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ORIGINAL ARTICLE

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Na^+ transport by the neural glutamine transporter ATA1

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Abstract Transfer of glutamine between astrocytes and neurons is an essential part of the glutamate–glutamine cycle in the brain. Here we have investigated how the neural glutamine transporter (rATA1/GlnT) works. Rat ATA1 was expressed in *Xenopus laevis* oocytes and examined using two-electrode voltage-clamp recordings, ion-sensitive microelectrodes and tracer flux experiments. Glutamine transport via rATA1 was electrogenic and caused inward currents that did not reverse at positive holding potentials. Currents were induced by a variety of neutral amino acids in the following relative order Ala>Ser/Gln/Asn/His/Cys/Met >MeAIB/Gly>Thr/Pro/Tyr/Val, where MeAIB is the amino acid analogue *N*-methylaminoisobutyric acid. The uptake of glutamine and the corresponding currents depended on Na^+ and pH. Hill-coefficient and flux studies with $^{22}NaCl$ indicated a cotransport stoichiometry 1 Na^+ per transport cycle. The transporter also showed uncoupled Na^+ transport, particularly when alanine was used as the substrate. Although substrate uptake increased strongly with increasing pH, no change of intracellular pH was observed during transport. A decrease of the intracellular pH similarly inhibited glutamine transport via ATA1, suggesting that the pH dependence was an allosteric effect on the transporter.

Keywords Amino acid transport · Glutamate–glutamine cycle · Glutamatergic neurons · System A

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Introduction

The glutamate–glutamine cycle is considered to be the major pathway for glutamate recycling in the brain [7]. NMR studies in vivo suggest that this cycle occurs in the intact brain [18] and that the energy demand of the brain is coupled to the recycling of the neurotransmitter glutamate via glutamine [19]. The majority of neurotransmitter glutamate released by neurons is indeed taken up by astrocytes (e.g., [1]), where it is converted to glutamine by the astrocyte-specific enzyme glutamine synthetase [15]. In order to be recycled, glutamine must be transferred from astrocytes to neurons. Although the uptake of glutamate by astrocytes has been studied in detail (see [17] for a review) a clear picture of the transfer of glutamine from astrocytes to neurons is only beginning to emerge (see [7] for a review).

Only a few studies have addressed glutamine transport in neurons [20, 23]. Glutamine transport has been studied in cultured cerebellar granule cells and cultured cortical neurons [20]. In both cell types glutamine uptake was largely Na^+-dependent, strongly inhibited by the amino acids asparagine, alanine, serine, cysteine, methionine, histidine and the amino acid analogue *N*-methylaminoisobutyric acid (MeAIB). These are typical features of the system A amino acid transporter [2, 9, 13]. Both cerebellar granule cells and cultured cortical neurons express additional glutamine uptake activities, namely system ASC and system L. In cerebellar granule cells in particular, glutamine transport is largely mediated by system A [20]. In a slightly different neuronal cell preparation, an additional glutamine-specific transporter has been described, namely system N^b [23].

In an attempt to clone new members of the vesicular neurotransmitter transporter family, plasma membrane transporters were identified with properties corresponding to the physiologically characterized amino acid

transport systems N and A [8, 10, 11, 14, 16, 21, 22, 24, 26, 27]. Three variants of system A, designated ATA1, ATA2 and ATA3, and two isoforms of system N (SN1 and SN2) were identified. As clones were isolated by several groups independently, ATA isoforms have also been designated as SAT1/2/3 or SA1/2/3 and system N isoforms have been abbreviated NAT1/2. ATA1 is particularly strongly expressed in glutamatergic neurons, suggesting its participation in glutamine uptake [24], and was hence first named GlnT. All members of the family are Na^+- and pH-dependent; however, controversy surrounds the transport mechanism. The pH sensitivity of ATA1 has been attributed to an increase in the maximum velocity [24], whereas for the related rSN1 the antiport of protons has been directly demonstrated in transfected cells by changes in the cytosolic pH that could be recorded during substrate transport [8]. It has not been investigated whether similar changes of the cytosolic pH are associated with substrate transport via ATA1. Similarly, the stoichiometry of the substrate/Na^+-cotransporter has not been investigated. For the related SN1 transporter, the cotransport of one or two Na^+ ions is discussed [8, 10], whereas for ATA2 the cotransport of one Na^+ with the substrate amino acid has been proposed [16, 21, 27].

For the glutamate–glutamine cycle, glutamine release has to occur from astrocytes, whereas neurons should accumulate glutamine. To clarify the role of Na^+ and H^+ in the transport of glutamine via the neural glutamine transporter GlnT/rATA1, we expressed its cRNA in *Xenopus laevis* oocytes and investigated the transport using two-electrode voltage-clamp recordings, intracellular pH electrodes and flux measurements. The data indicate that the pH dependence of rATA1 is caused by an allosteric mechanism and that one Na^+ is cotransported with the substrate. However, Na^+ transport is not always coupled to substrate translocation, particularly when alanine is used as the substrate.

Materials and methods

Materials

L-[U-^{14}C]glutamine (9.36 GBq/mmol), L-[U-^{14}C]alanine and ^{22}NaCl were purchased from Amersham/Pharmacia (Freiburg/Germany or Castle Hill, NSW, Australia). Unlabelled amino acids (all L-enantiomers) were purchased from Sigma/Fluka (Castle Hill, NSW, Australia). The cap-analogue m^{7}G(5′)ppp(5′)G was obtained from New England Biolabs (Schwalbach, Germany) or Life Technologies (Mulgrave, VIC, Australia). Collagenase A was purchased from Roche (Mannheim, Germany). All other chemicals were of analytical grade and were bought from E. Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), and Roche (Mannheim, Germany) or from Merck (Kilsyth, Victoria, Australia) or ICN Biomedicals (Aurora, Ohio, USA).

