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rBAT is an Amino Acid Exchanger with Variable Stoichiometry

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Abstract. The rBAT protein, when expressed in *Xenopus* oocytes, was previously shown to reproduce the selectivity of the Na^+-independent neutral and basic amino acid transport system called $b^{o,+}$. More recently, the capacity of rBAT to generate a transmembrane current was demonstrated when addition of neutral amino acids stimulated the efflux of cations (presumably basic amino acids) in rBAT-injected oocytes. In the present paper, aminoisobutyric acid (AIB), a neutral amino acid analogue, was shown to induce outward currents (efflux of basic amino acids) through rBAT similar to those caused by alanine in terms of affinity, maximal currents and *I-V* curves. Despite generating similar currents, the AIB transport rate was more than 30 times lower than that of alanine, thus challenging the assumption that rBAT functions as a classical exchanger. Experiments using a cut-open oocyte voltage clamp demonstrated that AIB was capable of stimulating rBAT-mediated currents from either side of the membrane. AIB, like alanine, was able to stimulate the efflux of radiolabeled alanine and arginine while no rBAT-mediated efflux was measurable in the absence of external rBAT substrates. These results demonstrate that (i) the presence of amino acids is required on both sides of the membrane for rBAT to mediate amino acid flux and thus rBAT must be some type of exchanger but (ii) rBAT-mediated amino acid influx is not stoichiometrically related to the efflux. A model of a ''double gated pore'' is proposed to account for these properties of rBAT, which contravene standard models of exchangers and other transporters.

Key words: Exchange — Cotransport — rBAT — Transporter — Stoichiometry — Oocyte

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Introduction

Expression cloning has permitted the isolation of cDNAs which are involved in amino acid transport without requiring extensive prior knowledge about the proteins that are coded for by the cloned cDNAs [5]. The cDNA coding for the rBAT protein was independently isolated in this manner by several groups [2, 6, 16, 19]. This protein was originally believed to function as a simple facilitated carrier of neutral and basic amino acids, similar to the $b^{o,+}$ amino acid transporter originally described in mouse blastocysts [18]. More recently, inward and outward currents were shown in rBAT-expressing *Xenopus* oocytes upon external addition of neutral amino acids [1, 3, 6]. From voltage clamp measurements of both intact and internally perfused oocytes, these currents appear to arise from the exchange of neutral amino acids for basic amino acids [6].

The operation of a strict exchanger mechanism is not easily differentiated from a simple carrier which experiences *trans* stimulation [14]; in either model, the substrate on the *trans* side is transported across the membrane and enters the *cis* solution during normal operation of the transporter, and it is this transport itself which permits or accelerates the transport of the *cis* substrate. The difference between the two types of transport is that an exchanger does not permit any transport in the absence of *trans* substrate.

In the present paper, internal perfusion experiments and efflux of radiolabeled substrate show that rBAT displays a fundamental property of exchangers: it requires substrate on the *trans* side to allow transport. However, aminoisobutyric acid (AIB), an analogue used by systems A and $b^{o,+}$, is capable of stimulating an efflux of intracellular basic amino acids which is more than an order of magnitude larger than the AIB uptake. This is in sharp contrast to alanine, which stimulates similar currents but is also transported into the cell at a level similar

to that of the stimulated current. Our results establish that the bilateral presence of amino acids is mandatory for rBAT-mediated transport while the amino acid influx and efflux are not stoichiometrically related. A simple model of a double-gated pore is proposed to explain the observations made.

Materials and Methods

Oocyte Injection and Maintenance

Oocytes were dissected from *Xenopus laevis* (Xenopus One, Ann Arbor, MI) and the follicular layers were removed by incubation in a Ca^{2+}-free Barth's solution containing 2 units/ml collagenase for 90 min with gentle agitation. rBAT RNA [6] was transcribed according to published procedures [9], with the modification that 3 mM $m^7G(5')ppp(5')GTP$ (New England Biolabs, Beverly, MA) and 0.3 mM rGTP were included to obtain capped RNA. The RNA was injected into oocytes in a volume of 46 nl, containing 0.2 μg/μl RNA in water; injection was performed one to two days after oocyte dissection. Oocytes were maintained in Barth's solution, supplemented with 0.1 mg/ml gentamycin, 100 U/ml penicillin, 0.1 mg/ml kanamycin, 2.5 mM Na pyruvate and 5% equine serum [12] for up to eight days.

