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Electrogenic amino acid exchange via the rBAT transporter**

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Abstract A cDNA clone was isolated from rabbit renal cortex using DNA-mediated expression cloning, which caused alanine-dependent outward currents when expressed in *Xenopus* oocytes. The cDNA encodes rBAT, a Na-independent amino acid transporter previously cloned elsewhere. Exposure of cDNA-injected oocytes to neutral amino acids led to voltage-dependent outward currents, but inward currents were seen upon exposure to basic amino acids. Assuming one charge/alanine, the outward current represented 38% of the rate of uptake of radiolabelled alanine, and was significantly reduced by prolonged preincubation of oocytes in 5 mM alanine. The currents were shown to be due to countertransport of basic amino acids for external amino acids using the cut-open oocyte system. This transport represents a major mode of action of this protein, and may help in defining a physiological role for rBAT in the apical membrane of renal and intestinal cells.

Key words: Amino acid transport; Counterexchange; Expression cloning; Oocyte; *Xenopus laevis*

1. Introduction

Amino acids cross cell membranes using specialized transport proteins, allowing the process to be selective and regulated [1]. Although amino acids have been shown to traverse some ionic channels [2], they generally are moved by transporters via facilitated diffusion or by cotransport if another solute (e.g. Na) is also carried. A third transport mode which has rarely been associated with amino acid transport is the exchanger mode where the influx of a substrate is tightly coupled to the efflux of a second substrate [1]. In recent years many transporters have been cloned and more information has become available for understanding how transporters utilize the energy contained in a solute concentration gradient to drive the transport of another solute.

A renal cDNA which is responsible for expression of Na^+-independent amino acid transport (rBAT) in *Xenopus* oocytes was recently isolated via expression cloning from rat and rabbit [3,4]. The amino acid transport activity is similar to the $b^{0,+}$ amino acid transport system previously found in mouse blastocysts [5]. rBAT is located in the brush border membranes of renal proximal tubules and, to a lesser extent, intestinal mucosa [6]. Two alternative transcripts (rBAT1 and rBAT2) have been identified for this protein in rabbits, which differ in length and sequence at both 5′ and 3′ untranslated regions [7]. Defects in this protein have been shown to be associated with cystinuria, suggesting that cystine transport is mediated by this protein in vivo [8]. The clinical importance of this protein warrants further examination of the mechanism of transport mediated by rBAT.

While using electrophysiological techniques to isolate amino acid cotransporter cDNAs from a renal cortex library, we found outward currents associated with external application of alanine to oocytes expressing cDNA from a 2.2 kb fraction of the library. We report here the isolation of the clone responsible for this current via expression cloning using nuclear injection of recombinant plasmids, identification of the insert as the rBAT cDNA and an analysis of the amino acid transport associated with expression of this protein in *Xenopus laevis* oocytes. The currents and radiolabel uptake mediated by rBAT identify the protein as a transporter which operates largely by exchanging neutral and basic amino acids.

2. Materials and methods

2.1. cDNA preparation

RNA was extracted with acid guanidinium phenol (RNA Stat-60, Tel-Test 'B' Inc., Friendswood TX); mRNA was purified with oligo-dT cellulose. cDNA was reverse transcribed with Superscript II (Life Technologies, Burlington, Ont., Canada) using an *Xho*I linker-primer (Stratagene, San Diego, CA). The cDNA was size-selected in 0.6% low-melting agarose and individual fractions were cut out of the gel. Each gel slice was digested with agarase and the DNA therein was ligated to *Eco*RI, *Xho*I-cut pMT21 [9] and used to transform XL-1 Blue MRF′ bacteria (Stratagene), which were grown in suspension in 0.3% agarose [10] to avoid selective amplification of plasmids. Plasmids were prepared from each suspension of bacteria using a Triton/lysis method followed by polyethylene glycol purification [11]. The cDNA insert isolated by expression cloning was subcloned into pBluescript for sequencing and RNA transcription [12].

2.2. Oocyte injection and maintenance

Aliquots of each plasmid preparation were diluted to 65 ng/μl in 50 mM KCl, 50 mM KH_2PO_4, pH 7.6, containing 6.5 ng/μl of a recombinant pMT21 bearing cDNA for expression of secreted alkaline phosphatase (pMT-SEAP) [13] for injection into oocytes.

