The glutamine transporter SNAT3 (SLC38A3, former SN1) plays a major role in glutamine release from brain astrocytes and in glutamine uptake into hepatocytes and kidney epithelial cells. Here we expressed rat SNAT3 in oocytes of Xenopus laevis and reinvestigated its transport modes using two-electrode voltage clamp and pH-sensitive microelectrodes. In addition to the established coupled Na\(^+\)-glutamine-cotransport/H\(^+\) antiport, we found that there are three conductances associated with SNAT3, two dependent and one independent of the amino acid substrate. The glutamine-dependent conductance is carried by cations at pH 7.4, whereas at pH 8.4 the inward currents are still dependent on the presence of external Na\(^+\) but are carried by H\(^+\). Mutation of threonine 380 to alanine abolishes the cation conductance but leaves the proton conductance intact. Under Na\(^+\)-free conditions, where the substrate-dependent conductance is suppressed, a substrate-independent, outwardly rectifying current becomes apparent at pH 8.4 that is carried by K\(^+\) and H\(^+\). In addition, we identified a glutamine-dependent uncoupled Na\(^+\)/H\(^+\) exchange activity that becomes apparent upon removal of Na\(^+\) in the presence of glutamine. In conclusion, our results suggest that, in addition to coupled transport, SNAT3 mediates four modes of uncoupled ion movement across the membrane.

The activity of ion-coupled membrane transporters can be associated with currents that depolarize or hyperpolarize the cell membrane. These currents may be due to electrogenic transport stoichiometry and/or to a non-stoichiometrical ion conductance (1). Transport-associated ion conductances have been identified in a number of transporters but have been particularly well studied in several neurotransmitter transporters (2–6). The currents associated with glutamate transporters of the SLC1 family have two components, a current elicited by the thermodynamically coupled cotransport of 3Na\(^+\) and 1H\(^+\) into the cell during glutamate uptake and an uncoupled conductance, which is carried by anions (7). The ratio between uncoupled and coupled current varies between the different glutamate transporter isoforms (8). Substrate-induced anion currents are also observed for the ASC-type neutral amino acid transporters, which are sequence related to the glutamate transporters (9, 10). The physiological relevance of these conductances has not been clarified but could be significant for EAAT4, which shows little transport but a high conductance (11). The physiological relevance has been better documented for a chloride conductance associated with the dopamine transporter (12, 13), which appears to modulate neuronal activity in the midbrain.

Interestingly, transport-associated conductances have also been observed in electroneutral transporters that do not carry out net charge movement (3, 9, 10, 14, 15). The serotonin transporter, for example, has several conductance states although its transport cycle involves the cotransport of 1Na\(^+\), 1Cl\(^-\), and the antiport of 1K\(^+\) with each serotonin\(^(+\)) molecule. Accordingly, the uptake of serotonin is unaffected by the membrane potential, but substrate application to oocytes expressing the rat 5-HT transporter results in significant inward currents (3). Very similar observations were made in the case of the glutamine transporter SNAT3. Uptake of glutamine is coupled to the cotransport of 1Na\(^+\) and the antiport of 1H\(^+\) and hence is unaffected by changes of the membrane potential (15, 16). However, inward currents are observed during transport (14, 15, 17). The carrier of these currents has been discussed controversially. Initially, the transporter was thought to be electrogenic (17), but this was refuted by two subsequent studies (14, 15). Further analysis of the currents showed that the reversal potential of the substrate-induced currents varies with pH, and as a result a proton conductance was proposed by Chaudhry et al. (14). Broer et al. (15), by contrast, did not detect such a shift and proposed a slippage mechanism in the transporter or the activation of oocyte endogenous nonspecific ion channels. To resolve these discrepancies, we reinvestigated ion transport activities associated with SNAT3 expression in oocytes in more detail. Our results suggest the presence of two substrate-induced conductances, one of which is carried by cations and the other by protons. In addition, we identified a substrate-independent cation conductance and a Na\(^+\)/H\(^+\) exchange activity.

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1 To whom correspondence may be addressed. E-mail: stefan.broeer@anu.edu.au.

2 To whom correspondence may be addressed. E-mail: deitmer@biologie.uni-ki.de.
**EXPERIMENTAL PROCEDURES**

**Oocytes and Injections**—Xenopus laevis oocytes (stages V and VI) were isolated by collagenase treatment as described (18) and allowed to recover overnight. The surgical removal of ovarian tissue was performed under anesthesia (20-min immersion in 1% MS-222) and was approved by the animal ethics committee of the University of Kaiserslautern. Rat SNAT3 was used as described previously (15). Oocytes were microinjected with 5–20 nl of rSNAT3 cRNA in water at a concentration of 1 μg/μl by using a microinjection device (WPI, Sarasota, Fl) or remained uninjected in the controls.

