CA activity. These results indicate that the CA used was neither degraded in the oocytes nor affected in its catalytic activity by the antibody.

We hypothesized that the facilitation of H^+ flux via the MCT1 by CA injected into the oocytes might be achieved by binding of CA to MCT1 at the C terminus, as has been shown for other anion transporters (24). We therefore deleted the C terminus of the MCT1 by cutting 56 amino acids from the C terminus by using site-directed mutagenesis. The mutated MCT1 (MCT1D56) was then expressed in oocytes as described under "Materials and Methods."

To check for the deletion of the C terminus, MCT1D56-expressing oocytes were stained with an antibody against the C terminus of the MCT1. The fluorescently labeled antibody showed no visible staining in the cell membrane or in the cytosol (data not shown). MCT1D56 showed, in comparison with the wild-type MCT1, a much reduced transport activity, indicated by a decrease in the lactate-induced rate of $[H^+]_i$ change by 70–85%. There was still detectable transport activity in MCT1D56-expressing oocytes, as measured by the change in pH_i and the labeled lactate flux, which was well above the control in native oocytes. The facilitation of the rate of $[H^+]_i$ change by CA, as observed in the wild-type MCT1, was completely suppressed in the MCT1D56 mutant (Fig. 5, *A–C*). The rate of $[H^+]_i$ change was the same in MCT1D56-expressing oocytes whether or not CA or H_2O had been injected (Fig. 5*D*). The CO_2-induced rate of H^+ flux was, however, still increased 4–5 times by the injection of CA in oocytes expressing the MCT1D56 mutant, as compared with oocytes injected with H_2O instead of CA (Fig. 5*E*). These results indicated that deletion of the C terminus of the MCT1 abolished the effect of injected CA on the lactate-induced, but not on the CO_2-induced, rate of acidification.

To demonstrate the direct binding between MCT1 and CA, MCT1 cDNA was translated *in vitro*, and the MCT1 protein was incubated in

