Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2011;27:79-90

Accepted: December 16, 2010

Substrate-dependent Interference of Carbonic Anhydrases with the Glutamine Transporter SNAT3-Induced Conductance

Alexandra Weise[1,a], Hans-Peter Schneider[1], Robert McKenna[2] and Joachim W. Deitmer[1]

[1]Abteilung für Allgemeine Zoologie, FB Biologie, University of Kaiserslautern, Kaiserslautern, [2]Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL, [a]Present adress: Department of Neurobiology, Heinrich-Heine-University of Düsseldorf, Düsseldorf

Key Words

SLC38A3 • Carbonic anhydrases • Asparagine • Membrane conductance

Abstract

The glutamine transporter SNAT3 (SLC38A3), which also transports asparagine and histidine, exchanges sodium for protons, and displays a non-stoichiometrical conductance, which is suppressed by the catalytic activity of carbonic anhydrase II (CAII). In this study, we show that this conductance of rat SNAT3, expressed in *Xenopus* oocytes, is also suppressed following co-expression with CAI, CAIII, CAIV, and CAII-H64A (mutant with impaired intramolecular H^+ shuttling). All CA isoforms and the CAII mutant displayed catalytic activity in intact oocytes, although *in vitro* studies had reported only very low catalytic activity of CAIII and CAII-H64A. The CA-mediated suppression of conductance was only observed, however, when glutamine, but not when asparagine, was the substrate. We hypothesized that this substrate specificity of the CA action might be due to the different ion selectivity induced by the different amino acid substrates, which induce currents carried by sodium and/or protons. The ion selectivity and conductance was dependent on both pH and extracellular sodium concentration for glutamine and asparagine; however the sodium dependence of the conductance, when asparagine was the substrate, was significantly greater at higher sodium concentrations, which might explain the difference in the sensitivity of the conductance to CAs. Given the presence of CAs in most cells, substrate sensing of SNAT3 would be indicated by different membrane potential changes.

Introduction

Glutamine is the most abundant amino acid, and a major carrier of ammonium in mammalian cells and tissues. The transport of glutamine across cell membranes is mediated by several amino acid carriers with different specificity for glutamine and other neutral amino acids. In rodent astrocytes, we identified both, Na^+-dependent and Na^+-independent carriers of glutamine, including system N, SNAT3, *SLC38A3*; [1, 2]. Glutamine efflux from astrocytes is believed to be required by neurons to synthesize glutamate and GABA. The members of this mem-

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015-8987/11/0271-0079$38.00/0

Accessible online at:
www.karger.com/cpb

Dr. Joachim W. Deitmer
Abteilung für Allgemeine Zoologie,
FB Biologie, University of Kaiserslautern
Postfach 3049, D-67653 Kaiserslautern (Germany)
E-Mail deitmer@biologie.uni-kl.de

brane transporter family have been characterized in some detail [3-6], revealing that SNAT3 is an electroneutral Na^+-glutamine/H^+ exchanger with an associated conductance, when heterologously expressed in *Xenopus* oocytes. Hence, activation of SNAT3 with substrate results in a current and an increased membrane conductance. This conductance has been identified to be due to monovalent cations or H^+, depending on the extracellular pH, being uncoupled from substrate transport [7]. Recently, we have also shown that this glutamine-induced associated conductance is suppressed, if carbonic anhydrase isoform II (CAII) had been injected or co-expressed in frog oocytes [1]. Accordingly, activation of SNAT3 with glutamine in tissues would not be expected to generate a current, since virtually all cells and tissues express catalytically active CAII [8].

The substrate glutamine is transported significantly faster than asparagine, while asparagine induces a larger current and conductance than glutamine [3, 4, 7]. We compared the membrane conductance of rat SNAT3, heterologously expressed in *Xenopus* oocytes, as induced by either glutamine or asparagine at different Na^+ and H^+ gradients, and tested different CA isoforms on the membrane conductance. We found that the effect of different cytosolic CA isoforms, CAI, CAII, and CAIII, and the extracellular carbonic anhydrase CAIV, which displayed catalytic activity in intact oocytes, suppressed the membrane conductance, when glutamine, but not when asparagine, was the substrate. Our results suggest that the different ions involved in the conductance when glutamine or asparagine is the substrate, modulate the sensitivity of the transporter conductance to the catalytic activity of CA. This may enable SNAT3-expressing cells to discriminate between the two substrates.

Material and Methods

Molecular biology

Rat SNAT3-DNA was isolated from *E. coli* XL-1 blue transfected with pGEM-He-Juel-SNAT3 [6]. Isolation of the plasmid was carried out using the Plasmid MiniPrep Kit II (PeqLab Biotechnologie GmbH, Erlangen, Germany). Isolated plasmids were linearised and cleaned up with the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany), followed by *in vitro* transcription (mMessage mMachine™, Ambion Inc., Huntington, Cambridge, UK) and another clean-up (RNeasy® MinElute™-Cleanup, Qiagen). The cRNA was stored at –80°C. CAII-, CAIII-, CAII-H64A- and CAIV-DNA for coexpression was isolated from *E.coli* DH5α either transfected with pGHJ-CAII-WT (wild type), pGHJ-CAIII-, pGHJ-CAII-H64A or pGHJ-CAIV. Isolation to transcription followed the protocol for SNAT3-DNA. Enzymes used for linearization were NotI (Roche Diagnostics GmbH/Mannheim) or SalI (Sigma-Aldrich/Munich).

Oocytes

Female frogs (*Xenopus laevis*) were supplied by Xenopus Express, Haute-Loire, France. Oocytes were isolated as described before [9], separated by collagenase treatment (Collagenase A, Roche, Mannheim, Germany) and left to recover overnight. Oocytes of the stages V and VI were selected and injected with either 11 ng SNAT3-cRNA (23 nl) or equivalent volume DEPC-H_2O. For co-expression, cells were injected with 11 ng SNAT3-cRNA mixed with 11 ng cRNA of CAII-WT, CAII-H64A, or CAIII (46 nl) 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 instead of SNAT3-RNA or with the same amount of CA-cRNA alone as used for co-expression (23 nl). In the experiments with co-expression of SNAT3 and CAIV, we used a concentration of 6.9 ng SNAT3-RNA and 2.07 ng CAIV-RNA.

For experiments with injected protein of CAI or CAII, we used the CAI- and CAII-protein from human or bovine erythrocytes, supplied by Sigma-Aldrich, Munich. SNAT3-expressing oocytes as well as control oocytes were injected the day before experiments with protein in a concentration of 2 µg/µl. Oocytes were stored in HEPES-buffered salt solution (NaCl 82.5 mmol, KCl 2.5 mmol, Na_2HPO_4 1 mmol, HEPES 5 mmol, $MgCl_2$ + 6 H_2O 1 mmol, $CaCl_2$ + 2 H_2O 1 mmol; pH 7.8, Gentamycin, 2 mg/l) at 18°C, and the medium was exchanged every day. This buffer -without antibiotics- was also used in the experiments as the non-bicarbonate buffer, with pH adjusted to 7.0, 7.4, 7.9 or 8.4.For measurements with CO_2-buffered solution, the composition differed in NaCl and $NaHCO_3$ content (in mM): NaCl – 5.5, $NaHCO_3$ – 77 for pH 7.9, and NaCl – 58.5, $NaHCO_3$ – 24 for pH 7.4, and NaCl -72.5, $NaHCO_3$ -10 for pH 7.0, respectively. Amino acids were applied in concentrations of 10 mM, the carbonic anhydrase inhibitor 6-ethoxy-2-bezothiazolsulfonamide (EZA, Sigma-Aldrich, Munich) at 10 or 30 µM. For experiments with reduced sodium, the normal sodium concentration was substituted by equimolar amounts of NMDG (N-Methyl-D-Glucamine, Sigma, Germany).