**pH Measurements with Microelectrodes**—Double-barrelled pH-sensitive microelectrodes to measure intracellular pH (pH$_i$) and membrane potential in frog oocytes were prepared as previously described (19). Briefly, the electrodes were pulled in two stages and silanized by filling a drop of 5% tri-N-butylchlorosilane in 99.9% pure carbon tetrachloride into the prospective ion-selective barrel and then baking the pipette on a hot plate at 475 °C for 4.5–5 min. For pH-selective microelectrodes a small amount of H$^+$-ionophore mixture (Fluka 95291) was backfilled into the tip of the silanized barrel and the remainder filled with 0.1 M sodium citrate, pH 6.0. The reference barrel was filled with 2 M potassium acetate. Electrodes were accepted for experiments if their response exceeded 50 mV/unit change in pH and if they reacted faster in the bath during calibration before and after each experiment than the fastest pH$_i$ changes recorded upon glutamine addition. On average, electrodes responded with a change of 54 mV to a change in pH by one unit. The recording arrangement was the same as described previously (20). The central and the reference barrels were connected by chlorided silver wires to the headstages of an electrometer amplifier. As described previously (21) 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 $\geq -25$ mV were used for experiments.

**Electrophysiological 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 was $\sim 1$ MΩ. For voltage clamp recordings, both electrodes were connected to the head stages of an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA). The experimental bath was grounded with a chloride-treated silver wire coated by agar dissolved in oocyte saline. Oocytes were clamped at $-40$ mV unless indicated otherwise. To measure ion conductances associated with SNAT3 expression, short voltage jumps were applied during recordings in increments of 20 mV ranging from $-100$ to $+20$ mV.

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**FIGURE 1.** Substrate dependence of SNAT3 expressed in oocytes. A, typical recording of the current and intracellular pH associated with activating SNAT3 with different concentrations of glutamine (0.2–5 mM), with histidine (5 mM), and with asparagine (5 mM). Conductance and current-voltage relationships were measured by applying voltage jumps in increments of 20 mV from $-100$ to $+20$ mV at different stages of the recording; in the intervening periods oocytes were held at $-40$ mV. Evaluation of the current-voltage relationships of glutamine-induced currents is shown in panel B ($n = 5$) and of histidine and asparagine-induced currents in panel C ($n = 5$). D, evaluation of the initial rate of proton change (ΔH$^{-}$/t) ($n = 5$), which is used as an indicator for the transport activity. E, evaluation of the conductance change (ΔG_m) associated with the SNAT3 transport activity ($n = 5$). The conductance depends on the substrate used and on the substrate concentration.

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3 The abbreviation used is: pH$_i$, intracellular pH.
**Site-directed Mutagenesis**—Site-directed mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as recommended by the manufacturer. The following primers were used (only sense primers shown, amino acid changing mutation in uppercase): N76H, ttc aat ctc agc Cac gcc atc atg ggc; G210A, atg cga cag ctt gCc tac ctg gcc tac Gcc agt ggc ttc tct; H300Q, gcc ttg tgc caA cct gag gtg ctg; T343A, ttc ggc tac ctg Gcc ttc tat gac ggg; R370Q, atc ttg tgt gtg cAG gtg gcc gtg ctg; and T380A, gcg gtc aca ctt Gcg gtt ccg atc gtt.

**Surface Biotinylation**—To determine plasma membrane expression, 15 oocytes were washed three times with 4 ml of ice-cold phosphate-buffered saline, pH 8.0. Subsequently, oocytes were incubated in 0.5 ml of sulfo-NHS-lc-Biotin (0.5 mg/ml; Pierce) solution in phosphate-buffered saline for 10 min at room temperature. The reagent was removed by washing oocytes four times with 4 ml of ice-cold phosphate-buffered saline, pH 8.0. Subsequently, oocytes were lysed by incubation in 1 ml of lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1% Triton X-100) for 30 min on ice. The lysate was centrifuged at top speed in a tabletop centrifuge for 15 min at 4 °C, and the supernatant was mixed with 30 μl of streptavidin-coated agarose particles (Pierce). The suspension was incubated at 4 °C overnight with slight agitation. Agarose particles were washed four times with 1 ml of lysis buffer and the pellet subsequently resuspended in 10 μl of SDS-PAGE sample buffer. Samples were boiled for 5 min and an aliquot of 30 μl loaded on the gel. After gel electrophoresis, proteins were blotted onto nitrocellulose membranes. SNAT3 was detected using an anti-peptide antibody at a 1:1000 dilution that was raised against a rat SNAT3 peptide (Zymed Laboratories, South San Francisco, CA). Antibody binding was detected by enhanced chemiluminescence using the ECL system according to the manufacturer’s instructions using the provided secondary antibody at a dilution of 1:2000 (Amersham Biosciences).