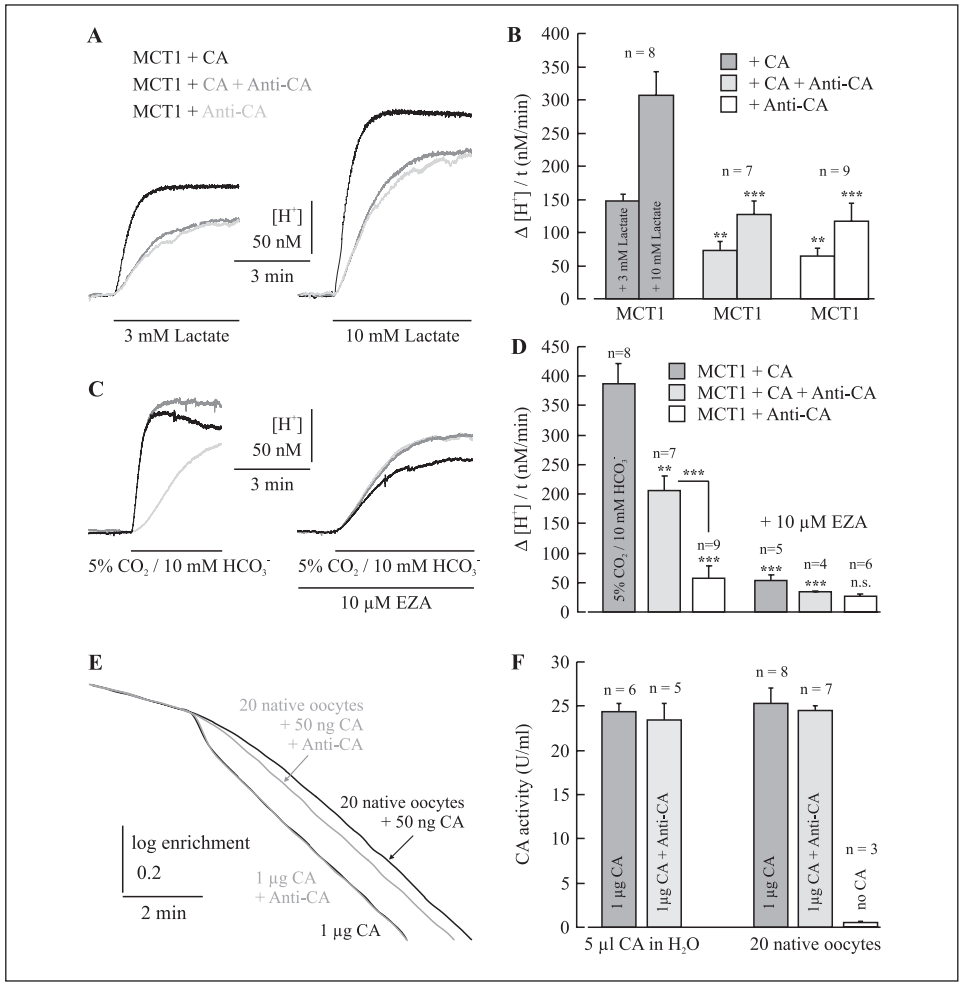

FIGURE 4. **Carbonic anhydrase antibody abolishes enhancement of MCT1 activity.** *A–D*, superimposed traces of $[H^+]$ changes (*A* and *C*) and plots of the rates of $[H^+]$ changes (*B* and *D*) as induced by 3 and 10 mM L-lactate (*A* and *B*) and by CO_2/HCO_3^- in the absence and presence of the CA inhibitor EZA (10 μM) (*C* and *D*) measured in oocytes expressing MCT1 with injected CA alone, or together with the CA antibody (*CA + Anti-CA*), or injected with the anti-CA alone. In *B* and *D*, **, $p < 0.01$ and ***, $p < 0.001$. *E* and *F*, activity measurement of CA using mass spectrometry with CA added (1 μg) and injected into 20 oocytes (50 μg/oocyte 20–24 h before) with and without anti-CA antibody and with no CA added (*F*). The beginning of the trace in *E* shows the rate of degradation of the ^{18}O-labeled substrate in the non-catalyzed reaction.

RIPA-CaMg buffer with immobilized CA. After extensive washing, bound MCT1 protein was eluted by SDS sample buffer. As shown in Fig. 6), MCT1 (43 kDa) was retained on CA. No binding of MCT1 was observed when the *in vitro* translated protein was incubated with BSA-coupled Sepharose or GST bound to glutathione-Sepharose or on blocked NHS-Sepharose (Fig. 6, *lanes 2*, *4*, and *6*). Recently it has been shown that the binding of the sodium bicarbonate cotransporter (NBC) or the chloride/bicarbonate exchanger 1 (AE1) to CA depends on an acidic amino acid cluster followed by hydrophobic amino acids (11, 12, 24). At the C terminus of MCT1 are two acidic amino acid clusters (amino acids 456–458 and 489–491). To test whether these residues are crucial for the interaction, we *in vitro* translated the MCT1 mutant, which lacks the last 56 amino acids of the C terminus (Fig. 6*A*, *lane 9*), and incubated the protein with CA-Sepharose. Interestingly, the *in vitro* translated mutant MCT1D56 protein (34 kDa) was also retained on CA (Fig. 6*A*, *lane 11*), meaning that the binding domain is not localized within the 56-amino acid stretch of the C terminus.

DISCUSSION

CA binding to various acid/base transporters, such as the AE, NBC, and sodium/hydrogen exchanger, has been shown to result in considerable augmentation of transport activity in these proteins (14, 25, 26). The present study shows that the activity of the monocarboxylate transporter MCT1, expressed in many different tissues (including the brain), is also enhanced by CA. There is evidence that this enhancement by CA is because of direct interaction between the enzyme and the MCT1, as already demonstrated for AE and NBC (11, 12, 27). Our experiments identify MCT1 as a binding protein of CA isoform II. The full-length *in vitro* translated MCT1 was retained on carbonic anhydrase, which was immobilized on Sepharose beads. The CA interaction sites of AE1 and NBC have been identified as a hydrophobic amino acid residue followed by acidic amino acids. Although there are some similar motifs in the C terminus of MCT1 (amino acids 437–494), these residues are obviously not required for the binding because the C-terminal mutant MCT1D56, which lacks the last 56 C-terminal amino acids, was also able to retain on CA beads. It is not known whether the amino acid motifs at the N terminus or the putative intracellular loops are responsible for CA interaction. Future experiments are necessary to identify the CA binding domain and analyze its impact on MCT1 transport activity by expressing MCT1 cDNA, in which the CA binding domain has been mutated or deleted.