Electrophysiology

Experiments were performed with microelectrodes impaled into oocytes as reported previously [10-11]. Current- and ion-selective double-barrelled microelectrodes were connected to HS-2A headstages (AxoClamp 2B amplifier, Axon Instruments, CA. U.S.A), gain 10x MG and 1x LU, respectively. For pH-sensitive electrodes, two borosilicate glass capillaries (1 mm and 1.5 mm in diameter) were twisted together and pulled to a micropipette. The tips of the 1.5 mm-micropipettes were backfilled with a mix of tri-N-butylchlorosilane and pure carbon tetrachloride and baked on a hotplate for 4 min 45 sec at 450°C. For filling of electrodes see [11]. Calibration of pH-sensitive electrodes was accomplished by changing the pH of extracellular solution by one pH unit, to which the electrodes responded with 52-54 mV/pH unit change.Oocytes were

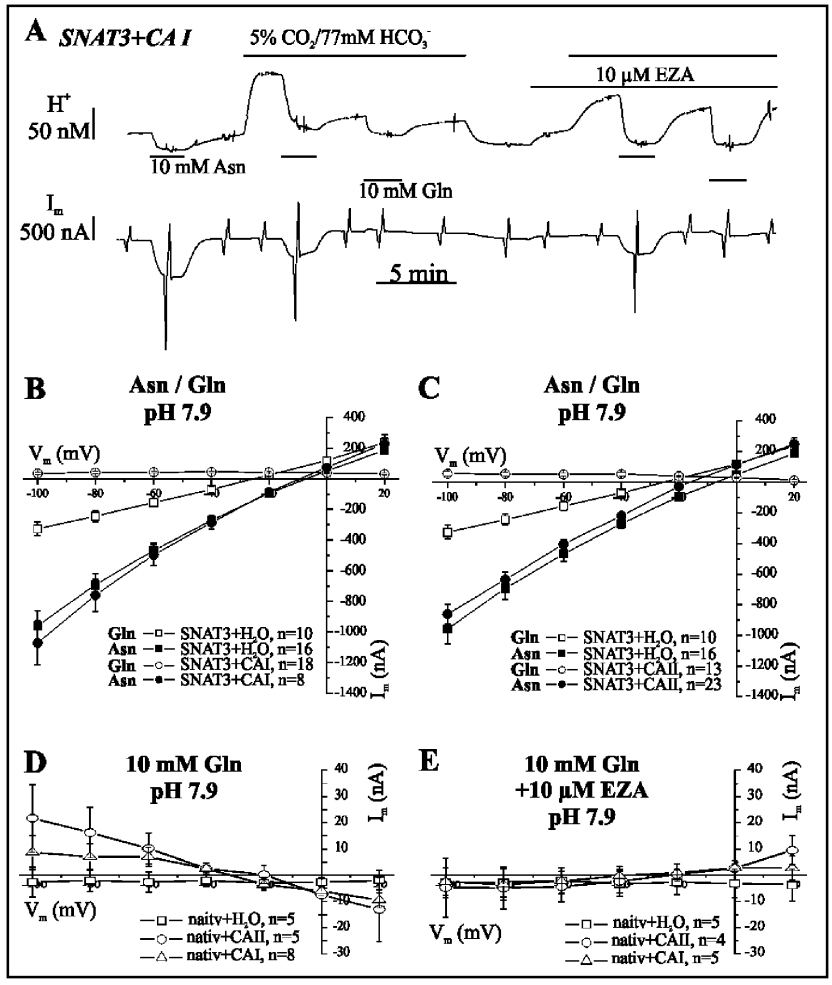

Fig 1. SNAT3-associated membrane conductance is suppressed by catalytic activity of CAI or CAII when glutamine, but not when asparagine is the substrate. (A) Original recording of the changes in intracellular H^+ and membrane current of a SNAT3+CAI-injected oocyte upon application of 10 mM asparagine (Asn) or glutamine (Gln) in HEPES- and in CO_2/HCO_3^--buffered solution (pH 7.9) with and without the carbonic anhydrase inhibitor EZA. (B, C) Current-voltage relationships in 10 mM asparagine and in 10 mM glutamine for SNAT3- and SNAT3+CAI-injected (B) as well as for SNAT3- and SNAT3+CAII-injected oocytes (C). The reversal potentials do not change when asparagine is the substrate with one of the cytosolic CA isoforms present. With glutamine as substrate, the conductance is suppressed in the presence of either CAI or CAII. (D, E) I/V curves of substrate-mediated currents in oocytes with and without injected CAI- or CAII-protein in normal saline, showing that the endogenous conductance induced by the amino acids is negligible.

voltage-clamped at –40 mV and perfused with either 10 mM glutamine or 10 mM asparagine added to HEPES-buffered oocyte saline or to 5% CO_2/HCO_3^--buffered saline. Intracellular pH (pH_i), current (I_m), and membrane potential (U_m) were recorded and displayed with software based on the program LabView (National Instruments Corporation, USA). The pH data were converted into changes of intracellular H^+ (ΔH^+, nM) and H^+ changes per time ($\Delta H^+/t$, nM/min) were analysed from the original traces. Changes of the membrane potential in steps of 20 mV from –100 to +20 mV were carried out to obtain current/voltage-relationships (I/V) of native oocytes and SNAT3-expressing oocytes with injected CAII protein or DEPC-H_2O, or of SNAT3 and CA-co-expressing oocytes. To gain the substrate-induced I/V-relationships, the current values without substrate were subtracted from the corresponding values in substrate. The substrate-induced slope conductance (ΔG_m) was calculated by using the values between -60 and -20 mV from the I/V-curves. Data were analysed and displayed by calculation software Origin (Origin Lab Corporation, Northampton/USA) or by EXCEL (Microsoft Corporation, USA).

Statistics

In the graphs, each data point represents the mean ± standard error of the mean (S.E.M.). For I/V-diagrams the mean values of control oocytes (H_2O-injected) are subtracted from values of each SNAT3-expressing oocyte, thus data points represent the mean ± S.E.M. of differences, which represent only SNAT3-induced changes in membrane current (all non-substrate-mediated currents subtracted). For calculation of significance, the Student's *t*-test, and if appropriate, a paired *t*-test was used. In the figures, a significance level of $p = 0.05$ is marked with *, $p = 0.01$ with **, and $p = 0.001$ with ***.

Results

The effect of CAI and CAII on membrane currents and conductance

We had shown that CAII, either injected as protein or co-expressed in oocytes, suppresses the membrane conductance associated with SNAT3 activity induced by addition of glutamine to the external solution [1]. Since glutamine and asparagine induced quite different conductances of SNAT3, we compared these substrates in the presence of different CA isoforms. CAII- or CAI-protein was injected into *Xenopus* oocytes expressing SNAT3, and intracellular pH and membrane current were recorded at a holding potential of -40 mV. In Fig. 1A an