Cell culture

Neuron-rich primary cultures were derived from embryonic rat brains (E16) as described by Löffler et al. [12]. In brief, embryos were removed from the uteri of pregnant rats after 16 days gestation. Total brains were passed successively through two nylon nets of 135 µm and 20 µm mesh width. One million cells were seeded onto poly-D-lysine-coated 35-mm dishes. After 3 days in culture, cells were treated for 24 h with cytosine arabinosid at a final concentration of 0.5 µM to kill dividing cells. Subsequently, cells were incubated in glia-conditioned neuron culture medium. The cultures were used at a culture age of 5–7 days. These cultures contain some astroglial cells but no oligodendroglial or ependymal cells as determined by cell-specific markers [12].

Cloning of rat ATA1

The rat neuronal glutamine transporter rATA1 was cloned by high-fidelity RT-PCR. For this, total RNA, isolated from rat neuron-rich primary cultures, was reverse transcribed with Superscript II reverse transcriptase. For PCR two primers were constructed which flanked the coding sequence of rATA1. Oligonucleotide ATA1s (5′ GAC ATT CGG ATT GAT TTC TTG A 3′) corresponded to bases 196–217 of the rat ATA1 cDNA sequence [24]. Oligonucleotide ATA1a (5′ GTT GGT CGC TGC TGG TTT 3′) corresponded to bases 1691–1708. The cDNA was amplified in a 30-cycle PCR reaction (45 s 95°C – 45 s 50°C – 480 s 72°C) using Pfu-DNA Polymerase (Promega, Mannheim, Germany). The amplified band was extracted from the agarose gel, the ends were phosphorylated with T4 polynucleotide kinase and the cDNA was subsequently ligated into the *Sma*I site of the oocyte expression vector pGEM-He-Juel [5]. The rat SN1 cDNA was kindly provided by Robert H. Edwards (Departments of Neurology and Physiology, UCSF School of Medicine, San Francisco). The coding region [8] was excised with *Bam*HI and *Hin*dIII and subcloned into the oocyte expression vector pGEM-He-Juel.

Oocytes and injections

Xenopus laevis females were purchased from the South African *Xenopus* facility (Knysna, Republic of South Africa). Oocytes (stages V and VI) were isolated by collagenase treatment as described [25] and allowed to recover overnight.

Plasmid DNA was linearized with *Not*I and transcribed in vitro using the T7 mMessageMachine Kit (Ambion, Austin, Tex., USA). Template plasmid was removed by digestion with RNase-free DNaseI. The complementary RNA (cRNA) was purified twice by phenol/chloroform extraction followed by precipitation with 0.5 vol 7.5 M ammonium acetate and 2.5 vol of ethanol to remove unincorporated nucleotides. The integrity of the transcript was checked by denaturing agarose gel electrophoresis. Oocytes were microinjected with 10 nl rATA1 cRNA in water at a concentration of 1 µg/µl, by using a microinjection device (WPI, Sarasota, Fla., USA) or remained uninjected in the controls.

Flux measurements

For each determination, groups of seven to ten cRNA- or non-injected oocytes were washed twice with 4 ml ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES/NaOH, pH 7.4). They were then incubated at room temperature in a 5-ml polypropylene tube containing 100 µl of the same buffer containing 5 kBq labeled amino acid plus unlabeled substrates as required. Transport was stopped after the appropriate interval by washing oocytes three times with 4 ml ice-cold ND96 buffer. Single oocytes were placed in scintillation vials and lysed by addition of 200 µl 10% SDS. After lysis, 3 ml scintillation fluid was added, and the radioactivity determined by liquid scintillation counting. For efflux experiments, oocytes were preloaded with 100 µM [U-^{14}C]glutamine for 15 min, as described above, and washed three times with 4 ml ice-cold incubation buffer. Efflux was initiated by replacing the ice-cold incubation buffer by 1 ml buffer at room temperature. Aliquots of 100 µl were removed at intervals for counting. The efflux curves were calculated by integration of

the measured radioactivity in the supernatant over time and corrected for the number of oocytes incubated.

Electrophysiological measurements

Two-electrodes voltage-clamp recordings were performed at a holding potential of –60 mV if not otherwise stated as described recently [25]. The data were filtered at 10 Hz and recorded with a MacLab digital-to-analog converter and software for data acquisition and analysis (AD Instruments, Castle Hill, Australia). During measurements oocytes were superfused with ND96-buffer at a flow rate of 20 ml/min and a complete exchange of the bath was reached within about 10 s. For the detection of transport-associated currents, amino acids were added to the superfusate at a final concentration of 3 mM.

pH-sensitive electrodes

pH-sensitive electrodes were made and calibrated as described previously [6]. In brief, borosilicate electrodes were pulled, silanized (hexamethyldisilazane, Fluka Chemicals) and baked at 200°C for 15 min. A column of H^+ cocktail (hydrogen ionophore II-cocktail A, Fluka Chemicals) of ≅300 µm in length was established at the tip of the electrode. The electrode was backfilled with a solution of 100 mM KCl buffered with 10 mM HEPES at pH 7.0. The electrode was calibrated using solutions with pH 6.0, 7.0, and 8.0. Only electrodes with a linear slope >50 mV/pH unit and stable calibration before and after the experiment were used. Signals were preamplified with an electrometer (FD223, WPI, Sarasota, Fla., USA) and subsequently recorded with a feedback amplifier connected to a MacLab digital-to-analog converter. On the basis of the calibration curve for the pH-sensitive electrode, the intracellular pH of oocytes was calculated as the difference between the membrane potential in millivolts measured simultaneously with a 3 M KCl microelectrode and the potential of the pH-sensitive electrode.

Calculations

Kinetic parameters were estimated by fitting uptake data to the Hill-equation $v=V_{max}[S]^n/(K_m{}^n+[S]^n)$, where v is uptake velocity, n the Hill coefficient and [S] is the substrate concentration, using the non-linear regression algorithms of Microcal Origin (Microcal Software, Northampton, USA). For radioactive flux measurements each data point represents the difference between the mean uptake activity (±SD) of seven rATA1- or rSN1-expressing oocytes and seven non-injected oocytes. The SD of this difference was calculated using Gauss' law of error propagation.