**Statistical Analysis**—The figures display typical recordings from individual oocytes combined with a statistical analysis of current-voltage relationships derived from these recordings. Data are presented as means ± S.E. of the mean. The number of oocytes (n) from separate experiments used to calculate the means is given in the figure legends. Statistical differences were tested using Student’s t test in Origin 7.0. Means were defined as statistically different at an error probability of *, p < 0.05; **, p < 0.01; or ***, p < 0.005.

**RESULTS**

**Coupled Transport of Na⁺ and H⁺ Together with Glutamine**—When expressed in *X. laevis* oocytes, SNAT3 carries out an electroneutral transport of glutamine, asparagine, and histidine coupled to the cotransport of 1Na⁺ and an antiport of 1H⁺ (15). Accordingly, superfusion of oocytes with glutamine caused a concentration-dependent alkalinization of the oocyte cytosol (Fig. 1, A, lower trace, and D).
sodium dependence of SNAT3-associated conductance at different external pH. FIGURE 4.

Ion Dependence of the Transport-associated Conductance—To identify the ion species mediating the SNAT3-associated ion conductance, we determined the reversal potential at different pH (Fig. 3, A and B). At a concentration of 10 mM glutamine, the conductance increased from 5.5 to 7.5 and 15 μS, when the pH was raised from 6.4 to 7.4 and 8.4, respectively. This increase most likely reflects the increase of the transport velocity from pH 6.4 to 8.4 (15). The reversal potential of the conductance changed from −14.8 to −9.2 and to −30.7 mV when the extracellular pH was changed from 6.4 to 7.4 and 8.4, respectively. For a conductance to be carried entirely by protons a reversal potential of 0 mV would be expected at pH 7.4 (pH ≈ pH_7), moving to −60 mV at pH 8.4 and +60 mV at pH 6.4. The results therefore suggest that protons contribute to the conductance above pH 7.4 but not at acidic pH. The presence of a proton conductance at pH 8.4 was further supported by the observation that the reversal potential of the glutamine-induced conductance was not affected by changes of the Na⁺ concentration (Fig. 4, A and B). Moreover, the magnitude of the currents was not affected by changes of the Na⁺ concentration, although the velocity of substrate-dependent transport depends on the Na⁺ concentration. At pH 7.4, by contrast, a significant change of the reversal potential was observed at different Na⁺ concentrations, and the amplitude of the conductance depended on the Na⁺ concentration (Fig. 4, A and C). The observed changes were smaller than expected for a Na⁺-selective conductance and also the reversal potential was more negative than expected for a Na⁺ conductance, suggesting that the conductance is nonspecific for cations.

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**FIGURE 4. Sodium dependence of SNAT3-associated conductance at different external pH.** A, typical recording of the current and intracellular pH associated with activating SNAT3 by 10 mM glutamine at different external Na⁺ concentrations (85, 28.5, and 8.5 mM) and at external pH 7.4 and 8.4. Current-voltage relationships were measured by applying voltage jumps in increments of 20 mV from −100 to +20 mV at different stages of the recording; in the intervening periods oocytes were held at −40 mV. B and C, evaluation of current-voltage relationships at different external Na⁺ concentrations of glutamine-activated currents at pH 8.4 (E, n = 8) and at pH 7.4 (E, n = 6). Note the shift in reversal potential (E_reversal) of the currents at pH 7.4, but not at pH 8.4.

**Ion Conductances Linked to SNAT3 Activity**—In addition, a cation conductance is observed, the nature of which has been discussed controversially (14, 15). To measure coupled transport and conductance in parallel, we recorded the intracellular pH and the conductance at the same time (Fig. 1). Oocytes were held at −40 mV, which is close to the resting potential. As a result, holding currents were close to 0 nA in the absence of substrate. The conductance was measured by applying short voltage jumps in increments of 20 mV from −100 to +20 mV during the recordings (Fig. 1A, upper trace). The coupled transport was measured by recording intracellular pH (lower trace). Similar to the alkalinization, the transport-associated conductance increased with increasing glutamine concentrations (Fig. 1, B and E). Conductance and pH changes, however, showed a different substrate specificity. At a concentration of 5 mM, glutamine caused the largest pH changes, followed by histidine and asparagine (Fig. 1, A and D). The conductance, by contrast, followed the order asparagine (2.2 μS) > glutamine (1.0 μS) > histidine (0.7 μS) (Fig. 1, B, C, and E). This result suggests that the velocity of coupled transport is governed by different principles than the ion conductance. Non-injected oocytes showed a basic conductance that was not affected by addition of glutamine (Fig. 2), demonstrating that the conductance and alkalinization is associated with SNAT3 expression.