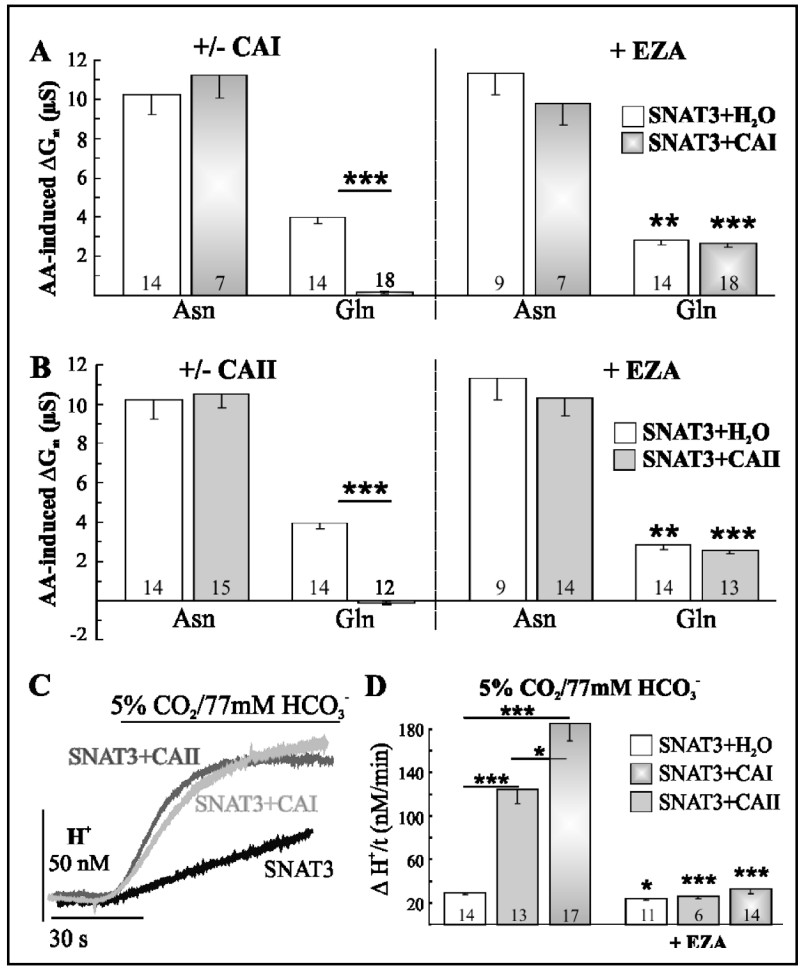

Fig 2. Substrate-induced membrane conductance as modulated by CAI and CAII in absence and presence of a CA inhibitor. (A, B) The amino acid-induced conductance for SNAT3-expressing cells without (white bars) and with injected CAI (grey bars) (A) or CAII (B), as shown in Fig. 1 (left panels), and after blocking the catalytic activity of CAs with ethoxyzolamide (EZA, 10 μM; right panels). (C, D) Intracellular rise of the proton concentration during application of CO_2/HCO_3^--buffered solution indicate catalytic activity by increased rate of H^+ rise (C), and corresponding statistics of the rate of H^+ change for oocytes containing SNAT3, SNAT3+CAI, and SNAT3+CAII with and without EZA (D).

original trace for a SNAT3-expressing, CAI-injected oocyte is shown. We started the measurement in HEPES-buffered saline, pH 7.9, and then changed to CO_2/HCO_3^--buffered saline, pH 7.9, in the absence and in the presence of the CA inhibitor ethoxyzolamide (EZA, 10 μM). We chose the high pH of the solution, because the amino acid-induced conductance is larger at these pH values [7], and we used CAI to evaluate, if its lower catalytic activity as compared to CAII may also suppress the membrane conductance in the presence of glutamine. Glutamine and asparagine both induced a drop in the intracellular H^+ concentration due to uptake of substrate in exchange of H^+. Introduction of CO_2 induced an intracellular acidification, which was rapid with CAI injected, but slow with no CAI, or when enzymatic CAI activity was inhibited by EZA (Fig. 1A). The rate of acidification is an indication of the rate of conversion of CO_2 to H^+ and HCO_3^- and hence of the catalytic CA activity. Native oocytes have very little or no intrinsic CA activity [12, 13].

The deflections in the current trace indicate the currents at varying membrane potentials between -100 and +20 mV, from which I/V relationships were plotted (Fig. 1B-E). These I/V curves indicated larger currents, when asparagine was the substrate as compared to glutamine.

When CAI had been injected, the current was suppressed in glutamine (Fig. 1B), similarly as could be shown when CAII was injected into SNAT3-expressing oocytes (Fig. 1C; see also [1]). Surprisingly, while the currents and conductance were suppressed in oocytes when glutamine was the substrate, the currents induced by asparagine were entirely unaffected by injection of either CAII or CAI (Fig. 1A-C). In native oocytes, or in oocytes injected with either CAII- or CAI-protein without SNAT3, only negligible currents were elicted by glutamine (Fig. 1D, E) or asparagine (not shown).

Inhibition of CAII and CAI by EZA partially recovered the glutamine-induced currents, but had no effect on the asparagine-induced currents (Fig. 1A-C). From the I/V curve, the slope conductance was calculated and plotted for the SNAT3 substrates (Fig. 2A, B). The plots show that first, the SNAT3-associated membrane conductance in asparagine was more than twice that in glutamine and second, that for both, CAII and CAI, the

ΔH^+ (nM)	HEPES			5% CO_2/77 mM HCO_3^-			
	Asn		Gln	Asn		Gln	
SNAT3	-30.3 ± 3.2 (n=16)	n.s.	-21.9 ± 2.6 (n=9)	-56.2 ± 7.7 (n=15)	n.s.	-64.4 ± 7.9 (n=9)	** (Asn) *** (Gln)
SNAT3+ CAII-WT	-33.4 ± 5.1 (n=16)	n.s.	-27.1 ± 3.6 (n=11)	-53.9 ± 11.6 (n=16)	n.s.	-75.3 ± 18.9 (n=11)	n.s. (Asn) * (Gln)
ΔI_m (nA)	HEPES			5% CO_2/77 mM HCO_3^-			
	Asn		Gln	Asn		Gln	
SNAT3	-483 ± 51 (n=12)	**	-241 ± 36 (n=9)	-172 ± 43 (n=15)	*	-40 ± 14 (n=9)	** (Asn) *** (Gln)
SNAT3+ CAII-WT	-406 ± 29 (n=17)	***	-224 ± 11 (n=11)	-216 ± 21 (n=16)	***	51 ± 4 (n=11)	*** (Asn, Gln)

Table 1. Transport activity (delta H^+) and membrane current (delta I_m) of SNAT3- and SNAT3+CAII-wild type-expressing oocyte in HEPES and in CO_2/HCO_3^--buffered saline with pH of 7.9.

conductance was suppressed in glutamine, but not in asparagine. Inhibition of the catalytic CA activity by EZA recovered the glutamine-induced conductance, both when CAI or CAII protein had been injected. The activity of the injected CAII- and CAI-protein was confirmed by an increased rate of intracellular acidification during application of CO_2 as compared to oocytes expressing SNAT3 alone (or native oocytes), which was sensitive to EZA (10 µM; Fig. 2C, D). A robust increase in the rate of acidification was obtained with CAII or CAI injected, and was about 30% larger with CAII than with CAI present. This significant difference in the rate of acidification between CAII and CAI, indicating the catalytic activity of the two CA isoforms, was not reflected by the strength of suppression in glutamine-induced conductance; both CA isoforms suppressed this conductance by more than 95%.

Table 1 summarizes the results for substrate transport (ΔH^+ in nM) and membrane current (ΔI_m in nA) for extracellular pH of 7.9 in HEPES- and in CO_2/HCO_3^--buffered solution. Larger transport in bicarbonate-buffered solution was due to a more expedient proton gradient as a result of the CO_2-induced intracellular acidification, as compared to the HEPES buffer system. This relationship was also shown in an independent set of experiments with radio-labeled glutamine [1], where the transport of 0.5 mM ^{14}C-Gln was 440 pmol/oocyte*5min for SNAT3+H_2O-injected oocytes in HEPES and 700 pmol/oocyte*5min in CO_2-buffered solution (p=0.001). SNAT3+CAII-injected oocytes had comparable values, with 450 pmol/oocyte x 5min in HEPES and 650 pmol/oocyte x 5min in CO_2-buffered solution (p=0.01). However, a higher total transport did not result in larger associated current, which confirmed that the proton-dependent conductance is uncoupled from substrate transport in SNAT3 [7].