Materials Used

Isotopes used were α-[1-^{14}C]-AIB (Dupont-NEN, Boston, MA), L-[2,3-^{3}H]-alanine (Amersham, Oakville, Ont.) and L-[2,3-^{3}H]-arginine (Dupont-NEN). The nonradioactive AIB (Sigma, #A-1879) was commercially analyzed for purity by reverse-phase high pressure liquid chromatography (Service de séquences de peptides de l'est du Québec, Ste.-Foy, QC). The AIB peak in the chromatograph was single and symmetrical and no contamination by other amino acids could be detected. All chemicals used were of reagent grade.

Two-Microelectrode Voltage Clamp

Oocyte currents were measured with standard two-microelectrode voltage clamp techniques as previously described [6,11]. Briefly, a commercial amplifier (Oocyte Clamp model #OC-725, Warner Instrument, Hamden, CT) and a data acquisition system (RC Electronics, Santa Barbara, CA) were used to send voltage pulses (150 msec duration) and to simultaneously record current and voltage signals. The oocyte was superfused with Saline-Barth's solution at approximately 1.5 ml/min. The composition of this solution is as follows (in mM): NaCl, 88; KCl, 3; $MgCl_2$, 0.82; $CaCl_2$, 0.74; HEPES/NaOH pH 7.5.

After microelectrode impalements, a membrane potential stabilization period of 1 to 5 min was observed. Oocytes whose membrane potential was less negative than −35 V were discarded. The membrane potential was then clamped to −50 mV. The voltage range covered by the 150 msec pulses was from −175 to +75 mV and traces were analyzed by averaging the signal in a window of 20 msec positioned after the decay of capacitative transients. The measurements are taken in the absence and in the presence of a particular substrate and the substrate-specific current is determined by subtraction [6].

In some experiments, the uptake of labeled substrate into oocytes was compared with the amount of current that was measured in parallel experiments using the same batch of oocytes. This was performed by obtaining the substrate-specific current when the oocyte potential was clamped at the resting potential measured in the presence of an amino acid as previously described [4]. The current has been transformed into pmoles/min, assuming that the charged substrate crossing the membrane was monovalent.

Transport Experiments

Uptake experiments were performed in 1 ml KCl-Barth's (Barth's solution where half of the NaCl has been replaced with KCl) using gentle agitation, as previously described [6]. Agitation was found to be crucial with this type of exchanger since amino acids exiting the oocyte into the unstirred layer of external medium can reduce (by competition) the uptake of external substrates. Uptake experiments were performed at four to eight days following injection, and were terminated by washing 5 times with ice-cold KCl-Barth's containing 5 mM substrate, prior to dissolving the oocyte in 0.5 ml 10% SDS and addition of scintillation cocktail. For efflux experiments, oocytes were loaded with labeled substrate by injection of a 46 nl bolus containing amino acids (either 2 pmoles alanine or 2 pmoles arginine or 81 pmoles AIB) into each oocyte; the substrate was dissolved in injection buffer, which contains 50 mM KCl, 50 mM K-phosphate buffer, pH 7.5. Each oocyte was rinsed 5 times with Barth's solution and incubated for 20 min in Barth's solution, during which time the solution was replaced four times. The oocytes were then exposed to 400 μl of KCl-Barth's and gently agitated; the bathing solution was removed and replaced at 1-min intervals, to enable the measurement of radiolabeled substrate released into the bathing solution.

Cut-Open Oocyte Measurements

Current measurements through a cut-open oocyte system [15] were performed using a custom built amplifier and a commercially available chamber (Dagan, Minneapolis, MN) with, as recently described [7], a low access resistance glass pipette (200 kΩ) for both internal perfusion and membrane potential measurement. The extracellular solution consisted of (in mM): 80 mannitol, 43.8 Na cyclamate, 6.2 NaCl, 3 N-methyl-glucamine (NMG), 1 $MgCl_2$, 0.9 $CaCl_2$ and 5 HEPES, pH 7.6. The intracellular solution consisted of (in mM): 75 mannitol, 43.8 Na cyclamate, 6.2 NaCl, 6 NMG, 1 $MgCl_2$, 1 EGTA, 0.9 $CaCl_2$ and 5 HEPES, pH 7.6. The osmolarity of both solutions was 180 mOsm. The intracellular perfusion rate was adjusted to 5 μl/min by maintaining the flow entering the perfusion pipette at 40 μl/min and the flow leaving the pipette at 35 μl/min.