Results

Superfusion of rATA1-expressing oocytes with glutamine or alanine strongly depolarized the membrane potential. When the membrane potential was clamped at –60 mV, superfusion caused inward currents, typically in the order of 100 nA at saturating concentrations (Fig. 1). Inward currents were not only caused by glutamine but by other amino acids as well (Fig. 2). The preferred substrate of the transporter was alanine, which always induced the greatest currents. A second group, comprised of glutamine/aspargine/histidine/serine/cysteine and methionine, caused currents 60% to 80% of those induced by alanine. Glycine and the system-A-specific *N*-methylated amino acid analogue MeAIB caused smaller currents of about 20–40% of those induced by alanine. Threonine, tyrosine, proline and valine caused small but significant currents. Cationic, anionic, and hydrophobic amino acids did not induce currents in rATA1-expressing oocytes different from non-injected control oocytes

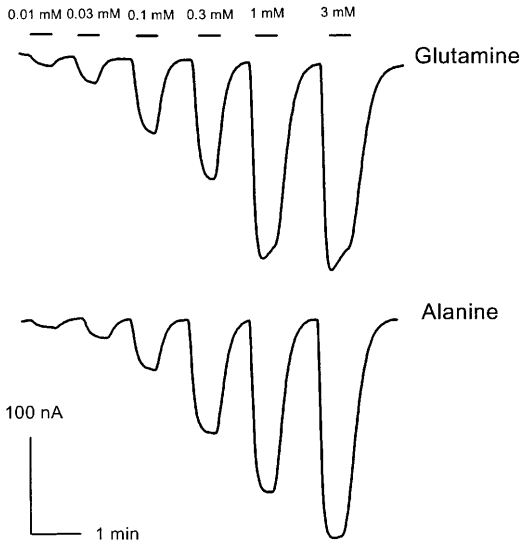

Fig. 1 Alanine and glutamine induce inward currents in ATA1-expressing oocytes. Oocytes were injected with 10 ng rATA1 cRNA. After an expression period of 3 days oocytes were superfused with ND96 supplemented with different concentrations of glutamine or alanine. The concentrations in the superfusate are indicated in the figure, superfusion intervals are indicated by *bars*. Removal of substrates caused a reversible decrease of the currents to background values. The oocytes were clamped at a holding potential of –60 mV

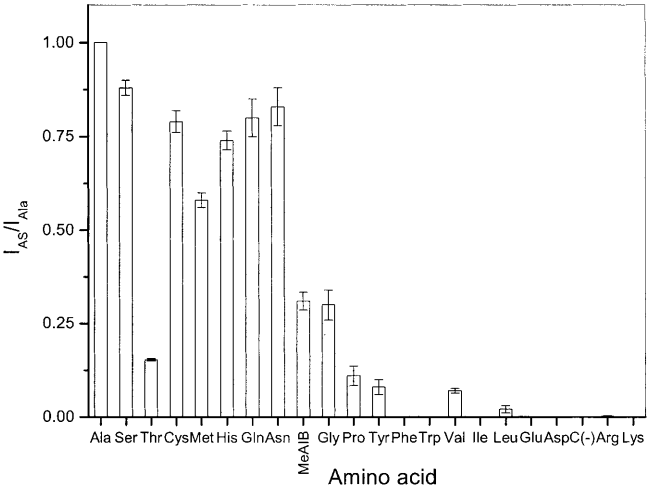

Fig. 2 Substrate specificity of rATA1. Oocytes were injected with 10 ng rATA1 cRNA. After an expression period of 3 days oocytes were superfused with the indicated amino acids. All amino acids were used at a concentration of 3 mM with the exception of cystine [*C(–)*; 0.3 mM]. Amino-acid-induced currents (I_{AS}) were normalized to the maximal current, which was induced by alanine (I_{Ala}). The oocytes were clamped at a holding potential of –60 mV. (*MeAIB* Amino acid analogue *N*-methylaminoisobutyric acid)

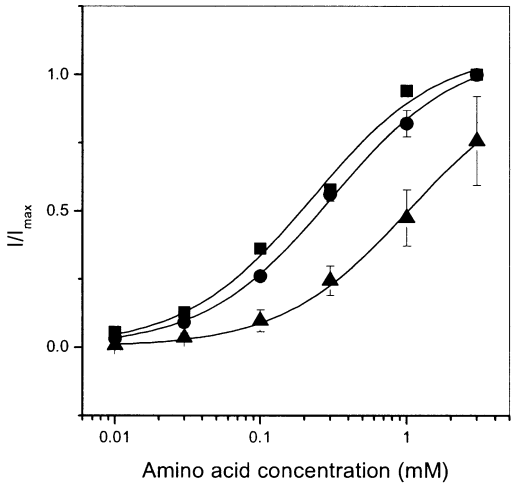

Fig. 3 Kinetic constants of alanine and glutamine transport via rATA1. Oocytes were injected with 10 ng rATA1 cRNA. After an expression period of 3 days oocytes were superfused with different concentrations of alanine (●), glutamine (■) or MeAIB (▲) in ND96 buffer. The oocytes were clamped at a holding potential of –60 mV. Standard errors are indicated by *bars* or were smaller than the size of the *symbol*

(Fig. 2). The half-maximal values for alanine- and glutamine-induced currents (designated $K_{0.5}$ throughout the study), determined at pH 7.4 and at a Na^+ concentration of 100 mM, were found to be 306±26 µM and 230±53 µM, respectively (Fig. 3). These values were similar to the apparent K_m values for uptake determined using labeled alanine and glutamine. In the flux experiments, also conducted at pH 7.4 and at a Na^+ concentration of 100 mM, K_m values of 513±58 µM and 506±108 µM were determined for alanine and glutamine, respectively. The similarity of the data gained by two different methods suggests that the currents are indeed triggered by substrate binding to the transporter. For the system-A-specific inhibitor MeAIB, a K_i of 1.3±0.5 mM was determined for its inhibition of alanine uptake, which coincides very well with the $K_{0.5}$ value of 1.0±0.1 mM as determined by analyzing MeAIB-induced currents (Fig. 3).