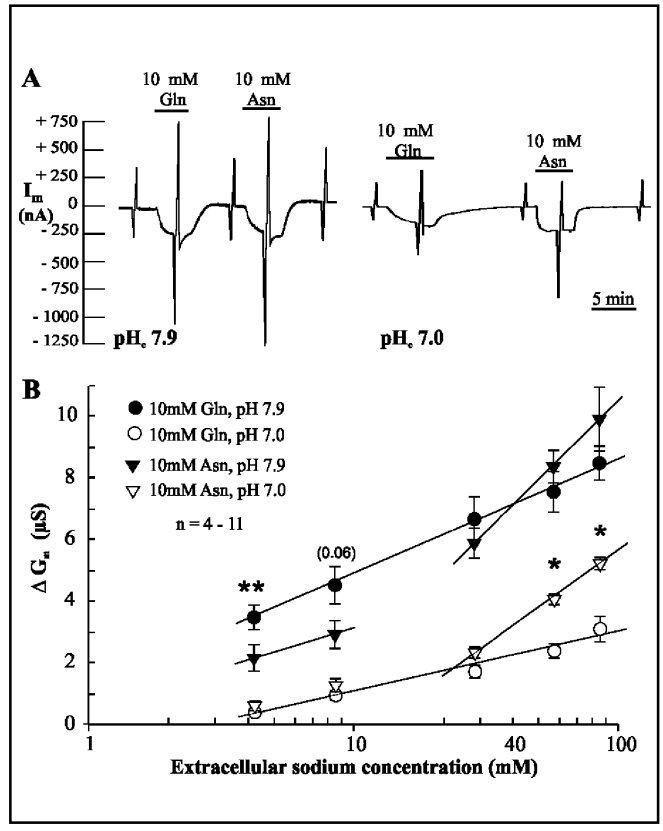

Fig 3. Currents and conductance associated with SNAT3 activity for the substrates glutamine and asparagine at different extracellular sodium concentration and at two different extracellular pH (7.0 and 7.9). (A) Original traces of the membrane current of SNAT3-expressing oocytes as induced by the substrates Gln and Asn in HEPES-buffered saline at extracellular pH (pH_e) 7.9 and 7.0, and at normal Na^+-concentration (85.5 mM; deflection in the traces are currents at different holding potentials between -100 and +20 mV to obtain current-voltage relationships). (B) Semi-logarithmic plot of the substrate-induced slope conductance as determined at different extracellular Na^+-concentrations and at two pH_e values. Note the linear relationship when Gln is the substrate, and the discontinuous relationship when Asn is the substrate.

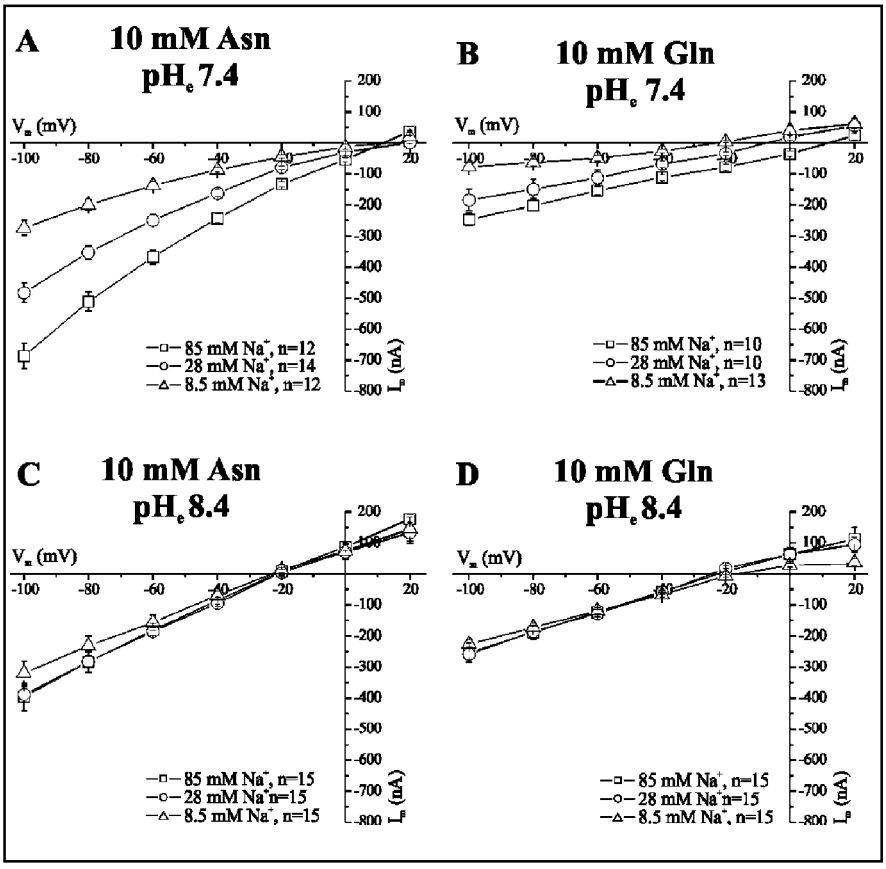

Fig 4. The sodium-dependence of the current-voltage relationship for SNAT3-expressing oocytes in HEPES-buffered solution. Currents were recorded at pH 7.4 (A, B) and pH 8.4 (C, D), for asparagine (A, C) and for glutamine (B, D), respectively at three different Na^+ concentrations as indicated. The currents were sodium-dependent at pH 7.4, where also the reversal potentials varied with the sodium concentration, but sodium-independent at pH 8.4.

	pHe 7,4/40 nM Asn	pHe 7,4/40 nM Gln	pHe 8,4/4 nM Asn	pHe 8,4/4 nM Gln
pH_i	7.67 ± 0.06	7.81± 0.06	7.85 ± 0.05	7.95 ± 0.03
(measured in 85, 28 & 8.5	7.55 ± 0.04	7.7 ± 0.06	7.84 ± 0.04	7.9 ± 0.02
mM Na^+)	7.43 ± 0.09	7.55 ± 0.06	7.74 ± 0.05	7.89 ± 0.04
E_{H+} (mV)	16	24	-32	-26
	9	18	-33	-29
	2	9	-38	-30
E_{Na+} (mV)	72	72	72	72
	33	33	33	33
	13	13	13	13
E_{rev} (mV / measured)	10	10	-25	-25
	20	-10	-25	-25
	20	-20	-25	-20

Table 2. Intracellular pH, H^+ and Na^+ equilibrium potentials (assuming 5 mM intracellular Na^+), and reversal potentials of SNAT3-associated currents, as determined from the current-voltage plots shown in Fig. 4, at different sodium concentrations and pH.

In order to characterize the different modes of conductance induced by glutamine and asparagine in SNAT3-expressing oocytes, we measured the membrane current in different external Na^+ concentrations at two different pH values (7.9, 7.0, see Fig. 3 A). The currents and the conductance as induced by 10 mM amino acid substrate at a holding potential of -40 mV were considerably larger at pH 7.9. The asparagine-induced current was always larger and faster at external pH 7.0 as compared to the currents induced by glutamine (Fig. 3A).

At normal Na^+ concentration, the conductance was larger in asparagine than in glutamine at both pH values, but the difference was more pronounced at pH 7.0 than at pH 7.9 (see Fig. 3B). The slope conductance plotted

against the extracellular Na^+ concentration revealed, however, an increased slope of 6.1 and 9.0 µS/ tenfold change in the sodium concentration ($10*[Na^+]_o$) for the asparagine-induced conductance at higher Na^+ concentrations (>28.5 mM) at pH 7.0 and pH 7.9, respectively, while the glutamine-induced conductance increased linearly with the extracellular Na^+ concentration with 1.8 and 3.3 µS/ ($10*[Na^+]_o$), respectively. At low extracellular Na^+ concentration (<28.5 mM), the conductance increase as induced by asparagine was similarly small as induced by glutamine with 2.1 and 2.6 µS/($10*[Na^+]_o$), respectively. (Fig. 3B). These results indicate that, at both low and high pH value, the SNAT3-associated conductance depends on extracellular Na^+ concentration (as does the transport activity). If asparagine was the substrate, the conductance, especially at the lower pH, was carried more by sodium than by protons, as compared to glutamine, and was more strongly rectifying.

To determine the reversal potentials, which might help to identify the ion species that are involved in the uncoupled conductance, we took current-voltage relationships at three different sodium concentrations in HEPES-buffered solution adjusted to pH_e 7.4 (40 H^+) nM or 8.4 (4 nM H^+). The I/V plots indicated that both slope conductance and reversal potential changed with the extracellular sodium concentration at pH 7.4, but not at pH 8.4 (Fig. 4), suggesting that Na^+ ions contribute to the transport-associated conductance at pH 7.4, but not at pH 8.4, when H^+ are the major carriers, as reported previously [7]**.** The ionic equilibrium potentials for H^+ and Na^+, and the reversal potentials of the associated conductance, are summarized in Table 2. The intracellular pH was measured and the intracellular Na^+ concentration was assumed to be 5 mM in the oocytes [6]**.** Both, Fig. 4 and Table 2, show that the conductance induced by asparagine has a different Na^+ dependence than that induced by glutamine at pH 7.4, with the asparagine-induced conductance revealing a stronger Na^+ dependence, with the reversal potential being 30 to 40 mV more positive at higher extracellular Na^+ concentrations.