Throughout this report we use the term "transport" for the coupled transport process involving amino acid substrate, Na⁺ and H⁺. To avoid confusion, we refer to the uncoupled unidirectional movement of ions as "conductions," although technically these are transport processes as well. Uncoupled Na⁺/H⁺ exchange refers to an antiport activity that does not affect glutamine transport by SNAT3 but is only visible during glutamine transport.
At pH 6.4 and reduced Na\textsuperscript{+} concentrations, currents were too small to be evaluated.

One of the hallmarks of SNAT3 is its ability to mediate transport when Na\textsuperscript{+} ions are replaced by Li\textsuperscript{+} ions (16, 22, 23). Accordingly, we observed significant alkalinization during superfusion of SNAT3-expressing oocytes with glutamine in Na\textsuperscript{+}-free LiCl buffer (Fig. 5). The glutamine-induced conductance was also observed in LiCl buffer. Upon replacing NaCl by LiCl, the reversal potential changed from −5.6 to +21.4 mV at pH 7.4, suggesting that Li\textsuperscript{+} can permeate the conductance pathway (Fig. 5B). At pH 8.4, by contrast, the reversal potential remained unaltered at −30 mV in the presence of both cations (Fig. 5C).

**Substrate-independent SNAT3-associated Conductance**—Glutamine transport via SNAT3 strongly increases with pH due to an allosteric proton binding site on the transporter (15). As pointed out above, the transport-associated conductance also increases with pH. In the absence of substrate, by contrast, the conductance appeared to be pH independent (Fig. 6A). Removal of Na\textsuperscript{+} in the absence of substrate, in addition, revealed a substrate-independent conductance that increased with pH (Fig. 6B). Similar to the substrate-induced conductance, the current was not carried by Cl\textsuperscript{−}, because it remained unaltered when Cl\textsuperscript{−} was replaced by gluconate\textsuperscript{−} (Fig. 6C). Addition of glutamine in the absence of Na\textsuperscript{+} did not change the conductance, suggesting that it is induced by the dissociation of Na\textsuperscript{+} from the transporter. Analysis of the voltage dependence of this conductance showed that it mediates outward rectifying currents at depolarized voltages with a reversal potential of about −60 mV in the absence of Cl\textsuperscript{−} and Na\textsuperscript{+} (Fig. 6D). When currents at pH 7.4 were subtracted from the currents at pH 8.4, the reversal potential moved to −80 mV (Fig. 6E). Native oocytes showed only a small conductance that hardly reacted to changes of the pH upon removal of Na\textsuperscript{+} or addition of glutamine (Fig. 2). This demonstrates that the substrate-independent conductance observed at high pH was not caused by an oocyte endogenous ion channel, unless it is up-regulated by expression of the transporter. A reversal potential in the range of −60 to −80 mV is consistent with the presence of a potassium-selective channel (24). In agreement with this notion, the reversal potential changed from −66 to −49 and −41 mV when the K\textsuperscript{+} concentration was changed from 2.5 mM to 10 and 20 mM, respectively (Fig. 7A). Thus, it appears that SNAT3-expressing oocytes show a potassium conductance that under physiological conditions is suppressed by the presence of Na\textsuperscript{+}. Non-injected oocytes displayed a similar conductance, albeit at lower magnitude (Fig. 7B).

**Sodium-Hydrogen Exchange Activity**—Removal of Na\textsuperscript{+} in the absence of glutamine resulted in a slow and negligible acidification of the oocyte cytosol, suggesting that endogenous substrate concentrations are too low to sustain reverse operation of SNAT3 (Fig. 8A). After preloading of oocytes with glutamine, however, removal of Na\textsuperscript{+} resulted in a fast and long-lasting acidification well beyond the resting pH (Fig. 8A). Release of the preloaded glutamine should bring the pH back to its resting value but not any further, because only very slow compensatory proton movements are exerted by oocyte endogenous regulatory mechanisms (21). No additional currents are observed upon removal of Na\textsuperscript{+} in the presence of glutamine (pH 7.4), excluding electrogenic influx of protons through the SNAT3-associated conductance (Fig. 8B, open circles). The acidification overshoot observed here suggests the presence of an electro-neutral Na\textsuperscript{+}/H\textsuperscript{+} exchanger activity that is activated either by elevated intracellular levels of glutamine or by an alkaline cytosolic pH. Once activated, the Na\textsuperscript{+}/H\textsuperscript{+} exchanger remained active even after removal of glutamine, resulting in a shift of the resting pH of the oocyte. Re-addition of external Na\textsuperscript{+} in the presence of glutamine returned the cytosolic pH to the value observed upon the first addition of glutamine (Fig. 8A). Subsequent removal of glutamine induced a similar intracellular