Data Analysis

All data are expressed as mean and standard error unless otherwise specified; *n* represents the number of oocytes used to derive the data. Statistical comparisons were performed using Student's *t*-test. The kinetic constants were derived by fitting the measured currents to a Michaelis-Menten curve using nonlinear regression (FigP, version 6.0, Elsevier-Biosoft, Milltown, NJ).

Abbreviations

AIB: aminoisobutyric acid; MeAIB: methylaminoisobutyric acid; rBAT: related to $b^{o,+}$ associated transport. (Barth's solution contained (in mM): 91 NaCl, 1 KCl, 0.82 $MgSO_4$, 0.33 $Ca(NO)_3$, 0.41 $CaCl_2$, 5 HEPES pH 7.6. EGTA: ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetracetic acid.)

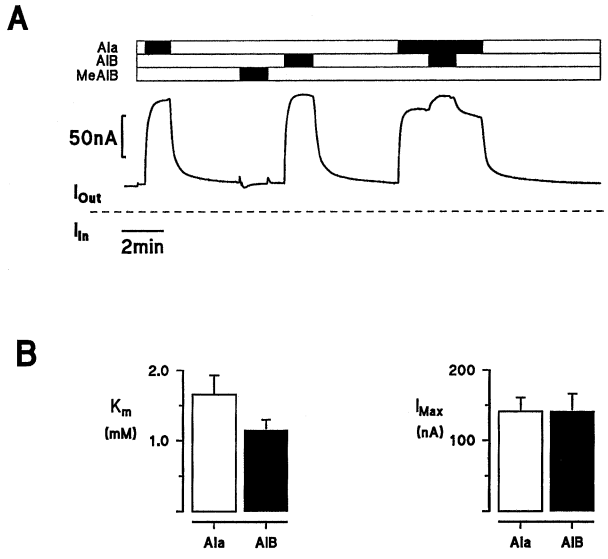

Fig. 1. (*A*) Comparison of rBAT currents induced by AIB and alanine. A *Xenopus* oocyte expressing rBAT was exposed to 5 mM concentrations of AIB, MeAIB and alanine in Saline-Barth's solution. The oocyte membrane potential was clamped at −50 mV and perfusion with each solution was performed until the outward current (I_{out}) reached a steady value. (*B*) Kinetic parameters for alanine and AIB currents. K_m and V_{max} values were determined for the outward currents induced by 5 mM alanine or AIB in Saline-Barth's solution at a holding potential of −50 mV, using a simple Michaelian model. The results were derived using 5 oocytes from 5 different donors.

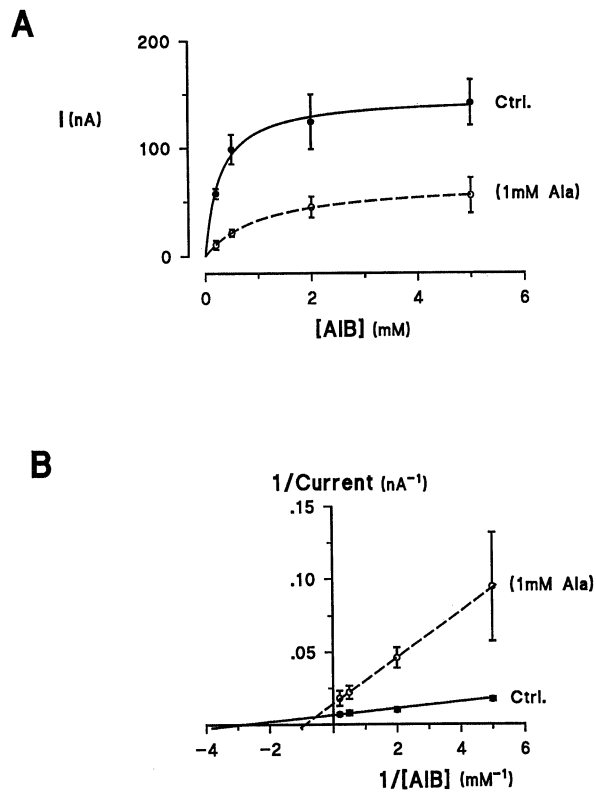

Fig. 2. (*A*) Inhibition of AIB-induced currents by alanine. rBAT-mediated currents at 0 mV were induced by the addition of various concentrations of AIB (control curve); these are compared to currents induced by addition of AIB to bathing solutions that already contained 1 mM alanine. Data shown represent means ± SE from 3 oocytes, each from a separate donor. (*B*) Analysis of alanine-AIB competition. The AIB-induced currents illustrated in (*A*) are represented in a Lineweaver-Burke plot where the intercept with the abscisssa is equal to $-1/K_m$. The maximal AIB-induced current is simply reduced by the amount of current already stimulated by 1 mM alanine.