Effect of other CA isoforms and mutants

We also co-expressed SNAT3 with CAIII and with the CAII-H64A mutant. CAIII, which lacks the H^+ shuttling histidine at position 64, has very little enzymatic activity *in vitro*, as has also been determined for the mutant CAII-H64A [14-16]. The current traces in Fig. 5A show that the asparagine-induced conductance is not affected by either CA, while the glutamine-induced conductance is suppressed in the presence of both CAIII and CAII-H64A (Fig. 5A, B). The rate of acidification upon introduction of CO_2 indicated similar CA activity for CAII-WT, CAIII and CAII-H64A, which was sensitive to EZA, except for CAIII, which is only partially inhibited by EZA

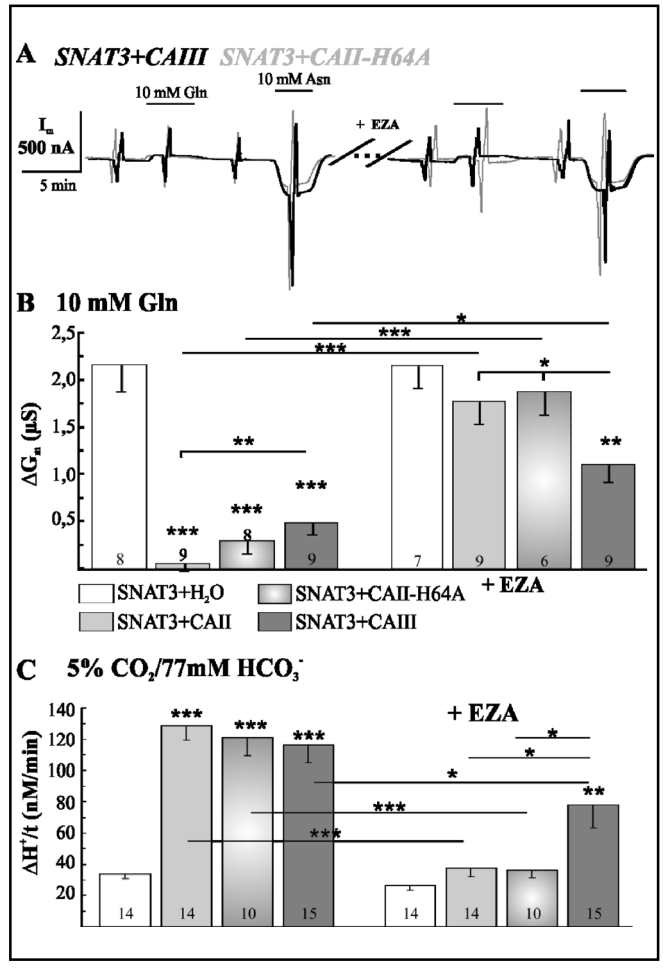

Fig 5. The CAII-H64A mutant and the isoform CAIII showed catalytic activity in oocytes and suppressed the SNAT3-associated, glutamine-induced conductance. (A) Superimposed original traces of membrane current recording (I_m) in CO_2/HCO_3^--buffered solution for SNAT3+CAIII- and SNAT3+CAII-H64A-expressing oocytes. Gln and Asn are applied repeatedly, first without and then with EZA. (B) Glutamine-induced membrane conductance for SNAT3, SNAT3+CAII, SNAT3+CAIII and SNAT3+CAII- H64A-expressing cells. The suppressed conductance in the presence of CA activity is fully or partly (CAIII) rescued after inhibition of CA activity by EZA (10 µM). The significance refers to the values in oocytes expressing SNAT3 alone, if not otherwise indicated by horizontal lines. (C) Changes in the rate of intracellular proton rise during application of CO_2/HCO_3^--buffered solution for the same types of oocytes as in B. CAIII shows significant catalytic activity in oocytes, but inhibition by EZA is incomplete.

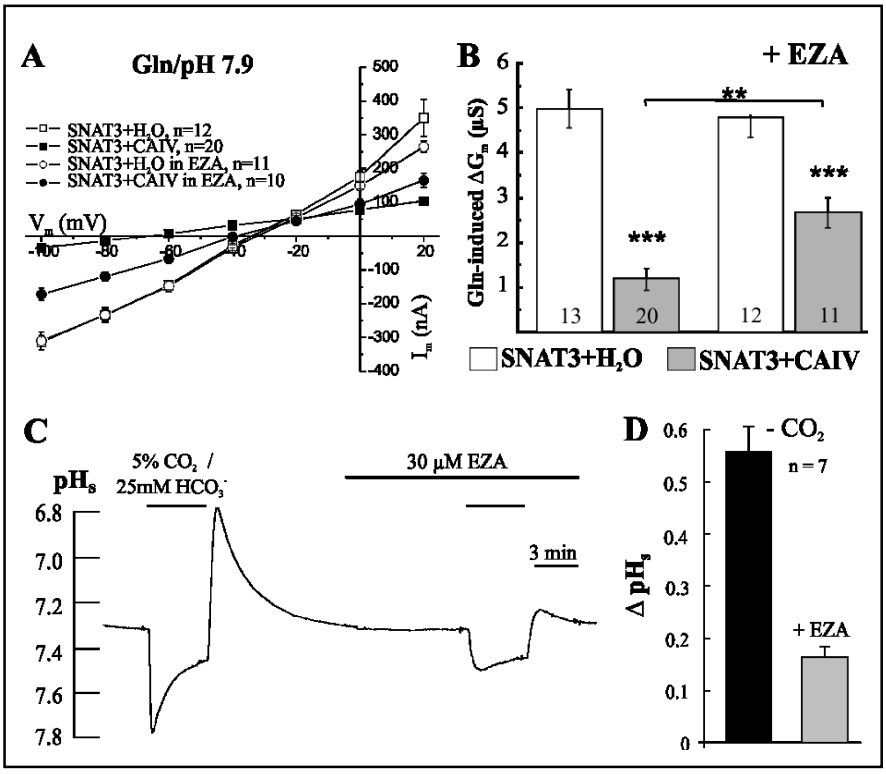

Fig. 6. The extracellular carbonic anhydrase IV (CAIV) also reduced Gln-induced, SNAT3-associated membrane conductance. (A, B) Current/voltage relationships (A) and the corresponding Gln-induced slope conductance (B) for SNAT3- and SNAT3+CAIV-expressing oocytes in the absence and presence of the CA-inhibitor EZA. (C, D) Original trace of the changes in surface pH (pH_s) of oocyte expressing CAIV (C), measured with pH-selective microelectrode gently pushed onto the surface of a cell (at pH 7.4). The pH transients are recorded upon addition and removal of CO_2/HCO_3^- which are greatly reduced in the presence of 30 μM EZA. The large pH_s transient upon removal of CO_2/HCO_3^- (D) was taken as a measure for extracellular catalytic CA activity.

(Fig. 5C; [17]). The high catalytic activity of CAIII and the shuttling mutant CAII-H64A in intact oocytes was surprising, since both have little activity *in vitro* as compared to CAII. However, the glutamine-induced conductance of cells co-expressing SNAT3+CAIII was still measurably larger than in cells co-expressing SNAT3+CAII (Fig. 5B). In the presence of EZA, SNAT3+CAIII-expressing oocytes showed still a significant suppression of glutamine-induced conductance conductance (1.2 ± 0.2 μS, n=8, p<0.05-0.01), at least compared to the other SNAT3- and CA-expressing oocytes (2.2 ± 0.2 μS, n=7 for SNAT3 alone, 1.8 ± 0.2 μS, n=9, for SNAT3+CAII- and 1.9 ± 0.2 μS, n=6 for SNAT3+CAII-H64A-coexpressing oocytes), which is probably due to the lower sensitivity of CAIII to the inhibitor. The results for asparagine are not shown, because they were not different for the different co-expressions with CAIII and CAII-H64A, and in the absence and presence of EZA.