Oocytes were taken from *Xenopus laevis* (Xenopus One, Ann Arbor, MI) and the follicular layers were removed as described [14], with the exception that defolliculation was performed in Ca^{2+}-free Barth's solution. 4.6 nl of plasmid DNA was injected into the center of the oocyte's animal hemisphere using a Drummond microinjector. Oocytes were maintained in Barth's solution (in mM: 88 NaCl, 3 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$ and 5 HEPES, pH 7.6), supplemented with penicillin, streptomycin, Na-pyruvate and 5% serum [14] for two days. The oocytes were rinsed in serum-free Barth's solution before being

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**The groups which have previously cloned the cDNA for this protein have so far suggested five different names for it. We shall employ the name rBAT (related to $b^{0,+}$ amino acid transport system) so as to remain consistent with the other group which isolated the cDNA from rabbit tissue [4].

The nucleic acid sequence reported in this paper has been submitted to the EMBL Data Bank with accession number Z 46844.

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incubated separately for 24 h in 200 μl of Barth's solution, in which alkaline phosphatase activity was measured [12]; only oocytes with visible expression of alkaline phosphatase were further analyzed.

In a number of experiments, no difference was seen in rBAT currents whether oocytes were injected with 300 pg of pMT21-rBAT plasmid or with 10 ng of in vitro transcribed rBAT RNA. All oocytes were henceforth injected with RNA due the superior survival of these oocytes. Neither was any difference found between uninjected oocytes and water-injected oocytes.

2.3. Two-micrelectrode voltage clamp

Oocyte currents were measured with the two microelectrode voltage-clamp technique using a commercial amplifier (Oocyte Clamp model #OC-725, Warner Instrument Co., Hamden, CT). The current and voltage microelectrodes were filled with 1 M KCl and had resistances ranging from 7 to 14 MΩ. The bath was referenced through an agar bridge containing 1 M KCl and the bath current electrode was an Ag/AgCl pellet. The oocyte was perfused with saline–Barth's solution, (modified Barth's solution such that Cl^- was the only anion) at approximately 1.5 ml/min. A membrane potential stabilization period was observed for 4–10 min. Oocytes whose membrane potential was less negative than −35 V were discarded. The membrane potential was then clamped to −50 mV. A data acquisition system and commercial software (RC Electronics, Santa Barbara, CA) were used to send voltage pulse protocol and simulataneously record current and voltage signals. 11 alternate 150 ms duration pulses separated by 500 ms periods at the resting potential (−50 mV) were used. The voltage range covered was from −175 mV to +75 mV. Current and voltage traces were analyzed by averaging the signal in a window of 20 ms positioned after the decay of capacitative transients. Where required, the currents in nA (nanocoulomb/s) were converted to pmol/min, assuming 1 charge/basic amino acid molecule (96,500 coulomb/mole).

2.4. Cut-open oocyte measurements

The cut-open oocyte measurements [16] were performed using a commercially available chamber (Dagan Corp., Minneapolis, MN) with, as recently described [17], a low access resistance glass pipette (200 kΩ) for both internal perfusion and membrane potential clamp. The extracellular solution used consisted of 6.2 mM NaCl, 43.8 mM Na-cyclamate, 0.9 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6, 80 mM mannitol and 3 mM *N*-methyl-glucamine (NMG) to reach 180 mOsm. The intracellular solution consisted of 6.2 mM NaCl, 43.8 mM Na-cyclamate, 0.9 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6, 75 mM mannitol, 1 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N'*,N'-tetracetic acid (EGTA) and 6 mM *N*-methyl-glucamine (NMG). A 40 μl/min flow entering the perfusion pipette was led to two pathways: a 35 μl/min flow was removed into a drain system, while 5 μl/min flow perfused the intracellular space. This flow rate is 6 times higher than that previously proposed [16], and permits a complete change of the internal milieu in 2–3 min.

2.5. Oocyte loading and transport experiments

Oocytes were preloaded with alanine by incubation in Barth's solution containing 5 mM alanine at 18°C for 2–3 h without agitation.

Transport experiments were performed as described previously [14] with the following modifications: oocytes were placed in KCl–Barth's solution (similar to Barth's solution as described above, but with 46.5 mM NaCl and 46.5 mM KCl) containing 0.5 mM substrate and incubated at room temperature for 5 min with gentle agitation. The uptake was stopped by washing five times with 4 ml of ice-cold, non-radiolabelled uptake solution containing 5 mM substrate.