Results

External application of 5 mM alanine or 5 mM AIB generated similar outward currents in rBAT-expressing oocytes whereas 5 mM MeAIB failed to stimulate any measurable currents (Fig. 1A). The effects of alanine and AIB were not additive since the current stimulated by cell exposure to 5 mM AIB decreased to less than 15% of its original value when AIB was added in the presence of 5 mM alanine. Figure 1B shows the average MichaelisMenten parameters obtained in experiments where several concentrations of alanine and AIB could be applied to the same oocyte ($n = 5$ oocytes). At −50 mV, the affinity for AIB ($K_m = 1.2 \pm 0.1$ mM) was slightly higher than that for alanine ($K_m = 1.7 \pm 0.3$ mM) while the maximal currents were identical (141 ± 20 μA for alanine and 141 ± 25 μA for AIB). Based on these kinetic parameters, if AIB and alanine compete for the same site, one can predict that the AIB-induced current measured in the presence of 5 mM alanine should be 13% of the current seen when AIB is added alone, which agrees with the results shown in Fig. 1A. Control (non-injected) oocytes showed no significant currents upon exposure to either alanine or AIB (I < 2 nA).

The interaction between alanine, AIB and rBAT was further examined in three different experiments where the induction of current by various concentrations of AIB were measured in the presence or absence of 1 mM alanine (Fig. 2). These measurements are reported at a membrane potential of 0 mV, to augment the magnitude and precision of the recorded currents (Fig. 3), especially at low substrate concentrations. The AIB-induced currents were clearly diminished when AIB was added to a solution that already contained alanine (Fig. 2A). It should be reiterated that each of the currents illustrated here actually represents the AIB-induced currents which are determined by subtracting the currents measured in the presence and absence of AIB. For this reason, standard kinetic analysis of competitive inhibition [13] cannot be employed since the I_{max} of the AIB-induced currents in the presence of alanine is automatically diminished by the amount of current originally generated by 1 mM alanine (*see* Fig. 2B) and the amount of current induced by alanine will decrease as the external concentration of AIB increases. Despite these limitations, it can

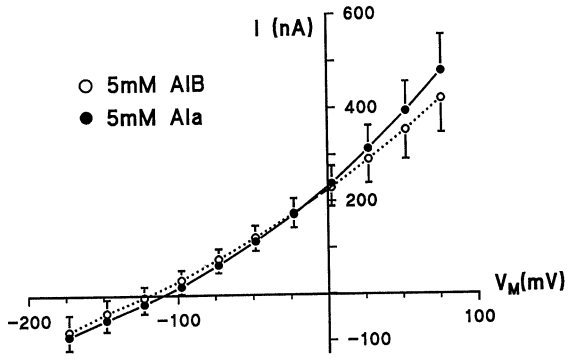

Fig. 3. Current-voltage curve for AIB and alanine-induced currents through rBAT. The current-voltage relationship was measured in 4 rBAT-expressing oocytes using both alanine and AIB.

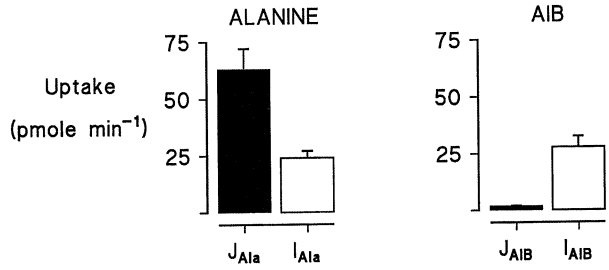

Fig. 4. Comparison of substrate-induced currents and uptakes. The rBAT-mediated transport of alanine and AIB (J_x, calculated after subtraction of uptake into control oocytes) is shown along with the rBAT-mediated currents measurements (I_x) induced by these substrates, measured using other cells from the same batch of oocytes ($n = 3$ different donors). Current levels have been converted from nA to pmoles charge per minute. The alanine uptake in noninjected oocytes was 7.0 ± 0.3 pmoles/min.