We also co-expressed SNAT3 with the extracellular carbonic anhydrase CAIV. As we found for the intracellular CAs, co-expression with CAIV had no effect on the SNAT3-associated membrane conductance, when asparagine was the substrate (data not shown). With glutamine as substrate, however, the conductance was greatly reduced, as shown by the I/V curves and the slope conductance (Fig. 6A, B). Ethoxyzolamide, applied at a concentration of 10 μM, could, however, only partially recover the conductance. In order to confirm extracellular expression and catalytic activity of CAIV, the surface pH was measured with a pH-sensitive microelectrode, gently pushed against the outer surface of oocytes injected with CAIV-cRNA (Fig. 6C, D). Upon application of CO_2/HCO_3^--buffered solution at constant bath pH of 7.4, the membrane surface pH transiently alkalinized from 7.3 to near 7.8, and after withdrawal of the CO_2/HCO_3^--buffer transiently acidified to 6.8. On average, upon removal of CO_2, the surface pH transient amounted to -0.56 ±0.05 pH units, which was reduced in the presence of EZA (30 μM) to 0.16 ±0.02 pH units (n=7), which was not different from the pH transients in native oocytes. Thus, although these experiments do no exclude intracellular activity displayed by CAIV, they indicate CAIV expression with the catalytic site at the extracellular face of the cell membrane.

Discussion

The present study shows that the associated conductance of SNAT3 depends on the amino acid substrate in magnitude, ion selectivity and sensitivity to carbonic anhydrases. We found that CAII suppresses glutamine-induced conductance [1], while it has no effect on the asparagine-induced conductance. We also tested the in-

tracellular cytosolic carbonic anhydrase isoforms CAI and CAIII, as well as a CAII-mutant, defective of its H^+-shuttling properties by replacement of the histidine residue at position 64 with alanine, and we tested the membrane-bound, extracellularly active isoform, CAIV. Despite their differences in conversion rates tested *in vitro*, and differences in the location of their active centre, either cytosolic or extracellular, all catalytically active CA isoforms suppressed the glutamine-induced, uncoupled, SNAT3-associated membrane conductance, but did not affect the currents and conductance elicited by the substrate asparagine.

We could show a linear relationship of the conductance with rising extracellular sodium concentration for two different extracellular pH values (7.0 to 7.9), when glutamine was the substrate. Asparagine revealed a biphasic relationship (Fig. 3B), with a similar Na^+ dependence at low extracellular Na^+ as glutamine, and a steep increase in sodium affinity at higher extracellular Na^+ concentration (>28 mM). Hence, the large conductance in the presence of asparagine at normal Na^+ concentration is carried mostly by Na^+, and less by H^+. The IV-curves and reversal potentials (Fig. 4, Tab. 2) support this conclusion. At pH 8.4 no shift of reversal potentials due to lower extracellular sodium concentration was observed (Fig. 4). Interestingly, at pH_e 7.4, in asparagine the reversal potential even shifted to more positive potentials by a reduction of sodium. In glutamine, in contrast, the reversal potentials change to more negative values with lowering the extracellular Na^+ concentration; since it is more negative than both H^+ and Na^+ equilibrium potentials, this suggests that other cations, presumably K^+, may contribute to the total current/conductance [7]. These ionic differences might be the reason why CAs would not interfere with the SNAT3-associated conductance activated by asparagine, although the mechanism of the substrate specificity of the CA effect on the SNAT3-associated membrane conductance remains unresolved at this point. Asparagine transport by SNAT3 may indeed serve as a Na^+-signaling gate in astrocytes, similar to the anion conductance of the glutamate transporter of the excitatory amino acid transporter (EAAT) family in neurons [18]. The physiological relevance of the anion conductance of the EAAT was suggested as an inhibitory signal at presynaptic terminals [19, 20]. The sodium conductance of SNAT3 associated with asparagine as substrate may represent a gate for Na^+ removal from astrocytes after being Na^+-loaded by glutamate uptake via GLT-1 and GLAST.

We analyzed the maximum transport activity of SNAT3-expressing cells, measured with H^+-sensitive microelectrodes, and compared these values to the maximal current amplitude detected during transport activity. Asparagine induced a significantly larger current than glutamine in both buffer systems. This aspect might have led to the conclusion by Fei et al. [5] that there is an electrogenic mode for SNAT3, since they worked with asparagine as substrate. Experiments with glutamine [6] predicted a precise 1:1 stoichiometry for Na^+ and H^+, when a proton and a sodium gradient were available to fuel transport process. Table 1 shows that asparagine transport (measured as ΔH^+) did not differ from the transport values for glutamine. Higher transport in bicarbonate-buffered solution was due to a larger proton gradient as compared to the HEPES-buffered solution. This increase in substrate transport did not, however, result in a larger SNAT3-associated current. A considerable acid uptake into astrocytes would be expected in bicarbonate-buffered solution during release of glutamine, because more protons would be taken up than ions flowing back by associated conductance as compared to non-bicarbonate buffer. This acidification is in good agreement for the need of the astrocyte to re-acidify after depolarization-induced alkalinisation [21].

Glutamine is required to refill synaptic neurotransmitter pools, both at glutamatergic [2, 22] as well as at GABAergic synapses [23-25]. A similar role for asparagine as a precursor for aspartate as neurotransmitter is not known. Studies in the rat dorsal horn with immunogold labeling technique for aspartate and glutamate as neurotransmitters in relation to the synaptic vesicle density gave only a positive result for glutamate in A- and C-fibers and supported the hypothesis that aspartate is a low level metabolic amino acid [26]. Pawlik et al. [27] discussed a stimulatory action of the low-affinity substrate asparagine on glutamine transport; especially during starvation, when glutamine is needed in higher amounts for urea synthesis following protein anabolism. Indeed Hutson et al. [28] described an amino acid-dependent up-regulation of asparagine synthase (AS) during starvation. AS catalyses the transaminase conversion of aspartate and glutamine to asparagine and glutamate, respectively [29-30]. Supplying cells with asparagine might involve enhanced SNAT3 activity, if more glutamine than usual is consumed.

CAIII-co-expressing oocytes showed significant catalytic activity, as estimated from the increased rate of acidification (see Fig. 5C), although CAIII was reported to have a very low activity *in vitro* of only 0.3 % of that

of CAII [31]. With a lysine at position 64 and a phenylalanine at position 198, which are both very inefficient proton shuttles [32-33], CAIII also lacks three of six charged residues from the cluster in the CAII-N-terminus [34]. With these properties of CAIII, it is remarkable that CAIII showed significant catalytic activity at all, when expressed in oocytes. This discrepancy might be due to cytosolic factors rescuing activity of CAIII in intact oocytes, or interfering with the conversion rate by using another catalytic center of the CAIII-protein, the phosphomonoesterase activity [35]. It appears that the activity of CAI and CAIII has been restored or activated by a yet unknown factor in the cytosol. Scozzafava and Supuran [36] have shown an increase of catalytic activity of CA by micromolar concentrations of histamine or activators, e.g. carnosine and imidazole derivates, in human erythrocytes. The activity of CAIII was also shown to be increased by small mobile buffers, especially imidazole, in membrane-inlet mass spectrometry and stopped-flow spectrophotometry [37-38]. In the present study, CAIII showed a weaker enzymatic interference with the protons transferred by the conductance during SNAT3-mediated glutamine transport as compared to CAII or CAI.