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**FIGURE 5.** Substrate-dependent and substrate-independent conductance in the presence of LiCl. A typical recording of the current and intracellular pH associated with activating SNAT3 by 10 mM glutamine at different external pH values (7.4, 8.4) and in the presence and absence of external Li\textsuperscript{+} (in exchange for Na\textsuperscript{+}). Current-voltage relationships were measured by applying voltage jumps in increments of 20 mV from −100 to +20 mV at different stages of the recording; in the intervening periods oocytes were held at −40 mV. Evaluation of the current-voltage relationships of SNAT3 in the presence and absence of Li\textsuperscript{+} at pH 7.4 (B, n = 11) and pH 8.4 (C, n = 11). Note the shift in reversal potential (E\textsubscript{rev}) of the currents at pH 7.4, but not at pH 8.4.
acidification as the removal of external Na\(^+\). A significant steady-state conductance increase was, however, only recorded in the presence of both glutamine and Na\(^+\)/H\(^+\) at pH 7.4 (Fig. 8A at points b and d and Fig. 8B).

Glutamine induced significantly larger changes of the intracellular pH than equivalent concentrations of asparagine (Fig. 1A). To investigate whether this difference results from a lack of uncoupled Na\(^+\)/H\(^+\) exchange activity, we repeated this protocol also with asparagine as the substrate for SNAT3. In this experiment we compared the pH changes when external Na\(^+\) was removed in the presence of glutamine or asparagine (Fig. 8, C and D). As shown, the cytosol acidified much faster and to a lower pH value in the presence of asparagine than in the presence of glutamine (Fig. 8C, lower trace). The rate of H\(^+\) change induced by asparagine was ~40% of that induced by glutamine, being 8.8 ± 1.5 nM H\(^+\)/min and 23.1 ± 2.8 nM H\(^+\)/min, respectively (n = 8; p < 0.001; Fig. 8D). Removing asparagine resulted in a similarly low rate of cytosolic acidification of 5.5 ± 1.0 nM H\(^+\)/min as observed upon removal of Na\(^+\) in the presence of asparagine. As shown before, the conductance increase in asparagine was larger than in glutamine and was only evident in the presence of Na\(^+\) (Fig. 8C, upper trace).

Site-directed Mutagenesis—To identify the molecular basis of the ion conductances, we tried to identify critical residues for this function. SNAT3 belongs to the SLC38 family of system A and N transporters, which are all Na\(^+\)-coupled transporters (25). The SLC38 family is further related to the SLC36 family of proton amino acid transporters (26) and to the vesicular GABA (\(\gamma\)-aminobutyric acid) transporter (SLC32), which is a proton exchanger (27). To identify residues critical for Na\(^+\) binding in SNAT3, we identified residues that are conserved in the SLC36 family, but not in the proton-dependent transporters of the SLC32 and SLC38 families. We further looked for residues that have been observed to form cation binding sites in other channels and transporters (28), such as threonine, serine, asparagine, and glycine residues. Using this approach we decided to generate the following mutants, N76H, G210A, S215A, H300Q, T343A, and T380A (Fig. 9). We also included an arginine residue in the predicted helix 8 (R370Q), which could be involved in substrate binding. Among these mutants, all but two, N76H and T380A, showed wild-type transport activity at pH 7.4 (Fig. 10A). N76H and T380A retained ~20% activity; they are located in predicted helices 1 and 8, respectively (Fig. 11). Surface biotinylation experiments showed that...
Transport Modes of Glutamine Transporter SNAT3

A SNAT3-expressing oocytes

B H2O-injected oocytes

FIGURE 7. Substrate-independent current of SNAT3-expressing oocytes is partly carried by potassium. Current-voltage relationships of the substrate-independent current in the absence of Na⁺ in SNAT3-expressing oocytes (A, n = 6) and in H2O-injected oocytes (B, n = 4) at different external K⁺ concentrations (2.5, 10, 20 mM). All measurements were made in the absence of Na⁺ comparing conductance at pH 7.4 and 8.4. The current-voltage relationships were measured by applying voltage jumps in increments of 20 mV from −100 to +20 mV at different stages of the recording; in the intervening periods oocytes were held at −40 mV.