be shown that the K_m and I_{max} values can still be used to obtain information about the type of interaction between the two substrates. If the two substrates are purely competitive, the K_{AIB} must increase by a factor (1 + [Ala]/K_{Ala}) when measured in the presence of alanine while the maximal current measured after AIB addition must be reduced by the amount of current already generated by alanine, i.e., I_{max}/(1 + [Ala]/K_{Ala}) where I_{max} and K_{ALa} are, respectively, the maximal current and the affinity for alanine. As illustrated in Fig. 2 (at 0 mV), the presence of 1 mM alanine increased the K_m for AIB from 0.28 ± 0.04 mM to 1.0 ± 0.3 mM while the maximal current decreased from 148 ± 27 nA to 69 ± 22 nA. These changes represent factors of 3.6 and 2.1 for the affinity and the maximal current respectively. When considering individual experiments, the data is consistent with a K_{Ala} varying between 0.4 and 1.0, which is fully compatible with the alanine affinity measured at 0 mV of 0.64 ± 0.3 mM ($n = 4$), in agreement with a purely competitive interaction between alanine and AIB. Best-fit analysis of these data to Hill equations yielded a Hill number of 1.07 ± 0.02 for the currents induced by AIB alone, and 1.15 ± 0.28 for currents induced by AIB in the presence of alanine. Neither is significantly different from a Hill number of 1.

To compare the current-voltage relationships for AIB- and alanine-induced currents, we utilized 4 paired experiments where both substrates had been tested in the same oocytes. The two substrate-induced currents demonstrated the same voltage dependence over an extended voltage range (Fig. 3). They showed similar reversal potentials which were reproducibly seen in rBAT-injected oocytes and are likely to result from accumulation (within the external unstirred layer) of positively charged substrate leaving the cell through rBAT [6].

Previous investigators have shown that rBAT is not capable (or barely so) of transporting AIB [2]. We verified that our cDNA clone of rBAT demonstrated a low level of AIB transport and we used oocytes from the same donors to compare the levels of current stimulated by alanine and AIB to their rates of uptake (Fig. 4). To increase the magnitude of current and flux levels at resting potential, oocytes were slightly depolarized (by about 20 mV) by using KCl-Barth solution [6]. While the two currents generated by alanine and AIB were not significantly different (24 ± 3 nA for alanine and 28 ± 5 nA for AIB, $n = 3$), the rBAT-specific transport rate of AIB was 30 times lower than the alanine transport rate. There was, however, a small but significant uptake of AIB through the expression of rBAT in *Xenopus* oocytes. Thus, AIB is capable of generating currents via basic amino acid efflux similar to those produced by alanine despite the fact that, unlike alanine, AIB is transported only slightly into the cell.

Further studies on the transport of AIB (*data not shown*) utilized injection of AIB into oocytes to examine the effects of intracellular AIB on AIB influx as well as to measure the efflux of radiolabeled AIB from oocytes in response to extracellular amino acids. The presence of intracellular AIB had no effect on subsequent uptake of radiolabeled AIB in either control or rBAT-expressing oocytes. Similarly, when oocytes were injected with radiolabeled AIB and the efflux was monitored, the amount of radioactivity appearing in the extracellular milieu per minute was not significantly different from the background radioactivity of the scintillation counter and no measurable efflux could be stimulated by external addition of 5 mM alanine, 5 mM AIB or .5 mM arginine. This further indicates that AIB is poorly transported by rBAT, and that no efflux is measurable when AIB is in competition with the large pool of intracellular amino acids.

To better examine the effects of extra- and intracellular AIB, we used the cut-open oocyte technique to control of both sides of the oocyte membrane. When 5 mM AIB was added to the external solution while 0.5 mM arginine was perfused internally, outward currents were