There are few reports on the specific expression of SNAT3 in muscle tissue, the only organ, where CAIII is highly expressed. Hundal et al. [39] described sarcolemmal glutamine transport mediated by a SNAT family member formerly named system N that was shown to be pH-independent and regulated by insulin -features described by Evans et al. [40] for SNAT2. This System A family member is recently under discussion as a transceptor, a membrane protein with a double role as a transporter and a receptor, being able to sense the nutrition state of preferred amino acids [41]. Onan et al. [42] found SNAT3mRNA in skeletal muscles, but did not measure SNAT3-protein expression. CAI, II and III are expressed in muscle tissue [43]; while immunostaining for CAIII was positive in all muscle fibers, CAII was found mainly in the connecting tissue, and CAI was stained weakly in the sarcolemma. It might be worth testing the significance of glutamine transporter SNAT3 in muscle tissue, first to determine the amount of transport mediated by this pH-dependent amino acid transporter and second to check how strong this protein is regulated by exercise-dependent metabolic changes, for example changes in membrane expression due to insufficient food supply during prolonged exercise or acidification due to increased physical excercise - processes where carbonic anhydrase might beneficially interact with this membrane protein.

CAII and CAIV are expressed in the kidney, as well as SNAT3. E.g., SNAT3 and CAIV are both expressed the at the basolateral membrane of the proximal tubulus [44-45], where glutamine uptake is mainly used to fuel urea production. It might be hypothesized that CAIV support the supply H^+, and CAII dissipates the H^+ accumulation ("pH microdomain") around SNAT3 transport pores by a 'push and pull' mechanism, already discussed by Purkerson & Schwartz [46] for other acid-base transporters in the kidney.

There are several cell types in the brain that might co-express SNAT3 and cytosolic CAII, like astrocytes in the granule cell layer and Bergmann glia dendrites in the molecular layer [47-50]. In the hippocampus, where SNAT3 is shown to be strongly co-localized with the GABA-transporter VGAT [47], inhibition of CAII modified the responses at the basket cell - pyramidal cell synapse of CA1 region [51-52]. In the eye, SNAT3 and CAII are expressed in Müller glial cells of rat and mouse [53-54]. The transport of glutamine appears to be directly related to the recycling of the neurotransmitter glutamate and GABA, and probably serve for the availability of these transmitters during increased activity.

CAIV was equally able to suppress glutamine-induced, but not asparagine-induced conductance. This extracellular CA isoform is known to contribute to extracellular local proton buffering, as e.g. in the hippocampus [55], where it was also reported to enhance anion exchanger 3 (AE3; [56]). By interacting with AE3, the membrane-bound CAIV was suggested to modify also intracellular pH changes. The activity of CAIV can be monitored by large extracellular pH transients near the membrane surface ("surface pH"), much larger than recorded in native oocytes and oocytes expressing CAII (unpublished observations).

In summary, our results indicate that all CA isoforms and the CAII mutant, which display significant catalytic activity in intact oocytes, also reduce the SNAT3-associated membrane conductance, when glutamine, but not when asparagine is the substrate. It is hypothesized that this substrate difference may be attributable to the different ion selectivity of the conductance. It remains to be studied, why these two substrates evoke a membrane conductance which is different in magnitude and ion selectivity, raising the possibility that the amino acid substrates themselves are part of the ion selectivity filter of the SNAT3-associated membrane conductance [57].

Acknowledgements

We are grateful to Dr. William S. Sly, St. Louis, MO, U.S.A., for the supply of the CAIV-cDNA construct, and thank Holger M. Becker for his comments on an earlier version of this manuscript. This work was supported by the Deutsche Forschungsgemeinschaft, GRK 845, and by RIMP of the TU Kaiserslautern.

References

1 Weise A, Becker HM, Deitmer JW: Enzymatic suppression of the membrane conductance associated with the glutamine transporter SNAT3 expressed in Xenopus oocytes by carbonic anhydrase II. J Gen Physiol 2007;130:203-215.

2 Deitmer JW, Bröer A, Bröer S: Glutamine efflux from astrocytes is mediated by multiple pathways. J Neurochem 2003;87:127-135.

3 Chaudhry FA, Reimer RJ, Krizaj D, Barber D, Storm-Mathisen J, Copenhangen DR, Edwards RH: Molecular analysis of system N suggests novel physiological roles in nitrogen metabolism and synaptic transmission. Cell 1999;99:769-780.

4 Chaudhry FA, Krizaj D, Larsson P, Reimer RJ, Wreden C, Storm-Mathisen J, Copenhangen D, Kavanaugh M, Edwards RH: Coupled and uncoupled proton movement by amino acid transport system N. The EMBO Journal 2001;20:7041-7051.

5 Fei YJ, Sugawara M, Nakanishi T, Huang W, Wang H, Prasad PD, Leibach FH, Ganapathy V: Primary structure, genomic organisation, and functional and electrogenic characteristics of human system N 1, a Na^+- and H^+-coupled glutamine transporter. J Biol Chem 2000;275:23707-23717.

6 Bröer A, Albers A, Setiawan I, Edwards RH, Chaudhry FA, Lang F, Wagner CA, Bröer S: Regulation of the glutamine transporter SN1 by extracellular pH and intracellular sodium ions. J Physiol 2002;539:3-14.

7 Schneider HP, Bröer S, Bröer A, Deitmer JW: Heterologous expression of the glutamine transporter SNAT3 in Xenopus oocytes with four modes of uncouples Transport. J Biol Chem 2007;282:3788-3798.

8 Tashian RE: Genetics of the mammalian carbonic anhydrases. Adv Genet 1992;30:321-356.

9 Becker HM, Hirnet D, Fecker-Trost C, Sültemeyer D, Deitmer JW: Transport activity of MCT1 expressed in Xenopus oocytes is increased by interaction with carbonic anhydrase. J Biol Chem 2005;280:39882-39889.

10 Bröer S, Schneider HP, Bröer A, Rahman B, Hamprecht B, Deitmer JW: Characterization of the monocarboxylate transporter 1 (MCT1) in Xenopus laevis oocytes by changes in cytosolic pH. Biochem J 1998;333:167-174.

11 Becker HM, Bröer S, Deitmer JW: Facilitated lactate transport by MCT1 when co-expressed with the sodium-bicarbonate cotransporter (NBC) in Xenopus oocytes. Biophys J 2004;86:235-247.

12 Becker HM, Deitmer JW: Carbonic Anhydrase II increases the activity of the human electrogenic Na^+/HCO_3^- cotransporter. J Biol Chem 2007;282:13508-13521.

13 Becker HM, Deitmer JW: Nonenzymatic proton handling by carbonic anhydrase II during H^+-Lactate cotransport via monocarboxylate transporter 1. J Biol Chem 2008;283:21655-21667.

14 Tu CK, Silverman DN, Forsman C, Jonsson BH, Lindskog S: Role of histidine 64 in the catalytic mechanism of human carbonic anhydrase II studied with a site-specific mutant. Biochemistry 1989;28:7913-7918.

15 Tu C, Qian M, An H, Wadhwa NR, Duda D, Yoshioka C, Pathak Y, McKenna R, Laipis PJ, Silverman DN: Kinetic analysis of multiple proton shuttles in the active site of human carbonic anhydrase. J Biol Chem 2002;277:38870-38876.

16 Jackman JE, Merz KM Jr, Fierke CA: Disruption of the active site solvent network in carbonic anhydrase II decreases the efficiency of proton transfer. Biochemistry 1996;35:16421-16428.

17 Supuran CT, Scozzafava A, Conway J: Carbonic anhydrase its inhibitors and aActivators. CRC Enzyme Inhibitor Series, CRC Press, 2004.

18 Kanai Y, Hediger MA: The glutamate/neutral amino acid transporter family SLC1: molecular, physiological and pharmacological aspects. Pflugers Archive - Eur J Physiol 2004;447:469-479.

19 Wersinger E, Schwab Y, Sahel JA, Rendon A, Pow DV, Picaud S, Roux MJ: The glutamate transporter EAAT5 works as a presynaptic receptor in mouse rod bipolar cells. J Physiol 2006;577:221-234.

20 Veruki ML, Morkve SH, Hartveit E: Activation of a presynaptic glutamate transporter regulates transmission through electrical signaling. Nature Neurosci 2006;9:1388-1396.