Even though the C terminus of MCT1 is not required for the binding of CA, it seems to play a crucial role in the interaction between these two proteins because C-terminal deletion removes the enhancement of transport activity by CA. That a direct interaction between MCT1 and CA is needed for the enhancement of H^+ flux over the transporter is also supported by the findings that the co-injection of CA together with an antibody against the enzyme suppresses the CA-induced increase in the rate of the lactate-induced acidification, possibly by sterically hindering the binding of CA to MCT1. Surprisingly, and in contrast to other acid/base transporters, blocking the CO_2 reaction center of CA by the sulfonamide ethoxyzolamide does not affect the augmentation of

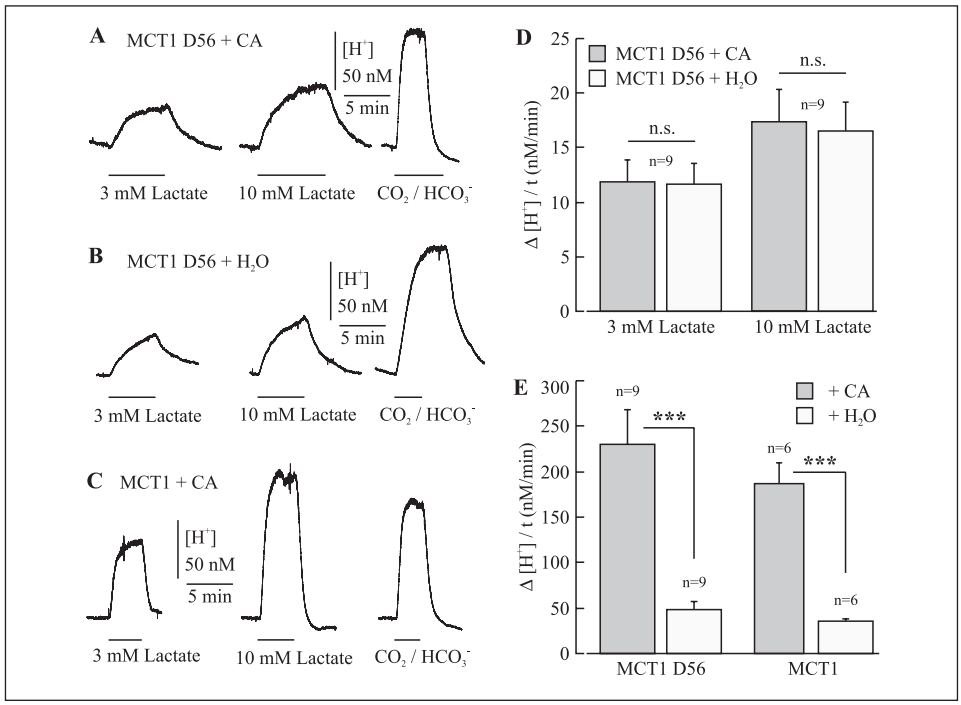

FIGURE 5. **C-terminal deletion of MCT1 removes transport enhancement by carbonic anhydrase.** Shown are changes of $[H^+]$ as induced by L-lactate and CO_2/HCO_3^- (*A–C*) and the rate of $[H^+]$ changes (*D* and *E*) in oocytes expressing the C-terminus-deleted mutant MCT1D56 with injected carbonic anhydrase (*CA*) (*A*) or with injected H_2O (*B*), as compared with wild-type MCT1 injected with CA (*C*). *E*, ***, $p < 0.001$.