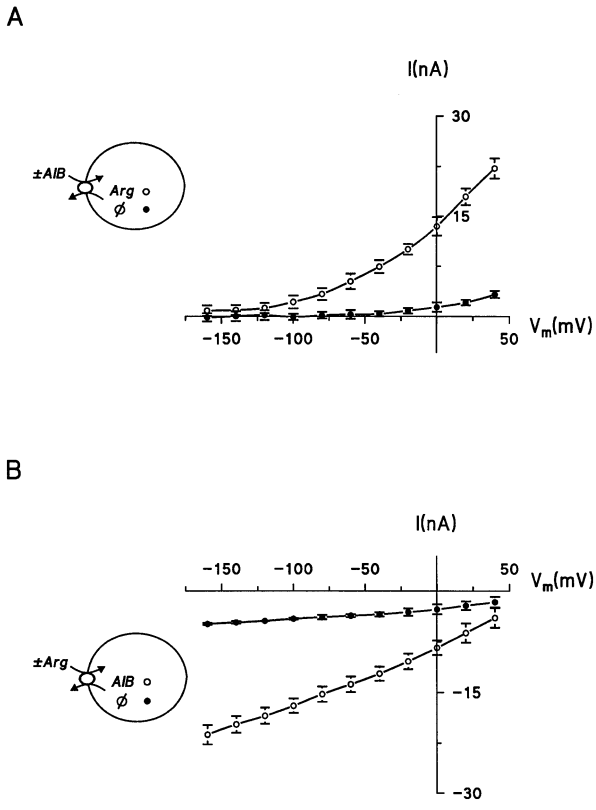

Fig. 5. (*A*) Effects of external AIB on rBAT currents in cut-open oocytes. Addition of 5 mM AIB to an, rBAT-expressing oocyte perfused internally with or without 0.5 mM intracellular arginine caused outward currents. The diagram to the left demonstrates the perfusion conditions on both sides of the membrane during the experiment; the intracellular chamber was perfused with a solution containing either arginine or no substrates. These currents were not observed in control oocytes. (*B*) Effects of internal AIB on rBAT currents in cut-open oocyte. Internal perfusion of rBAT-expressing oocyte membranes with 5 mM AIB permitted the production of large inward currents when 0.5 mM arginine was added externally. These currents were not observed in control oocytes.

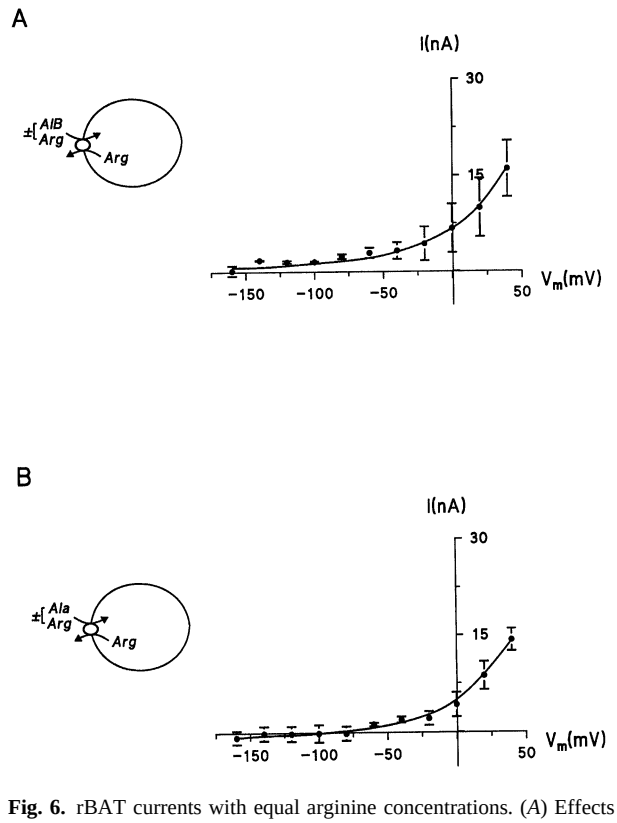

Fig. 6. rBAT currents with equal arginine concentrations. (*A*) Effects of external AIB. The cut-open oocyte system was used with internal perfusion of arginine (0.5 mM). After the external addition of AIB (5 mM) and arginine (0.5 mM), only outward currents were seen. (*B*) Effects of external alanine. Addition of alanine and arginine to the external bathing solution produced similar effects to those seen upon addition of AIB and arginine.

observed which mimicked the effects seen with the two microelectrode voltage clamp technique (Fig. 5A). If intracellular arginine was omitted, then a very small current was found upon addition of AIB to the external solution (Fig. 5A). An inward voltage-dependent current was found using an internal perfusate containing AIB and external application of arginine (Fig. 5B), suggesting that AIB binding sites are accessible at both sides of the membrane and that AIB is capable of stimulating arginine transport in either direction when it and arginine are bound to rBAT at opposite sides of the membrane. A small, voltage-insensitive inward current was seen when arginine was added to the external surface of rBAT-expressing oocytes in the absence of substrate at the internal surface (Fig. 5B).