2.6. Statistical analysis

Unless otherwise noted, error bars represent standard error of means and the *n* value represents the number of oocytes coming from at least two different donors. Statistical comparisons were done using Student's *t*-test.

3. Results

3.1. cDNA clone isolation

Injected recombinant pMT21 containing 2.2 kb renal cDNA induced outward currents (approx. 10 nA) in *Xenopus* oocytes clamped at −50 mV when exposed to 1 mM alanine. The magnitude of the induced currents increased throughout the expression cloning of this cDNA as the number of other cDNAs present in the injected sample was diminished. A single cDNA clone was isolated by this procedure.

3.2 cDNA sequencing

The sequence of the isolated cDNA (rBATx) demonstrates

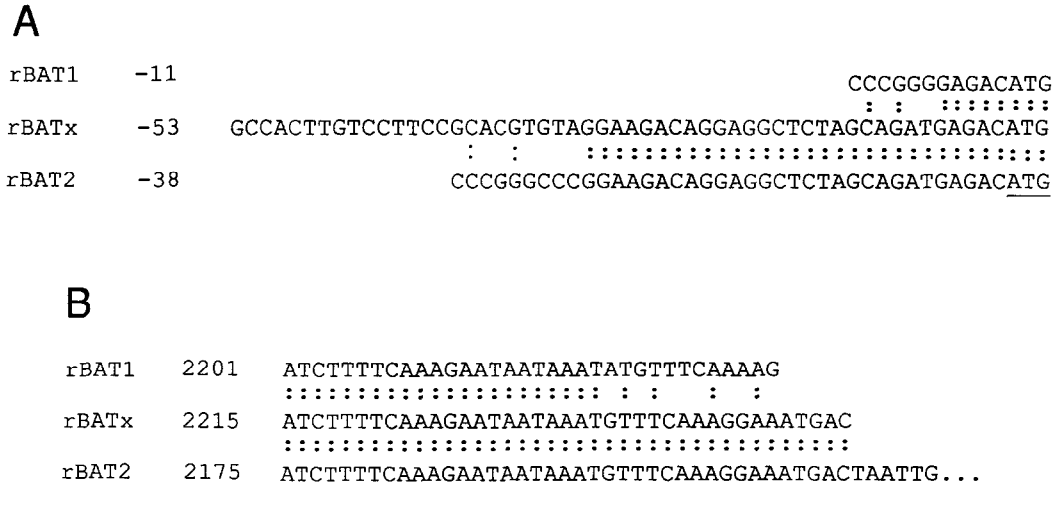

Fig. 1. Comparison of untranslated sequences between current-inducing cDNA and rBAT1 and rBAT2. The clone isolated in this work is labelled rBATx; sequence identity is indicated by colons. Numbers indicate sequence position of the nucleotide shown to the right of the number. (A) Complete untranslated 5′ region. The beginning of the translated region is shown by the underlined ATG codon. (B) Terminal portion of 3′ untranslated region; the preceding 159 base pairs of 3′ untranslated region are not shown. Polyadenylation begins immediately after the nucleotides shown for rBAT1 and rBATx, but the rBAT2 sequence contains another 1.6 kb prior to the beginning of polyadenylation (represented here by three dots).