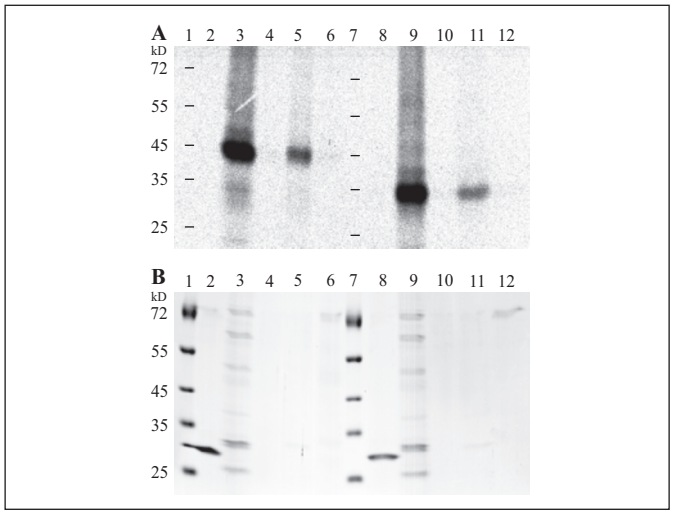

FIGURE 6. **MCT1 and MCT1D56 is retained on CAII-Sepharose.** Phosphorimaging analysis of MCT1 pulldown assay (*A*) and corresponding Coomassie stain (*B*) are shown. *In vitro* translated [^{35}S]MCT1 (*lanes 2–6*) and [^{35}S]MCT1D56 (*lanes 8–12*) were incubated with CAII-Sepharose (*lanes 5* and *11*), blocked Sepharose (*lanes 4* and *10*), BSA-Sepharose (*lanes 6* and *12*), and glutathione-Sepharose (*lanes 2* and *8*). After extensive washing, Sepharose was resuspended in SDS sample buffer, and eluted proteins were separated by 12–7% SDS-PAGE. Gels were dried and exposed to a phosphorimaging screen. Approximately 25% of the input of MCT1 (*lane 3*) and MCT1D56 (*lane 9*) was retained on CAII-Sepharose (*lanes 5* and *11*).

MCT1 transport activity by CA. This is supported by the finding that CA-mediated enhancement of MCT1 transport activity was independent of added CO_2/HCO_3^- but dependent on the C terminus of MCT1, which itself had no effect on the CA-enhanced rate of $[H^+]_i$ change induced by CO_2. Saline solution buffered with HEPES to pH 7.0 in the nominal absence of CO_2/HCO_3^- contains less than 70 μM HCO_3^-. This would result in 160 μM H^+ buffer capacity and, with a 5-fold increase in the rate of $[H^+]_i$ change by CA, would still result in less than 1 mM extra buffer capacity, which is less than 10% of the intrinsic buffer capacity. We therefore conclude that lactate-H^+ cotransport via MCT1 is enhanced by direct binding of CA to the MCT1, which might involve an allosteric conformation change of the MCT1. The enhancement of MCT1 transport activity by CA is not dependent on the CO_2 reaction center of CA, which catalyzes the reversible reaction of CO_2 and H_2O to H^+ and HCO_3^-; rather, it may be mediated by CA removing H^+, transported into the oocyte by the MCT1 directly from the inner mouth of the transporter, and thereby stabilizing the H^+ gradient into the cell.

Deletion of the C terminus of MCT1 resulted in a considerable loss of transport activity by up to 85%. In C terminus-deleted anion exchanger AE1 and human NBC3, it was found that the C terminus appears to be instrumental for processing of the transport protein into the membrane (27, 28). Therefore, C-terminal deletion may not necessarily be the primary cause for the reduction in transport activity of the MCT1 but may impair the incorporation of MCT1 into the membrane, which then results in loss of transport activity. Nevertheless, although greatly reduced, significant transport activity could still be measured in the MCT1D56 mutant. Enhancement of transport activity by CA, however, was abolished in the mutant.

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 (29) and cultured oligodendrocytes (30), that membrane-attached CA can create pH microdomains and local buffering. In smaller cells ($<$20 μM), these CA-mediated pH microdomains may significantly affect the 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 buffer capacity. As discussed by Ro and Carson (30) these gradients would be influenced by the partition coefficient between freely diffusing and metabolon-associated CA.

In summary, our study provides evidence for another interaction between CA and an acid/base transporter, a member of the MCT family. In contrast to other transporters, however, this transporter seems to be

insensitive to the inhibition of the CO_2 reaction center by sulfonamides and to the absence of CO_2/HCO_3^-. The hypothesis that CA removes H^+ imported by the MCT1 directly from the inner mouth of the transporter and thereby stabilizes the inwardly directed proton gradient needs to be clarified by additional experiments. The functional significance of CA-enhanced lactate transport has been shown in cell cultures, where extracellular CA isoform IV activity was needed to achieve maximum lactate influx into astrocytes and neurons (10). This underlines a role of CA activity in an increased lactate shuttling from glial cells to neurons and the interaction of acid/base-coupled transporters with H^+/HCO_3^- handling, accelerated by CA. The cooperation between CA and MCT might also be relevant in other tissues where these two proteins are expressed, such as in muscle and in epithelia, for the import and export of lactate/pyruvate or other monocarboxylates and ketone bodies and/or when lactate is accumulated under hypoxic/anoxic conditions.