We also used the cut-open oocyte to further examine the nature of the interaction between AIB and rBAT. The fact that AIB stimulates a current while barely being transported suggests that AIB could work as an allosteric activator transforming rBAT into an arginine transporter. To test this possibility, we measured AIB-induced current in the presence of arginine on both sides of the oocyte membrane. In the presence of 0.5 mM intracellular arginine, addition of 5 mM AIB and 0.5 mM arginine to the external solution failed to stimulate the inward current predicted for an arginine transporter at negative membrane potentials. Instead, a monotonic outward current was observed (Fig. 6A). Similar results were obtained with alanine, as shown in Fig. 6B. This suggests that the binding of AIB to the transporter does not simply transform it into an arginine permease or channel, but that it exclusively favours the transport of *trans* arginine.

While current measurements reveal that AIB can induce the efflux of cationic substrates and the cut-open oocyte demonstrates that arginine is one of these substrates, these techniques cannot reveal whether AIB is capable of promoting the efflux of neutral substrates using the rBAT protein. There is also no information as to whether amino acids can exit via rBAT in the absence of external substrates. To examine these issues, we injected

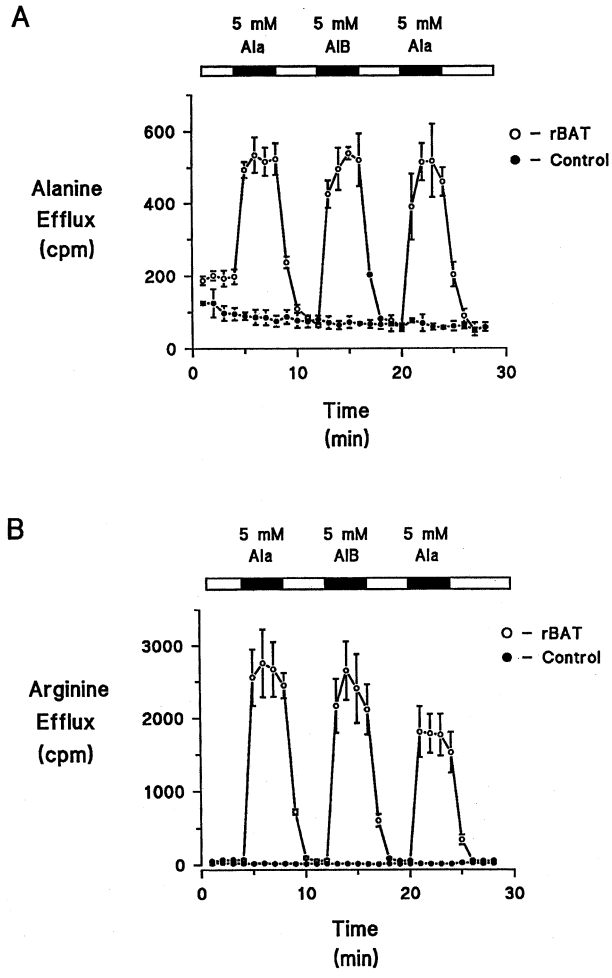

Fig. 7. (*A*) Stimulation of alanine efflux by AIB and alanine. Injection of radiolabeled alanine into *Xenopus* oocytes was followed by measuring the efflux of radiolabel when the oocytes were exposed to alanine or AIB. Values shown are the mean and standard deviation for a typical experiment, using two oocytes per curve; similar results were obtained from another donor. Oocytes contained approximately 27,000 cpm each. (*B*) Stimulation of arginine efflux by AIB and alanine. Injection of radiolabeled arginine into *Xenopus* oocytes was performed as for alanine injections, and efflux was monitored accordingly. Values shown are the mean and standard deviation from a typical experiment, using three oocytes per curve. The results are typical of two sets of experiments. Oocytes contained approximately 40,000 cpm each.