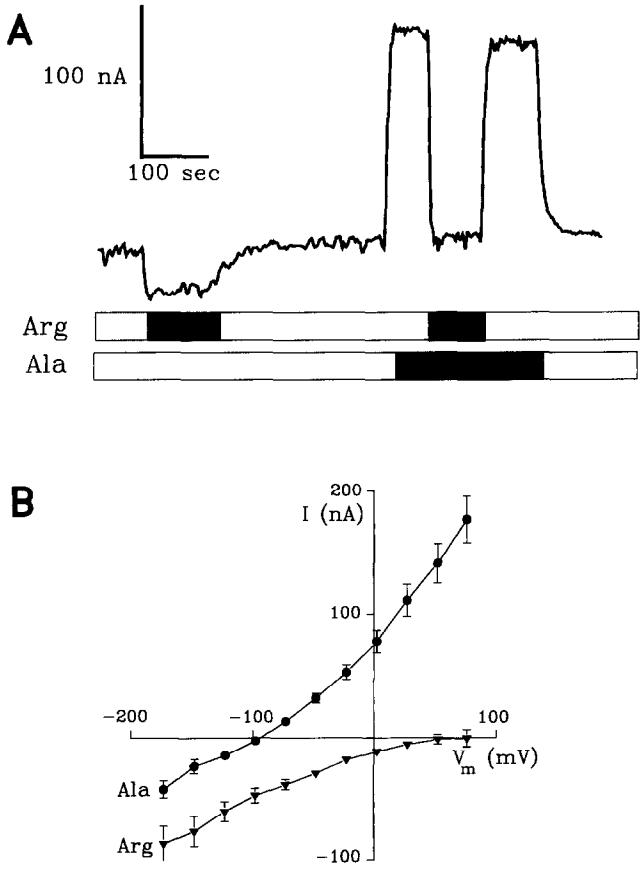

Fig. 2. Current recordings from voltage-clamped rBAT-expressing oocytes. (A) Typical tracing from oocyte; downward deflections represent outward currents. Black bars indicate the presence of arginine (0.5 mM) or alanine (5 mM). (B) Average currents induced by arginine (n = 11) and alanine (n = 10). Both amino acids were applied at 0.5 mM concentrations. The currents shown here were obtained by subtracting currents measured in the absence of substrate from currents measured in the presence of substrate.

that the cloned DNA coded for the rBAT protein. The 5′ untranslated region of the isolated cDNA is identical to that previously reported for the rabbit rBAT2 cDNA, aside from the initial 24 bp (see Fig. 1A). The translated region of the cDNA is virtually identical to that found in rBAT1 and rBAT2. This region in rBATx contains three nucleotide differences from rBAT1 (630, C > T; 637, C > G; 1230, G > T), changing one amino acid. All three sequence differences in rBATx are amongst the five differences found between rBAT1 and rBAT2. The 3′ untranslated region of our rBAT cDNA is identical to that of rBAT1 except that the terminal 15 nucleotides are identical to those found in the analogous position of rBAT2 (see Fig. 1B), following which the cDNA is polyadenylated, probably due to the same polyadenylation signal as used by rBAT1. By contrast, this region of rBAT2 contains two nucleotides that differ from rBAT1 and rBATx and it continues for another 1.6 kb of untranslated cDNA prior to polyadenylation.

3.3. Amino acid-induced currents

cDNA-injected oocytes do not show appreciably different currents than control (non-injected) oocytes when immersed in Barth's solution. In the presence of 1 mM Ala, outward currents of 60–100 nA are invariably seen at −50 mV in rBAT-expressing oocytes but not in control oocytes (see Fig. 2A); these currents are independent of bath [Na], [K] and [Cl]. Similar currents were seen upon exposure to leucine, isoleucine, serine and glutamine. In contrast, addition of 1 mM arginine to Barth's solution causes an inward current of 35 nA at −50 mV; the same effect is seen with lysine. No amino acid-induced currents were seen upon application of proline, methylaminoisobutyric acid, aspartic acid or glutamic acid. When both alanine and arginine are present, the net current cannot be described as an addition of the individual currents seen with these two amino acids (see Fig. 2A). This, along with the fact that these currents appear together in all oocytes injected with rBAT (and never separately), supports our contention that both currents are likely to be mediated through the same protein. This is in agreement with the cross-inhibition seen with these substrates during uptake experiments with rBAT-expressing oocytes [4].

We chose to continue our analysis of the electrogenic nature of rBAT activity using alanine and arginine as model neutral and basic amino acids. At −50 mV, the alanine-induced currents demonstrated a K_m value of 1.3 ± 0.2 mM (n = 11) while the arginine-induced currents had a K_m value of 9.0 ± 1.3 μM (n = 6).

3.4. Voltage dependence of amino acid-induced currents

Currents were measured in rBAT-expressing oocytes in the presence of 0.5 mM alanine or arginine at a range of potentials (see Fig. 2B); substrate-induced currents were obtained by subtracting currents measured in saline–Barth's solution immediately before or after exposure to substrate. Exposure to alanine caused outward currents that increased rapidly as the transmembrane potential became positive but approached zero or became inward at negative transmembrane potentials. The addition of arginine to the bath caused an inward current which was largest at negative transmembrane potentials.