FIGURE 8. Na⁺/H⁺ exchange activity in SNAT3-expressing oocytes. A, typical recording of the membrane current and intracellular pH associated with activating SNAT3 by 10 mM glutamine in the presence and absence of external Na⁺; reversal of transport activity by either removing Na⁺ or glutamine is indicated by large overshooting intracellular acidification. Current-voltage relationships were measured by applying voltage jumps in increments of 20 mV from −100 to +20 mV at different stages of the recording; in the intervening periods oocytes were held at −40 mV. B, evaluation of the current-voltage relationships in the presence and absence of Na⁺ or glutamine (n = 10). C, comparison of pH changes induced by 10 mM glutamine and 10 mM asparagine in the presence and absence of Na⁺. D, evaluation of the initial rate of acidification induced by the removal of Na⁺ in the presence of glutamine (n = 8) and in the presence of asparagine (n = 8).

all mutants, including N76H and T380A, are expressed at the cell surface, similar to the wild-type (Fig. 10B).

The T380A mutant showed reduced, but still significant, transport activity as indicated by the alkalinization observed during superfusion with 10 mM glutamine (Fig. 12A). Similar to the wild type, substrate-dependent and substrate-independent conductances were observed. The glutamine-dependent conductance was pH dependent and only significant at pH 8.4, but not at pH 7.4 (Fig. 12B). Removal of Na⁺ abolished the glutamine-induced conductance, demonstrating that, as in the wild type, it is only observed during active transport (Fig. 12C). The selective removal of the substrate-induced conductance in this mutant at pH 7.4 further supports the notion of two substrate-dependent conductances in wild-type SNAT3, namely a proton conductance (predominant at pH 8.4) and a cation conductance (predominant at pH 7.4). Mutation of Thr-380 removes the cation conductance at pH 7.4 but leaves the proton conductance at pH 8.4 intact. The substrate-independent conductance remained unaffected by this mutation. The T380A mutant showed very little transport activity when NaCl was replaced by LiCl (Fig. 12, E and F), suggesting that Thr-380 may also contribute to the ion selectivity of the coupled cotransport.

DISCUSSION

In this study we have demonstrated the presence of several modes of ion transport by the amino acid transporter SNAT3. The principal mode of the transporter is the electroneutral transport of glutamine, which is coupled to the cotransport of 1Na⁺ and the antiport of 1H⁺ (15, 16). In addition, we and others found ion movements that are uncoupled from substrate transport (14, 15). At pH 8.4, a substrate-induced proton conductance is associated with SNAT3 that has also been observed previously (14). This conductance requires active substrate translocation, as it is abolished both in the absence of Na⁺ and in the absence of substrate. At lower pH, the reversal potential of the substrate-induced conductance is much less affected by changes of the pH but instead becomes sensitive to changes of the Na⁺ concentration. The reversal potential of the conductance of about −30 mV, however, suggests that it is a nonspecific cation conductance; this is also supported by the observation that it is permeable to lithium. Surprisingly, it appears that at higher pH, namely a proton conductance at pH 7.4, and a cation conductance at pH 8.4, the conductance is much less affected by changes of the pH but instead becomes sensitive to changes of the Na⁺ concentration. The reversal potential of the conductance of about −30 mV, however, suggests that it is a nonspecific cation conductance; this is also supported by the observation that it is permeable to lithium. Surprisingly, it appears that at higher pH, namely a proton conductance at pH 7.4, and a cation conductance at pH 8.4, the conductance is much less affected by changes of the pH but instead becomes sensitive to changes of the Na⁺ concentration. 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ance. The size of the substrate-induced proton conductance appears to be governed by the rate of transport and the nature of the substrate side chain. The smaller the substrate side chain, the higher is the conductance. This suggests that the conductance is mediated by residues in the transport protein that are not directly involved in the ion cotransport or antiport. The proton conductance increases with the transport rate, indicating that it is associated with a distinct step in the transport cycle. However, the currents are activated by lower Na$_\text{+}$/H$_\text{+}$ concentrations than the transport (14).

This extreme Na$_\text{+}$ sensitivity has been considered evidence for an association of the conductance with the step after binding of Na$_\text{+}$/H$_\text{+}$ but before translocation occurs (14). Mutation of residue threonine 380 to alanine abolishes the cation conductance but leaves the proton conductance intact. The mutation also appears to affect the ion selectivity of the coupled cotransport, reducing the ability of Li$_\text{+}$ to drive transport. It is tempting to speculate that the proton conductance is associated with residues close to the substrate side chain, whereas the cation conductance is related to residues involved in ion cotransport in SNAT3.