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REFERENCES

1. Halestrap, A. P., and Meredith, D. (2004) *Pflugers Arch. Eur. J. Physiol.* **447,** 619–628
2. Bröer, S., Rahman, B., Pellegri, G., Pellerin, L., Martin, J.-L., Verelysdonk, S., Hamprecht, B., and Magistretti, P. J. (1997) *J. Biol. Chem.* **272,** 3096–3102
3. Bröer, S., Schneider, H.-P., Bröer, A., Rahman, B., Hamprecht, B., and Deitmer, J. W. (1998) *Biochem. J.* **333,** 167–174
4. Galić, S., Schneider, H.-P., Bröer, A., Deitmer, J. W., and Bröer, S. (2003) *Biochem. J.* **376,** 413–422
5. Deitmer, J. W. (2000) *BioEssays* **22,** 747–752
6. Magistretti, P. J., and Pellerin, L. (2000) *Rev. Med. Suisse Romande* **120,** 739–742
7. Schurr, A., West, C. A., and Rigor, B. M. (1988) *Science* **240,** 1326–1328
8. Schurr, A., Payne, R. S., Miller, J. J., and Rigor, B. M. (1997) *J. Neurochem.* **69,** 423–426
9. Cater, H. L., Benham, C. D., and Sundstrom, L. E. (2001) *J. Physiol.* (*Lond.*) **531,** 459–466
10. Svichar, N., and Chesler, M. (2003) *Glia* **41,** 415–419
11. Vince, J. W., and Reithmeier, R. A. (1998) *J. Biol. Chem.* **273,** 28430–28437
12. Gross, E., Pushkin, A., Abuladze, N., Fedotoff, O., and Kurtz, I. (2002) *J. Physiol.* (*Lond.*) **544,** 679–685
13. Alvarez, B. V., Loiselle, F. B., Supuran, C. T., Schwartz, G. J., and Casey, J. R. (2003) *Biochemistry* **42,** 12321–12329
14. Li, X., Alvarez, B., Casey, J. R., Reithmeier, R. A., and Fliegel, L. (2002) *J. Biol. Chem.* **277,** 36085–36091
15. Jeffrey, M., Wells, G. A., and Bridges, A. W. (1991) *J. Comp. Pathol.* **104,** 337–343
16. Becker, H. M., and Deitmer, J. W. (2005) *Pflügers Arch. Eur. J. Physiol.* **449,** S33 (abstr.)
17. Deleted in proof
18. Weiner, M. P., Costa, G. L., Schoettlin, W., and Cline, J. (1994) *Gene* (*Amst.*) **151,** 119–123
19. Deitmer, J. W. (1991) *J. Gen. Physiol.* **98,** 637–655
20. Becker, H. M., and Deitmer, J. W. (2004) *J. Biol. Chem.* **279,** 28057–28062
21. Silverman, D. N. (1982) *Methods Enzymol.* **87,** 732–752
22. Sültemeyer, D. F., Fock, H. P., and Canvin, D. T. (1990) *Plant Physiol.* **94,** 1250–1257
23. Becker, H. M., Bröer, S., and Deitmer, J. W. (2004) *Biophys. J.* **86,** 235–247
24. Pushkin, A., Abuladze, N., Gross, E., Newman, D., Tatishchev, S., Lee, I., Fedotoff, O., Bondar, G., Azimov, R., Ngyuen, M., and Kurtz, I. *J. Physiol.* (*Lond.*) **559,** 55–65
25. McMurtrie, H. L., Cleary, H. J., Alvarez, B. V., Loiselle, F. B., Sterling, D., Morgan, P. E., Johnson, D. E., and Casey, J. R. (2004) *J. Enzyme Inhib. Med. Chem.* **19,** 231–236
26. Sterling, D., Reithmeier, R. A., and Casey, J. R. (2001) *J. Biol. Chem.* **276,** 47886–47894
27. Loiselle, F. B., Jaschke, P., and Casey, J. R. (2003) *Mol. Membr. Biol.* **20,** 307–317
28. Cordat, E., Li, J., and Reithmeier, R. A. (2003) *Traffic* **4,** 642–651
29. Vaughan-Jones, R. D., Peercy, B. E., Keener, J. P., and Spitzer, K. W. (2002) *J. Physiol.* (*Lond.*) **541,** 139–158
30. Ro, H. A., and Carson, J. H. (2004) *J. Biol. Chem.* **279,** 37115–37123