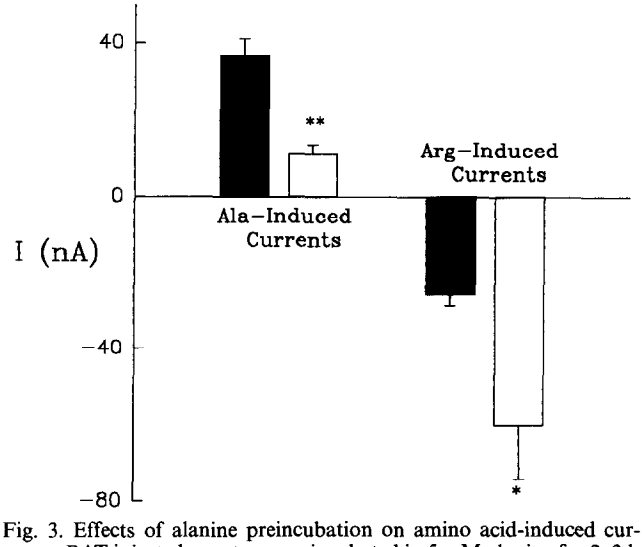

Fig. 3. Effects of alanine preincubation on amino acid-induced currents. rBAT-injected oocytes were incubated in 5 mM alanine for 2–3 h prior to measurement of currents induced by 0.5 mM alanine or arginine. Black bars indicate oocytes preincubated in the absence of alanine; white bars indicate oocytes preincubated in the presence of alanine. **Indicates 99% significance; *indicates 95% significance.

3.5. Amino acid loading of oocytes

The polarity of the alanine-induced currents seen with rBAT expression and the affinity of the system for arginine suggested that external addition of alanine may stimulate the efflux of cationic amino acids. To investigate this, we attempted to alter the normal oocyte internal amino acid levels [18]. Preloading of rBAT-expressing oocytes in 5 mM alanine should cause an increase in intracellular alanine and perhaps a decrease in intracellular cationic amino acids. Such a preincubation diminished the alanine-induced currents by 66% and augmented the arginine-induced currents by 131% (see Fig. 3). It is impossible to know how much of the induced change is due to augmentation of intracellular alanine, or to diminished intracellular basic substrates such as arginine and lysine. For this reason, we decided to forego further research with amino acid loading in favour of the cut-open oocyte method (see below).

3.6. Comparison of current and uptake

We next compared the magnitudes of the rBAT currents and the amount of transported [^{3}H]alanine in a series of parallel experiments (see Fig. 4). These experiments used a modified Barth's solution where half of the NaCl was replaced with KCl, so that the depolarized membrane potential should increase the amount of alanine transport. To mimic the membrane potential experienced by oocytes in the uptake experiment, the oocytes in the voltage-clamp experiment were exposed to alanine and the zero-current potential which they reached was measured; the oocytes were then clamped at that potential and the current passing through the rBAT protein was measured by removing alanine from the bath solution. The magnitude of the alanine-dependent outward current, when converted to pmoles charged substrate/minute, was approximately 38% of the amount of rBAT-transported alanine, which was estimated from the difference in alanine uptake between non-injected and rBAT-expressing oocytes.

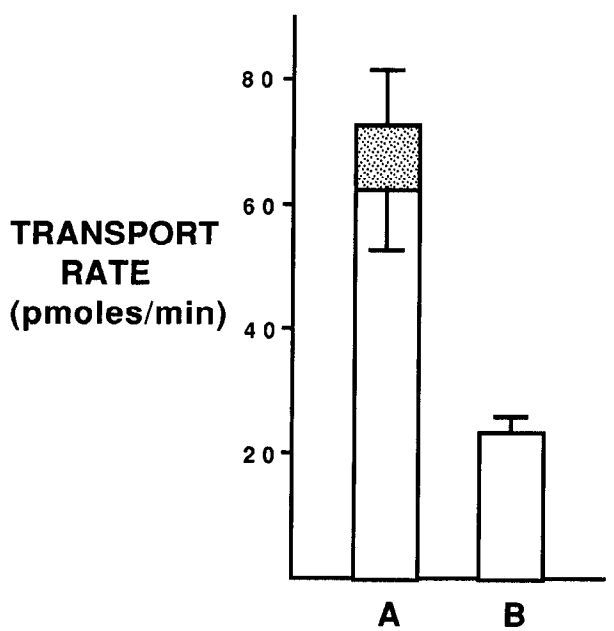

Fig. 4. Alanine uptake and alanine-induced currents. rBAT mRNA-injected oocytes were exposed to 0.5 mM alanine in KCl–Barth's solution for either alanine-induced current measurement via two-microelectrode recording, or for measurement of uptake of radiolabelled alanine. Each data point represents experiments using 6 oocytes from each of three separate donors. (A) Uptake of radiolabelled alanine. The bar represents total alanine uptake in rBAT-expressing oocytes and the stippled area represents alanine uptake in non-injected oocytes. The white area of the bar represents rBAT-mediated uptake of alanine. The rising and descending error bars represent the S.E.M. of the total and subtracted uptake values. (B) Amount of alanine-induced charge measured in rBAT-expressing oocytes, converted to pmol/min.