To elucidate the nature of the inward currents, the ion dependence of glutamine uptake was determined in flux studies. Transport of labeled glutamine was Na^+ dependent (Fig. 4A), replacement of Na^+ by Li^+ caused a partial reduction of glutamine uptake, whereas replacement by K^+, choline$^+$ or *N*-methyl-D-glucamine (NMDG) caused the almost complete loss of transport activity (Fig. 4A). Replacement of NaCl by Na-gluconate had no effect on glutamine transport, as determined by glutamine-induced currents (Fig. 4B) or by using labeled alanine and glutamine (data not shown). In agreement with published results [24, 26] and similar to the related transporters rSN1 and ATA2, the uptake of glutamine strongly increased with increasing pH (Fig. 5). Taken together, Na^+ and H^+ ions were the most likely candidates for involvement in the transport mechanism.

Substrate-induced currents decreased when the holding potential was shifted to positive values approaching 0 nA at membrane potentials >+40 mV (Fig. 6A, B). An efflux of preloaded labeled glutamine could be elicited by completely replacing extracellular Na^+ by NMDG (Fig. 6C), supporting the notion that the electrochemical gradient of Na^+ was used for uptake. A quantitatively similar efflux could be elicited by adding unlabeled substrates of the transporter, suggesting that the efflux of preloaded glutamine is enhanced by an exchange against unlabeled substrates. The two efflux processes, however, are likely to occur by two different mechanisms. The efflux of glutamine in the absence of Na^+ and extracellular substrate is a net effect. In contrast, the efflux elicited by unlabeled glutamine in the presence of Na^+ probably represents the exchange of labeled for unlabeled substrate. The similar velocities of both efflux processes suggests that the substrate translocation is the rate-limiting step in the transport cycle rather than the return of the unoccupied carrier. This notion was supported by the absence of trans-stimulation. Preloading oocytes with 10 mM MeAIB for 30 min did not alter the subsequent uptake activity for labeled alanine (100 µM; data not shown). The cotransport of glutamine or alanine together with Na^+ was directly demonstrated by flux studies with ^{22}NaCl in parallel experiments with oocytes of the same batch. To increase the specific activity, the extracellular NaCl concentration was reduced to 10 mM, while the remaining 86 mM was replaced by choline chloride. A cotransport stoichiometry of 1:1 was determined for glutamine, whereas for alanine a slightly higher stoichiometry of 1.6:1 was found (Table 1). When substrate-induced cur-

Table 1 Cotransport of Na^+ with glutamine and alanine in oocytes expressing rATA1. Oocytes were injected with 10 ng rat ATA1 cRNA. After an expression period of 3 days, the ND96 buffer was replaced by ND96 buffer containing 10 mM NaCl/86 mM choline chloride. Uptake of 1 mM [U-^{14}C]glutamine or 1 mM [U-^{14}C]alanine was followed over a time course of 20 min at room temperature. In parallel samples, the uptake of ^{22}NaCl was determined in the presence and absence of 1 mM unlabeled amino acid. Values are the mean (±SD) uptake activity of ten oocytes, which was corrected for the transport activity of non-injected oocytes under identical conditions

Conditions	Amino acid uptake (pmol/20 min per oocyte)	^{22}Na uptake (pmol/20 min per oocyte)	Ratio uptake (^{22}Na/amino acid)
None ND96	–	106±23	–
1 mM Alanine	427±66	671±75	1.6
1 mM Glutamine	321±46	312±53	0.97