21 Deitmer JW, Rose CR: pH regulation and proton signaling in glial cells. Prog Neurobiol 1996;48:73-103.

22 Albrecht J, Sonnewald U, Waagepetersen HS, Schousboe A: Glutamine in the central nervous system: function and dysfunction. Front Biosci 2007;12:332-343.

23 Sonnewald U, Kortner TM, Qu H, Olstad E, Suñol C, Bak LK, Schousboe A, Waagepetersen HS: Demonstration of extensive GABA synthesis in the small population of GAD positive neurons in cerebellar cultures by the use of pharmacological tools. Neurochem Int 2006;48:572-578.

24 Rae C, Hare N, Bubb WA, McEwan SR, Bröer A, McQuillan JA, Balcar VJ, Conigrave AD, Bröer S: Inhibition of glutamine transport depletes glutamate and GABA neurotransmitter pools: further evidence for metabolic compartmentation. J Neurochem 2003;85:503-514.

25 Dienel G, Ryder E, Greengard O: Distribution of mitochondrial enzymes between the perikaryal and synaptic fractions of immature and adult rat brain. Biochim Biophys Acta 1977;496:484-494.

26 Larsson M, Persson S, Ottersen OP, Broman J: Quantitative analysis of immunogold labeling indicates low levels and non-vesicular localisation of L-aspartate in rat primary afferent terminals. J Comp Neurol 2001;430:147-159.

27 Pawlik TM, Lohmann R, Souba WW, Bode BP: Hepatic glutamine transporter activation in burn injury: role of amino acids and phosphatidylinositol-3-kinase. Am J Physiol Gastrointest Liver Physiol 2000;278:G532-G541.

28 Hutson RG, Warskulat U, Häussinger D, Kilberg MS: An example of nutrient control of gene expression: amino acid-dependent regulation of asparagine synthetase. Clin Nutr 1996;15:327-331.

29 Horowitz B, Meister AJ: Glutamine-dependent asparagine synthetase from leukemia cells. Chloride dependence, mechanism of action, and inhibition. J Biol Chem 1972;247:6708-6719.

30 Meister A, Fraser PE: Enzymatic formation of L-asparagine by transamination. J Biol Chem 1954;210:37-43.

31 Elder I, Fischer Z, Laipis PJ, Tu C, McKenna R, Silvermann DN: Structural and kinetic analysis of proton shuttle residues in the active Site of human carbonic anhydrase III. Proteins 2007;68:337-343.

32 Forsman C, Behravan G, Jonsson BH, Liang ZW, Lindskog S, Ren XL, Sandström J, Wallgren K: Histidine 64 is not required for high CO2 hydration activity of human carbonic anhydrase II. FEBS Lett 1988;229:360-362.

33 Chen X, Tu C, LoGrosso PV, Laipis PJ, Silverman DN: Interaction and influence of phenylalanine-198 and threonine-199 on catalysis by human carbonic anhydrase III. Biochemistry 1993;32:7861-7865.

34 Sly WS, Hu PY: Human carbonic anhydrases and carbonic anhydrase deficiencies. Annu Rev Biochem 1995;64:375-401.

35 Koester MK, Pullman LM, Noltmann EA: The p-nitrophenyl phosphatase activity of muscle carbonic anhydrase. Arch Biochem Biophys 1981;211:632-642.

36 Scozzafava A, Supuran CT: Carbonic anhydrase activators: high affinity isozymes I, II, and IV activators, incorporating a beta-alanyl-histidine scaffold. J Med Chem 2002;45:284-291.

37 Tu CK, Paranawithana SR, Jewell DA, Tanhauser SM, LoGrasso PV, Wynns GC, Laipis PJ, Silverman DN: Buffer enhancement of proton transfer in catalysis by human carbonic anhydrase III. Biochemistry 1990;29:6400-6405.

38 An H, Tu C, Duca D, Montanez-Clemente I, Math K, Laipis PJ, McKenna R, Silverman DN: Chemical rescue in catalysis by human carbonic anhydrase II and III. Biochemistry 2002;41:3235-3242.

39 Hundal HS, Rennie MJ, Watt PW: Characteristics of L-glutamine transport in perfused rat skeletal muscle. J Physiol 1987;393:283-305.

40 Evans K, Nasim Z, Brown J, Clapp E, Amin A, Yang B, Herbert TP, Bevington A: Inhibition of SNAT2 by metabolic acidosis enhances proteolysis in skeletal muscle. J Am Soc Nephrol 2008;19:2119-2129.

41 Hundal HS, Taylor PM: Amino acid transceptors: gate keepers of nutrient exchange and regulators of nutrient signaling. Am J Physiol Endocrinol Metab 2009;296:E603-613.

42 Onan MC, Fisher JS, Ju J-S, Fuchs BC, Bode BP: Type I diabetes affects skeletal muscle glutamine uptake in a fibre-specific manner. Exp Biol Med 2005;230:606-611.

43 Moyle S, Jeffery S, Carter ND: Localisation of human muscle carbonic anhydrase isozymes using immunofluoreszence. J Histochem Cytochem 1984;12:1262-1264.

44 Moret C, Dave MH, Schulz N, Jiang JX, Verrey F, Wagner CA: Regulation of renal amino acid transporters during metabolic acidosis. Am J Physiol Renal Physiol 2007;292:F555-F566

45 Purkerson JM, Kittelberger AM, Schwartz GJ: Basolateral carbonic anhydrase IV in the proximal tubule is a glycosylphosphatidylinositol-anchored protein. Kidney Int 2007;71:407-416.

46 Purkerson JM, Schwartz GJ: The role of carbonic anhydrases in renal physiology. Kidney Int 2007;71:103-115.

47 Boulland J-L, Osen KK, Levy LM, Danbolt NC, Edwards RH, Storm-Mathisen J, Chaudhry FA: Cell-specific expression of the glutamine transporter SN1 suggests differences in dependence on the glutamine cycle. Europ J Neurosci 2002;15:1615-1631.

48 Bouland J-L, Rafiki A, Levy LM, Storm-Mathisen J, Chaudhry FA: Highly Differential expression of SN1, a bidirectional glutamine transporter in astroglia and endothelium in the developing rat brain. Glia 2003;42:260-275.

49 Ridderstrale Y, Wistrand PJ: Carbonic anhydrase isoforms in the mammalian nervous system. In Kaila K, Ransom B (eds): pH and brain function. New York, John Wiley, 1998, pp 21-43.

50 Ridderstrale Y, Wistrand PJ: Membrane-associated carbonic anhydrase activity in the brain of CAII-deficient mice. J Neurocytol 2000;29:263-269.

51 Sun MK, Nelson TJ, Xu H, Alkon DL: Calexcitin transformation of GABAergic synapses: from excitation filter to amplifier. Proc Natl Acad Sci USA 1999;96:7023-7028.

52 Sun MK, Nelson TJ, Alkon DL: Functional switching of GABAergic synapses by ryanodine receptor activation. Proc Natl Acad Sci USA 2000;97:12300-12305.

53 Umapathy NS, Li W, Mysona BA, Smith SB, Ganapathy V: Expression and function of glutamine transporters SN1 (SNAT3) and SN2 (SNAT5) in retinal Müller cells. Invest Ophthalmol Vis Sci 2005;46:3980-3987.

54 Vardimon L, Fox LE, Moscona AA: Developmental regulation of glutamine synthetase and carbonic anhydrase II in neural retina. Proc Natl Acad Sci USA 1986;83:9060-9064.

55 Svichar N, Esquenazi S, Waheed A, Sly WS, Chesler M: Functional demonstration of surface carbonic anhydrase IV activity on rat astrocytes. Glia 2006;53:241-247.

56 Svichar N, Waheed A, Sly WS, Hennings JC, Hübner CA, Chesler M: Carbonic anhydrases CA4 and CA14 both enhance AE3-mediated $Cl^{-}HCO^{3-}$ exchange in hippocampal neurons. J Neurosci 2009;29:3252-3258.

57 Bröer S, Schneider HP, Bröer A, Deitmer JW: Mutation of asparagine 76 in the center of glutamine transporter SNAT3 modulates substrate-induced conductances and Na^{+} binding. J Biol Chem 2009;284:25823-25831.