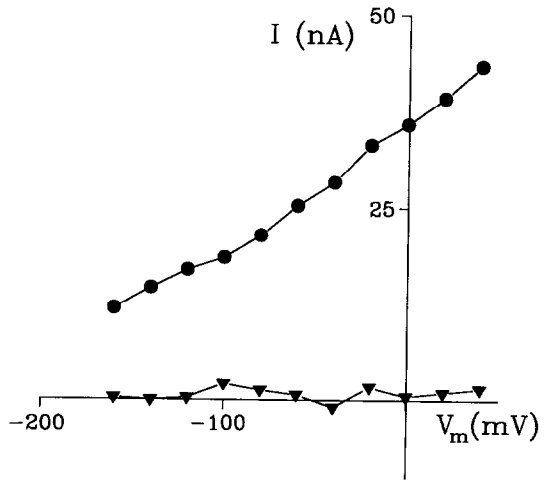

Fig. 5. Typical alanine–arginine exchange in cut-open oocytes. In the cut-open oocyte system addition of 0.5 mM alanine to the external solution does not lead to any change in currents (triangles). If 0.5 mM arginine is present in the internal bath, addition of 0.5 mM alanine to the external bath causes a large inward current (circles).

3.7. Cut-open oocyte currents

Since the control of rBAT currents by internal amino acids is not easily investigated using two microelectrodes, we chose to alter this directly using the cut-open oocyte system [16]. In the absence of intracellular amino acids, addition of alanine to the external bathing solution did not induce any apparent currents in rBAT-expressing oocytes (see Fig. 5). When 0.5 mM arginine was present intracellularly, addition of alanine to the external solution led to an outward current similar to that seen with the two-microelectrode system. This provides direct proof that the rBAT protein is capable of generating a current upon application of external amino acids provided that arginine (or, presumably, any other charged rBAT substrate) is present within the oocyte.

4. Discussion

We isolated a cDNA clone which led to outward currents when DNA-injected oocytes were exposed to alanine or most other neutral amino acids at −50 mV. Neutral amino acids which induced currents matched the neutral amino acids which were transported by the rBAT protein [4] while basic amino acids (also known to be transported by rBAT) generated inward currents. cDNA sequencing confirmed the clone as coding for the rBAT protein, but with a 5′ region closely related to that of rBAT2 and with a 3′ region similar in length and sequence to rBAT1. Our cDNA also contained a coding region that was intermediate between those found in rBAT1 and rBAT2; for these reasons we have called it rBATx, reflecting

what we expect will be a wide and gradual heterogeneity amongst rBAT transcripts.

The magnitude of the current induced by alanine was large at positive transmembrane potentials but at negative transmembrane potentials the alanine-induced current approached zero or exhibited a reversal potential. Possibly the inward current at highly negative potentials is caused by cationic, high-affinity substrates for rBAT in the unstirred layer surrounding the oocyte (the cationic substrates are present in the unstirred layer due to previous exchange for alanine). When baseline alanine currents are reduced by clamping the oocytes at more negative potentials followed by a series of voltage pulses, the inward current is significantly reduced (not shown); also, the inward portion of the alanine-induced current decayed a few minutes after the washout of alanine from the external bath. This is consistent with our hypothesis that the cationic substrate accumulating around the oocyte has a high affinity for rBAT.

rBAT is known to transport neutral and basic amino acids into cells which express it. Given that *Xenopus* oocytes already possess large intracellular concentrations of all amino acids, it is reasonable to suppose that the currents measured here represent an exchange of neutral for charged amino acids. Under these conditions, alanine influx through rBAT would be coupled to efflux of basic and neutral amino acids, leading to the outward currents observed here. Arginine influx, also coupled to an efflux of both neutral and basic amino acids, should demonstrate an inward current as seen here. The magnitudes of the currents depend on both the affinities of rBAT for neutral and basic amino acids as well as their intracellular concentrations. This hypothesis is supported by several lines of evidence. Preloading of the oocyte with alanine leads to a decrease in alanine-induced outward currents but to increased arginine-induced inward currents. This preloading results in increased intracellular alanine levels and, presumably, decreased intracellular arginine and lysine levels. Thus, altering the intracellular amino acid concentrations results in changes in the alanine and arginine-induced currents. An electrically silent partial exchange of alanine for neutral intracellular amino acids would explain why alanine uptake is more than twice the level of alanine-induced current.