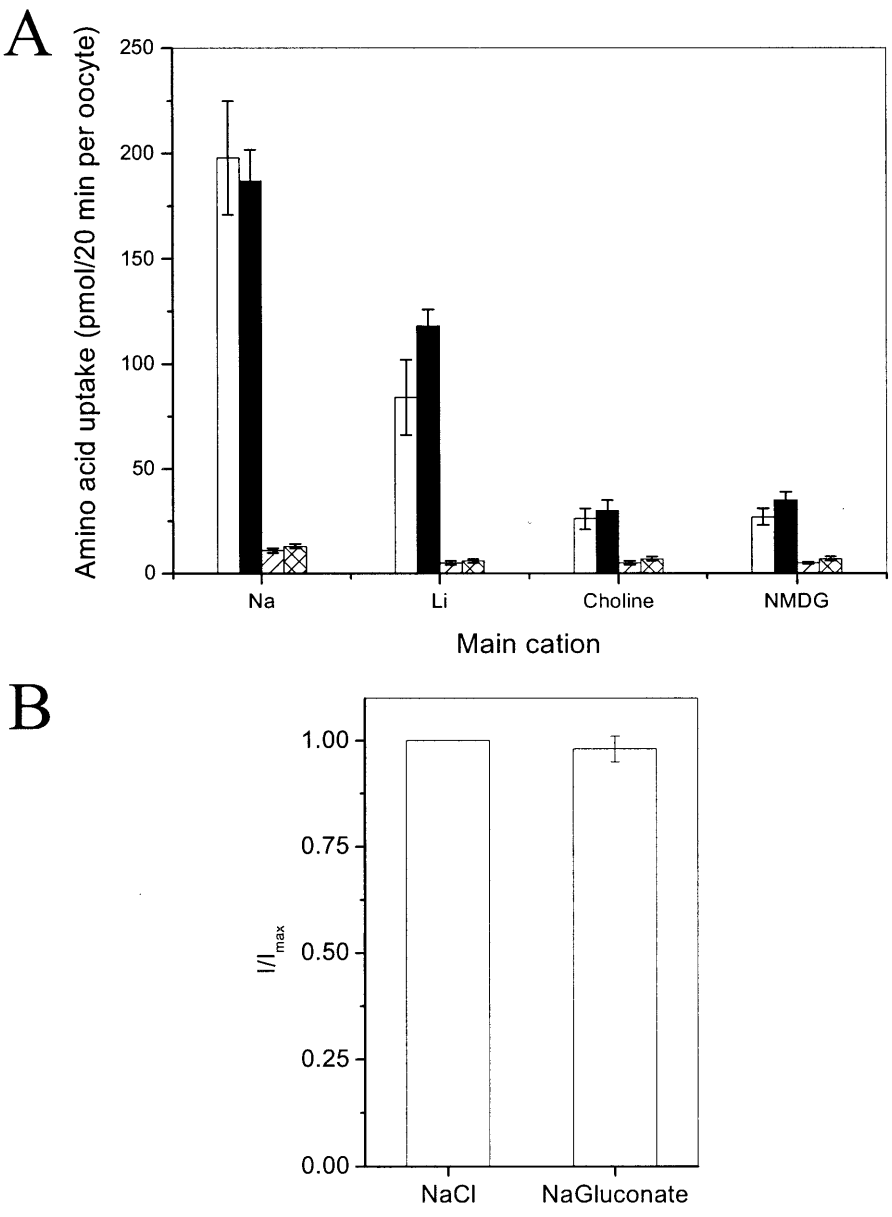

Fig. 4A, B Ion dependence of glutamine uptake and currents in rATA1-expressing *Xenopus laevis* oocytes. **A** Oocytes were injected with 10 ng rATA1 cRNA (*open* and *black bars*) or remained uninjected in the controls (*hatched* and *cross-hatched bars*). After an expression period of 3 days, the uptake of 100 µM [U-^{14}C]glutamine (*open* and *hatched bars*) or 100 µM [U-^{14}C]alanine (*black* and *cross-hatched bars*) was followed over a time course of 20 min in ND96 buffer, and in modified ND96 in which NaCl was replaced by LiCl, KCl, choline chloride or NMDG chloride. Each *bar* represents the mean uptake activity (±SD) of seven oocytes. **B** After an expression period of 3 days oocytes were superfused with glutamine (3 mM) in ND96 buffer and in modified ND96 in which NaCl was replaced by Na-gluconate. The oocytes were clamped at a holding potential of –60 mV

rents were converted into uptake of charges, the derived values were higher than the measured flux rates (data not shown). However, currents were recorded under voltage-clamp conditions at a holding potential of –30 mV, whereas flux studies were performed under current-clamp conditions. When the holding potential in voltage-clamp recordings was set to values that were recorded during superfusion with substrates, transport of charges and fluxes showed matching figures. In other words, a current of –10.8±2.4 nA (389 pmol charges/h) corresponded to a flux 319±20 pmol glutamine/h. Even in the absence of substrate, ATA1-expressing oocytes took up 134±29 pmol $^{22}Na^+$/20 min compared to a basal uptake of 26±4 pmol $^{22}Na^+$/20 min in non-injected oocytes. To further analyze the cotransport of Na^+, the dependence of glutamine transport on NaCl concentration was determined (Fig. 7). At a membrane potential of –30 mV, a $K_{0.5}$ value for Na^+ of 3.4±0.2 mM (1 mM Gln, pH 7.4) was determined. The $K_{0.5}$ value was reduced with hyperpolarization of the membrane potential, showing a value of 1.4±0.1 mM at –60 mV. In agreement with electrophysiological recordings an apparent K_m value of 5±2 mM was determined when the Na^+ dependence of [^{14}C]glutamine (1 mM) fluxes was determined. As pointed out above, flux experiments were carried out under current-clamp conditions, where the membrane potential is likely to approach values more positive than –30 mV. Under most conditions the Na^+ dependence could be fitted with hyperbolic curves, displaying a Hill coefficient of close to 1. However, at alkaline extracellular pH values significant alanine-induced inward currents were recorded even in the nominal absence of Na^+. Since the $K_{0.5}$ of Na^+ decreases at alkaline pH (see below), small traces of NaCl in the solution could be responsible for

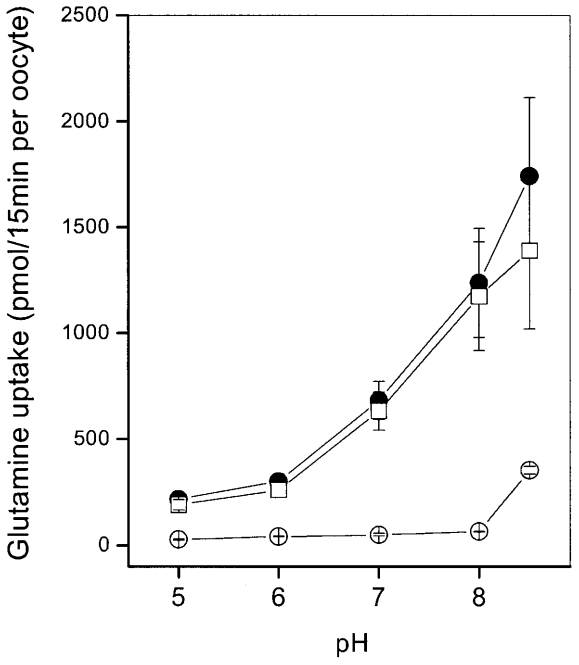

Fig. 5 pH dependence of glutamine uptake in rATA1-expressing oocytes. Oocytes were injected with 10 ng rATA1 cRNA (●) or remained uninjected in the controls (○). After an expression period of 3 days, the uptake of 100 µM [U-^{14}C]glutamine was determined over a period of 15 min in modified ND96 buffer of different pH values. The net transport activity (□) was calculated by subtracting the transport activities of non-injected from that of ATA1-expressing oocytes

this observation. In contrast to substrate-induced currents, the uptake of labeled alanine was entirely Na^+-dependent at pH 8.5 (data not shown).