In addition, we report here for the first time the presence of a substrate-independent cation conductance associated with SNAT3 that is activated by alkaline pH. This conductance is not observed in native oocytes and is associated with SNAT3 expression. Binding of Na$_\text{+}$/H$_\text{+}$ to the transporter abolishes the substrate-independent conductance and at the same time allows the transporter to switch to the substrate-induced conductances. The substrate-independent conductance is a cation conductance, which is not affected by replacement of chloride.

We also detected a Na$_\text{+}$/H$_\text{+}$/H$_\text{+}$/H$_\text{+}$ exchange activity associated with SNAT3 expression that is observed in the presence of glutamine, but not when asparagine is used as a substrate. The endogenous buffering capacity of oocytes is 12.9 ± 0.9 mM H$_\text{+}$/pH unit (29). At the V$_\text{max}$ of glutamine transport by SNAT3 at pH 7.4 (~1 nmol/10 min/oocyte), the proton concentration increases by ~2 mM/10 min in the oocyte cytosol (30), which should result in a pH change of ~0.16 pH units. However, pH changes are in the order of 0.4 to 0.8 pH units in about 5 min. The stronger than expected pH difference is most likely due to Na$_\text{+}$/H$_\text{+}$ exchange activity. It has previously been suggested that the difference of pH changes observed after application of asparagine or glutamine is related to the activation of the proton conductance, thereby blunting the coupled transport-linked pH changes (14). Asparagine induces smaller pH changes, which suggests that the conductance is related to the activation of the proton conductance. However, the currents are activated by lower Na$_\text{+}$/H$_\text{+}$ concentrations than the transport (14).
changes than glutamine because it has a more prominent conductance. Our results instead suggest that asparagine transport is not associated with uncoupled Na\(^+\)/H\(^+\) exchange and therefore only the smaller pH change caused by coupled proton antiport is observed. In agreement with the presence of a Na\(^+\)/H\(^+\) exchange activity, Bröer et al. (15) previously reported that uptake of \(^{22}\)Na was three times higher than uptake of glutamine, although the uptake of glutamine was unaffected by changes of the membrane potential. The difference between asparagine and glutamine-induced pH changes lends further support to the notion that the substrate side chain affects ion transport via SNAT3. Substrates can form part of a cation binding site as revealed by the high resolution structure of the bacterial leucine transporter LeuT\(_{\lambdaA}\) (31). In this structure, the substrate carboxyl group, two asparagine side chains (of the protein), and backbone groups coordinate Na\(^+\) at binding site 1. By analogy, the side

FIGURE 10. Transport activity and surface expression of SNAT3 mutants. Oocytes were injected with 30 ng of SNAT3 cRNA. A, after an expression period of 4–5 days glutamine uptake was measured as radioactively labeled substrate flux over a time period of 16 min. Each bar represents the mean ± S.D. of ten oocytes. One of three similar experiments is shown. B, in oocytes of the same group, surface proteins were biotinylated and bound to agarose particles. Biotinylated proteins were separated by SDS-PAGE and SNAT3 detected by Western blotting. SNAT3 forms two bands in oocytes, most likely corresponding to monomeric and dimeric protein.

FIGURE 11. Topological model of SNAT3. The topology of SNAT3 was predicted using the TMHMM program provided by the web server of the Technical University of Denmark. The schematic was generated using TOPO2 transmembrane protein display software, www.sacs.ucsf.edu/TOPO2/. Residues mutated in this study are encircled with a bold line. Charged residues in the transmembrane domains are shaded in gray.
The occurrence of several conductance states associated with expression of transporters in *X. laevis* oocytes has been observed before for the mammalian serotonin transporter (3, 32). Four distinct currents were observed: first, a transport-associated current that requires turnover of the transporter and is most likely carried by Na\(^+\)/H\(^+\) ions; second, a transient current that is also carried by Na\(^+\)/H\(^+\); third, a leakage current, which is observed in the absence of substrate; and fourth, a proton conductance, which is also observed in the absence of substrate. Both transporters use more than one ion species for coupled transport, and it appears likely that similar residues are involved in coupled and uncoupled ion movements.