It is difficult to comprehend the utility of having a relatively non-specific, Na-independent transporter such as rBAT in the brush border membranes of renal and intestinal epithelia, where transporters are generally involved in a net influx of amino acids. Such a transporter would be expected to allow efflux of the amino acids which accumulate within the cell. The wide variety of neutral and basic substrates accepted by this transporter suggests that the interplay of amino acid concentrations and membrane potential will be difficult to decipher, but may allow the transport mediated by this protein to serve a useful role without causing a serious loss of amino acids from the cell. The complex means by which the rBAT protein transports and exchanges such a wide variety of amino acids will require further study before the mechanism by which the transporter functions can be identified.

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References

[1] Stein, W.D. (1989) Methods Enzymol. 171 (Ch. 3), 23–62.
[2] Banderali, U. and Roy, G. (1992) Am. J. Physiol. 263, C1200–C1207.
[3] Tate, S.S., Yan, N. and Udenfriend, S. (1992) Proc. Natl. Acad. Sci. USA 89, 1–5.
[4] Bertran, J., Werner, A., Moore, M.L., Stange, G., Markovich, D., Biber, J., Testar, X., Zorzano, A., Palacin, M. and Murer, H. (1992) Proc. Natl. Acad. Sci. USA 89, 5601–5605.
[5] Van Winkle, L.J. (1993) Biochim. Biophys. Acta 1154, 157–172.
[6] Pickel, V.M., Nirenberg, M.J., Chan, J., Mosckovitz, R., Udenfriend, S. and Tate, S.S. 1993) Proc. Natl. Acad. Sci. USA 90, 7779–7783.
[7] Markovich, D., Stange, G., Bertran, J., Palacin, M., Werner. A., Biber, J. and Murer, H. (1993) J. Biol. Chem. 268, 1362–1367.
[8] Calonge, M.J., Gasparini, P., Chillarón, J., Chillón, M., Gallucci. M., Rousaud, R., Zelante, L., Testar, X., Dallapiccola, B., Di Silverio, F., Barceló, P., Estivill, X., Zorzano, A., Nunes, V. and Palacín, M. (1994) Nature Genet. 6, 420–425.
[9] Kaufman, R.J., Davies, M.V., Wasley, L.C. and Michnick, D. (1991) Nucleic Acids. Res. 19, 4485–4490.
[10] Kriegler, M. (1990) Gene Transfer and Expression: a Laboratory Manual, Stockton Press, New York.
[11] Ausubel, F.A., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1994) Current Protocols in Molecular Biology, Greene/Wiley-Interscience, New York.
[12] Gurevich, V.V., Pokrovskaya, I.D., Obukhova, T.A. and Zozulya, S.A. (1991) Anal. Biochem. 195, 207–213.
[13] Swick, A.G., Janicot, M., Cheneval-Kastelic, T., McLenithan, J.C. and Lane, M.D. (1992) Proc. Natl. Acad. Sci. USA 89, 1812–1816.
[14] Coady, M.J., Pajor, A.M., Toloza, E.M. and Wright, E.M. (1990) Arch. Biochem. Biophys. 283, 130–134.
[15] Quick, M.W., Naeve, J., Davidson, N. and Lester, H.A. (1992) Biotechniques 13, 357–361.
[16] Taglialatela, M., Toro, L. and Stefani, E. (1992) Biophys. J. 61, 78–82.
[17] Costa, A.C.S., Patrick, J.W. and Dani, J.A. (1994) Biophys. J. 67, 395–401.
[18] Taylor, M.A. and Smith, D.L. (1987) Dev. Biol. 124, 287–290.

Note added in proof

Similar research using rBAT1 has recently been published by Busch et al. in 'Opposite directed currents induced by the transport of dibasic and neutral amino acids in *Xenopus* oocytes expressing the protein rBAT', J. Biol. Chem., Vol. 269, pp. 25581–25586, 1994.