The uptake of alanine strongly increased with increasing pH. Since ^{22}Na fluxes suggested that the cotransport stoichiometry might exceed unity when alanine was used as a substrate, we analyzed whether protons were involved in the transport mechanism of rATA1. In the mechanism of the related glial glutamine transporter rSN1, amino acid transport is coupled to the exchange of Na^+ ions against protons. To determine fluxes of protons, we recorded changes of the cytosolic pH with intracellular pH electrodes during superfusion with alanine in oocytes expressing rATA1. No changes of the intracellular pH were observed in rATA1-expressing oocytes during the uptake of alanine. By contrast, a substantial alkalization was observed in rSN1-expressing oocytes (Fig. 8). The observed changes of the intracellular pH were reversible (data not shown) and not caused by the metabolism of glutamine (see [3]). In agreement with the observation that protons are not translocated by rATA1, we found that intracellular acidification similarly decreased the transport activity as a decrease of the extracellular pH. In these experiments the intracellular pH was acidified by incubation of oocytes in $NaHCO_3$ solutions. As the volume of the transport solution was too small to be kept in equilibrium with CO_2, solutions were transiently titrated to physiological pH values and used immediately. The addition of 30 mM $NaHCO_3$ (pH 7.4) to the transport buffer decreased alanine uptake from 180±28 to 140±27 pmol/20 min per oocyte. A stronger decrease of the cytosolic pH, achieved by adding 90 mM $NaHCO_3$, further decreased transport activity to 59±14 pmol/20 min.

The results presented above demonstrate that only Na^+ and the substrate amino acid participate in the transport mechanism of rATA1. The strong increase of transport velocity with increasing pH as seen in rATA1 partially resulted from a decrease of the $K_{0.5}$ for Na^+ from 31±9 mM at pH 6.0 to 3.5±1.5 mM at pH 7.4, as determined by the analysis of glutamine-induced currents at a holding potential of –50 mV. To further investigate the transport mechanism, the mutual influence of the substrate and cotransported ion was determined (Table 2). The apparent $K_{0.5}$ value for Na^+ depended only weakly on the substrate concentration. In contrast, a reduction of the Na^+ concentration significantly increased the $K_{0.5}$

Table 2 Mutual influence of Na^+ and glutamine or alanine in oocytes expressing rATA1. Oocytes were injected with 10 ng rATA1 cRNA or remained uninjected in the controls. After an expression period of 3 days, the apparent K_m values for Na^+ and glutamine, MeAIB or alanine were determined at different concentrations of substrate and Na^+. For analyzing Na^+ dependence, NaCl was stepwise replaced by NMDG chloride. Uptake of [U-^{14}C]glutamine or [U-^{14}C]alanine was followed over a time course of 20 min at room temperature

NaCl (mM)	As (mM)	Membrane potential (mV)	K_m Na^+ (mM)	K_m substrate (µM)
100	Gln varied	–30	–	239±28
100	Gln varied	–60	–	137±5
30	Gln varied	–60	–	300±60
10	Gln varied	–60	–	550±140
100	Ala varied	–60	–	306±26
Varied	Gln (1 mM)	–30	3.4±0.2	–
Varied	Gln (1 mM)	–60	1.4±0.1	–
Varied	Gln (0.2 mM)	–60	2.4±1.1	–
Varied	Ala (1 mM)	–60	1.7±0.5	–
Varied	Ala (0.2 mM)	–60	1.6±0.7	–
Varied	Gln (1 mM)	Variable (flux)	5.0±2.0	–
100	MeAIB varied	–60	–	1039±114
100	MeAIB varied	Variable (K_i)	–	1300±500