The physiological relevance of these ion conductances remains to be determined, some of which can only be observed under *in vitro* conditions such as Na\(^+\) removal. The conductances highlight that the coupling of ion cotransport to substrate translocation is incomplete. This phenomenon, which is observed in a number of transporters, has been termed “drive slip” (33) and may contribute to the conspicuous reversibility of SNAT3, which is important for its physiological function. SNAT3 is the molecular correlate of the system N transporter, which is involved in glutamine uptake and release in liver, kidney, and brain astrocytes (25). In mammalian cells, glutamine is transported by electrogenic and electroneutral transporters (34). As a result, it is difficult to identify whether glutamine-induced currents reflect the presence of an electrogenic transporter or an uncoupled conductance. To date, no study has attempted to detect SNAT3-associated conductances in expression systems other than oocytes. It has previously been suggested (14) that the proton conductance could reduce the alkalinization caused by coupled proton antiport, thereby allowing modulation of glutamine uptake or release. Our results, by contrast, indicate that the uncoupled Na\(^+\)/H\(^+\)/H\(^+\) exchange activity aggravates the alkalinization when glutamine is taken up and results in an influx of Na\(^+\)/H\(^+\) that is about three times higher than that of coupled transport. The excessive influx of Na\(^+\) during glutamine transport could have relevance for liver metabolism. Perfusion of liver with physiological concentrations of glutamine causes significant cell swelling (35), thereby affecting proteolysis in the liver (36). Uptake of glutamine into perportal hepatocytes is mediated by SNAT3 (16, 23), and the additional Na\(^+\) flux is likely to contribute to the swelling process. In brain astrocytes, where glutamine efflux is the prevalent mode of glutamine transport, the uncoupled Na\(^+\)/H\(^+\) exchange activity could modulate intracellular Na\(^+\).

**Figure 12.** Functional characterization of conductances associated with SNAT3 mutation T380A. A, recording of the current and intracellular pH associated with activating mutant SNAT3 with 10 mM glutamine at pH 7.4 and 8.4. B, evaluation of the current-voltage relationships of glutamine-induced currents (n = 5). C, recording of the current as in panel A when NaCl is replaced by NMDG-Cl. D, evaluation of the current-voltage relationships of the currents shown in panel C (n = 5). E, recording of the current as in panel A when NaCl is replaced by LiCl. F, evaluation of the current-voltage relationships of the currents shown in panel E (n = 3). Current-voltage relationships were measured by applying voltage jumps in increments of 20 mV from -100 to +20 mV at different stages of the recording; in the intervening periods oocytes were held at -40 mV.
REFERENCES

1. Sanders, M. S., and Amara, S. G. (1996) *Curr. Opin. Neurobiol.* 6, 294–302
2. Blakely, R. D., Defelice, L. J., and Galli, A. (2005) *Physiology (Bethesda)* 20, 225–231
3. Mager, S., Min, C., Henry, D. J., Chavkin, C., Hoffman, B. J., Davidson, N., and Lester, H. A. (1994) *Neuron* 12, 845–859
4. Galli, A., Blakely, R. D., and DeFelice, L. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 8671–8676
5. Ryan, R. M., and Vandenberg, R. J. (2002) *J. Biol. Chem.* 277, 13494–13500
6. Wadiche, J. I., and Kavanaugh, M. P. (1998) *J. Neurosci.* 18, 7650–7661
7. Fairman, W. A., Vandenberg, R. J., Arriza, J. L., Kavanaugh, M. P., and Amara, S. G. (1995) *Nature* 375, 599–603
8. Ingram, S. L., Prasad, B. M., and Amara, S. G. (2002) *Nat. Neurosci.* 5, 971–978
9. Fei, Y. J., Sugawara, M., Nakanishi, T., Huang, W., Wang, H., Prasad, P. D., Leibach, F. H., and Ganapathy, V. (2000) *J. Biol. Chem.* 275, 23707–23717
10. Broer, S. (2003) *Methods Mol. Biol.* 227, 245–258
11. Galli, A., Blakely, R. D., and DeFelice, L. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 8671–8676
12. Kilberg, M. S., Handlogten, M. E., and Christensen, H. N. (1980) *J. Biol. Chem.* 255, 4011–4019
13. Gu, S., Roderick, H. L., Camacho, P., and Jiang, J. X. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 3230–3235
14. Hille, B. (2001) *Ion Channels of Excitable Membranes*, 3rd Ed., pp. 441–470, Sinauer Associates, Inc., Sunderland, MA
15. Mackenzie, B., and Erickson, J. D. (2004) *Pflugers Arch. Eur. J. Physiol.* 447, 784–795
16. Boll, M., Daniel, H., and Gasnier, B. (2004) *Pflugers Arch. Eur. J. Physiol.* 447, 776–779
17. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) *Nature* 437, 215–223
18. Cao, Y., Mager, S., and Lester, H. A. (1997) *Cell* 99, 769–780
19. Haussinger, D., Lang, F., Bauers, K., and Gouaux, E. (2005) *Nature* 437, 215–223
20. Nelson, N., Sacher, A., and Nelson, H. (2002) *Nat. Rev. Mol. Cell. Biol.* 3, 876–881