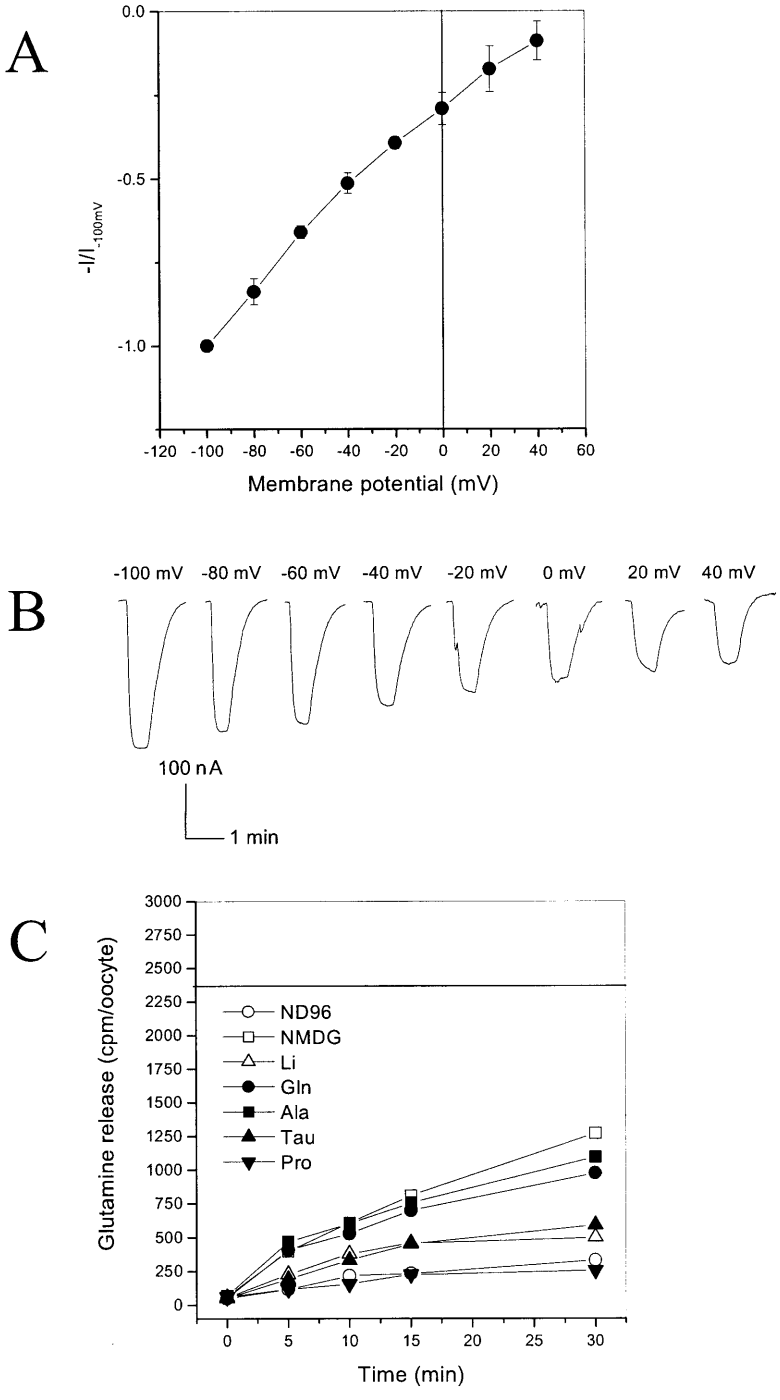

Fig. 6A–C Reversibility of rATA1 depends on the electrochemical gradient of Na^+. Oocytes were injected with 10 ng rATA1 cRNA. **A** After an expression period of 3 days oocytes were clamped at a holding potential of –60 mV. After reaching a constant inward current, voltage ramps from –100 to +40 mV were applied. Ramps were performed in the absence and presence of substrate (2 mM alanine). The depicted *curve* shows the difference between currents in the absence and presence of alanine. In other experiments oocytes were clamped at different voltages and the substrate-induced inward currents were separately recorded at each holding potential (**B**). The reversibility of glutamine transport via ATA1 was studied in flux experiments (**C**). For each curve 10 oocytes were preloaded with 100 µM [^{14}C]glutamine for 15 min. After washing, glutamine efflux was determined in ND96 buffer (○), in Na^+-free NMDG-substituted ND96 (□) in Li^+-substituted ND96 (△), or ND96 containing 3 mM glutamine (●), 3 mM alanine (■), 3 mM taurine (▲) or 3 mM proline (▼). The *curve* represents the combined efflux of all oocytes divided by the number of oocytes. The *horizontal line* indicates the level of preloading

value for glutamine from 137±5 µM at 100 mM NaCl to 550±140 µM at 10 mM NaCl (Table 2), suggesting an ordered binding of the substrates. The $K_{0.5}$ value for glutamine also depended on the membrane potential, increasing from 137±5 µM to 239±28 µM, when the holding potential was shifted from –60 mV to –30 mV, respectively.

Discussion

The transfer of glutamine from astrocytes to neurons in the brain critically relies on the presence of suitable transport systems in both cell types. It is likely that neuronal and glial glutamine transporters display different properties, being adapted to glutamine uptake and efflux, respectively. In this report we have investigated the mechanism, stoichiometry, and reversibility of a recently cloned neuronal glutamine transporter ATA1 [24]. Ex-

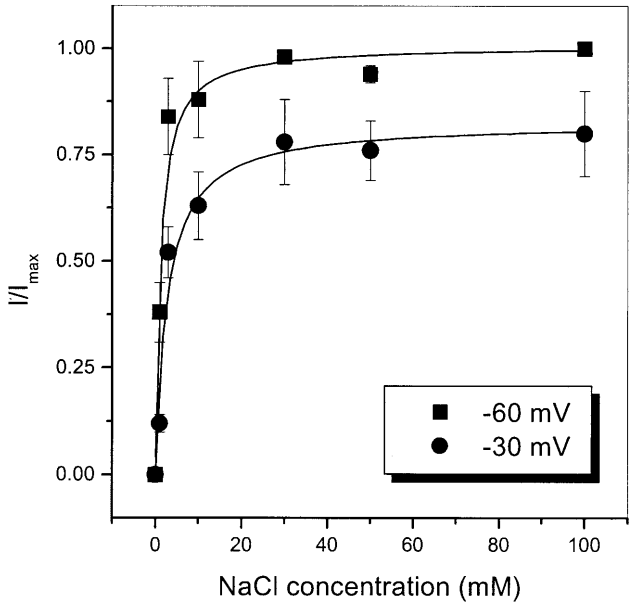

Fig. 7 Na^+ dependence of glutamine-induced currents in rATA1-expressing oocytes. Oocytes were injected with 10 ng rATA1 cRNA. After an expression period of 3 days oocytes were superfused with glutamine in modified ND96 buffer in which NaCl was gradually replaced by NMDG chloride. The oocytes were either clamped at a holding potential of –60 mV (■) or –30 mV (●)

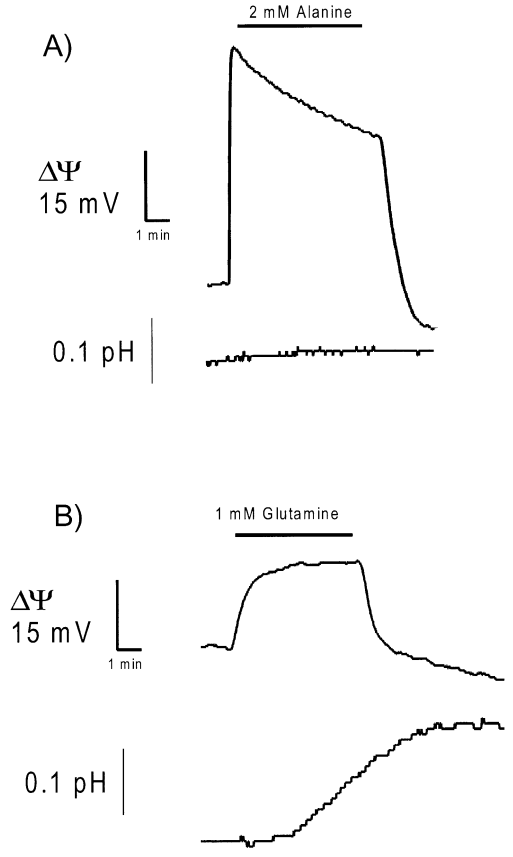

Fig. 8A, B Amino acid transport via rSN1 but not via rATA1 is accompanied by changes in the cytosolic pH. Oocytes were injected with 10 ng rATA1 (**A**) or 10 ng rSN1 cRNA (**B**). After an expression period of 3 days oocytes were superfused with 2 mM alanine (rATA1) or 1 mM glutamine (rSN1) in ND96 buffer. The membrane potential and the cytosolic pH were recorded under current-clamp conditions. Superfusion intervals are indicated by *bars*

pression of the transporter might not be confined to neurons, as we were able to amplify its cDNA from both neuronal and astroglial mRNA (data not shown), but it appears to be more abundant in neurons. The initial characterization of this transporter [24, 26] demonstrated its Na^+ dependence but did not address the cotransport stoichiometry or the involvement of protons, questions that became critical subsequent to the cloning of other members of this family. To investigate these issues, we compared flux studies with intracellular pH and voltage-clamp recordings.

Rat ATA1 is a non-specific Na^+-dependent transporter for neutral amino acids. The transporter appears to prefer amino acids with polar side-chains, particularly glutamine, asparagine, methionine, cysteine, serine, etc., whereas amino acids with hydrophobic side-chains like the branched-chain amino acids are not accepted as substrates. Surprisingly, serine is strongly preferred over threonine, a feature that has also been observed in flux studies with cultured cells [2]. Charged side-chains are not tolerated by rATA1.

Flux studies using $^{22}NaCl$ and Hill analysis indicated that ATA1 probably transports neutral amino acids in a 1:1 cotransport with Na^+. Protons are not translocated by the transporter itself as in the related transporter SN1. The delay of intracellular alkalization as observed in SN1-expressing oocytes after substrate addition (Fig. 8) is likely to be a result of the positioning of the intracellular pH electrodes [6]. The onset and the slope of changes of the intracellular pH thus varied between different oocytes; however, it was always observed in SN1-expressing oocytes but not in ATA1-expressing oocytes.

The main effect of an increased proton concentration on ATA1 is a reduction of the affinity of Na^+ on the exofacial side. The Na^+ concentration in turn affected the apparent K_m value for glutamine, whereas less influence of the glutamine concentration was detected on the apparent K_m of Na^+. This suggests that Na^+ binds to the transporter before the substrate amino acid, which in turn would cause the voltage dependence of the glutamine K_m value. Binding of glutamine is not expected to be affected by the membrane potential, whereas Na^+ binding is. The observed Na^+-cotransport stoichiometry of >1 when alanine was used as a substrate cannot be attributed to an exchange of Na^+, as recently demonstrated in the case of the glutamine transporter ASCT2 [4]. It appears more likely that uncoupled sodium fluxes are responsible for the observed stoichiometry of >1, a notion that is supported by increased substrate-independent Na^+ fluxes in ATA1-expressing oocytes.

Although a slow exchange and efflux activity was observed in ATA1-expressing oocytes, the transporter

mainly mediates net influx of Na^+ and substrate. The currents are generated by Na^+-cotransport and the release of substrate is only slightly stimulated by extracellular substrates. By contrast, in the obligatory amino acid exchanger ASCT2 [4], currents are carried by anions, the transport of which is not coupled to substrate translocation. Moreover, efflux is as fast as influx in the presence of extracellular substrates.

The characteristics of system-A-isoform ATA1 closely resemble those of ATA2 [16, 21, 27] and ATA3 [22]. However, ATA1 and ATA2 have a tenfold higher affinity for alanine than ATA3. In respect to the concept of the glutamate–glutamine cycle, it is worth noting that ATA1 has the highest affinity for glutamine of all isoforms. Differences were also detected in terms of substrate specificity. MeAIB is a slowly transported substrate of both ATA1 and ATA2, but is translocated in negligible quantities by ATA3 ([22], although it might act as an inhibitor of ATA3). Methylation of the α-amino group decreases the translocation rate for all isoforms, suggesting that the substrate translocation step is rate limiting for transport. Proline is a good substrate for ATA2 and ATA3 but almost not recognized by ATA1. Glutamine, in contrast, is a good substrate for ATA1 and ATA2 but is almost not recognized by ATA3. All isoforms display substrate and Na^+ K_m values that increase under depolarizing conditions. However, the strongest influence on the K_m for Na^+ in the case of ATA1 is exerted by the extracellular pH.

The properties of ATA1 fit well with its proposed function as a neuronal glutamine transporter. It accumulates glutamine inside the cytosol using the electrochemical gradient of Na^+ ions. In contrast to SN1, protons do not act as an opposing driving force, but rather regulate the transporter by changing the K_m for the cotransported Na^+ ion.

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Note added in proof Since submission of this paper Gu et al. (Journal of Biological Chemistry, Vol 276, pp 24137–24144) have reported isolation of a mouse cDNA sequence, the derived amino acid sequence of which is 97% identical to the sequence used in this study and is 92% identical to the human ATA1 [26]. The authors claim, providing little functional evidence, that the mouse cDNA encodes a transporter with functional properties of System N, and have hence named it NAT2. The results of this study are at variance with this notion demonstrating that ATA1 has functional properties of system A and does not represent a functional correlate of system N. The sequence isolated by Gu et al. in all likelihood is the mouse ATA1 transporter.

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