radiolabeled alanine into rBAT-expressing and control oocytes and permitted the wounds to heal for twenty min. Following this, the amount of radiolabel which was released into the bathing solution was measured at 1-min intervals, using incubation in KCl-Barth's solution with gentle agitation. There was a small efflux of radiolabeled alanine in control oocytes which remained fairly constant in the presence or absence of extracellular alanine or AIB (*see* Fig. 7), demonstrating that there is no relation between the efflux of alanine and the presence of extracellular amino acids in normal *Xenopus* oocytes. The efflux from rBAT-expressing oocytes in the absence of external substrates was similar to that seen in the control oocytes. However, the addition of 0.5 mM alanine or AIB to the external surface of the oocyte led to large, identical effluxes of radiolabeled alanine, which were only seen in the rBAT-expressing oocytes (Fig. 7A). Exposure of rBAT-expressing oocytes to external amino acids after they had been similarly loaded with radiolabeled arginine resulted in an efflux of labeled arginine that was dependent on the presence of external substrate. Alanine and AIB stimulated equivalent levels of arginine efflux through rBAT (Fig. 7B) and the arginine efflux in the absence of external substrate was the same in control and rBAT-expressing oocytes. Some control oocytes displayed a small increase in Arg efflux in the presence of alanine or arginine, but this was several orders of magnitude smaller than that seen in rBAT-expressing oocytes. Thus external AIB is capable of inducing the efflux of both cationic and neutral substrates of the rBAT protein, and amino acid efflux through rBAT is vanishingly small in the absence of external substrates.

Discussion

The association of outward currents with exposure of rBAT-expressing oocytes to AIB was a considerable surprise given that this substrate, although chemically similar to alanine, has been shown not to be appreciably transported by rBAT [2]. We repeated the uptake experiments performed by other groups and confirmed that there is a small but statistically significant level of AIB transport by rBAT. The currents measured here after AIB addition could not be due to AIB transport through System A or a similar cotransporter because they were only seen in oocytes expressing rBAT, they were outward (i.e., not due to cotransported Na^+ ions) and were not induced by MeAIB, a paradigm substrate for System A [4]. HPLC analysis confirmed that the AIB used in these experiments was not contaminated by other neutral amino acids or degradation products, which might otherwise have accounted for the currents observed. Thus, AIB binding to the rBAT transporter causes outward currents similar to those caused by alanine and other neutral substrates of this carrier [6].

AIB and alanine compete for binding to a single external site which induces outward currents and is presumably the site involved in rBAT-mediated uptake of alanine. We have also found that AIB and alanine are capable of competing with arginine for the induction of currents (*data not shown*), suggesting that the transporter must have a single external site which binds both neutral and basic amino acids, in agreement with the competitive inhibition observed elsewhere using radiolabeled uptake of neutral and dibasic amino acids [2, 19].

The cut-open oocyte measurements show that AIB can bind to rBAT from either side of the membrane, which initially appears somewhat contradictory to the results described here measuring the influx and efflux of radiolabeled AIB. However, efflux of AIB is limited by the competition between AIB and other internal amino acids for binding to rBAT, and there are considerable quantities of intracellular amino acids in *Xenopus* oocytes [17]. Also, the influx of AIB is extremely low (Fig. 4), despite the lack of external competing substrates, which suggests that the efflux of AIB may also be low. Thus, we believe that AIB can bind to the rBAT amino acid binding sites at either side of the membrane and generate currents, but with a relatively low transport rate in either direction.

The requirement for a *trans* substrate to stimulate amino acid influx or efflux through the rBAT protein is a typical feature of both *trans*-stimulated facilitated carriers and exchangers. The two differ in that an exchanger is unable to transport *cis* substrate in the absence of *trans* substrate. While the rBAT-specific amino acid efflux vanishes in the absence of external amino acids (*see* Fig. 7), a small arginine inward current persists during internal perfusion with an amino acid-free solution (*see* Fig. 5). This current, which is at our detection limit, may well be due to imperfect perfusion of the intracellular contents and we believe that our experimental evidence favors the contention that this protein operates as an exchanger.

Conversely, rBAT does not demonstrate all the characteristics of a ''classical'' exchanger [8], since AIB permits transport of a *trans* substrate without itself being transported in a stoichiometric ratio to the *trans* substrate. Neither the ''consecutive'' nor the ''simultaneous'' models of exchangers [10] can be reconciled with the results described here. The two phenomena demonstrated in this work for rBAT activity (variable ratio between amino acid influx and efflux and the mandatory presence of amino acids on both sides of rBAT) are the fundamental characteristics which must be accounted for by any model describing rBAT-mediated transport. This has prompted us to suggest the model of a double-gated pore, as shown in Fig. 8.

In this model a site (State I) is accessible at each side of the membrane (the sites are not necessarily identical) and two barriers exist, preventing substrates from crossing the bilayer. The binding sites on each side of the membrane are shown as being randomly filled by substrate (States II and III); the model is set up this way for reason of simplicity, and not to imply any knowledge of order at this step. Alanine, arginine and AIB are able to bind at either site though there may well be some rBAT substrates which can only bind at one site, given the wide variety of substrates for this protein. Binding of a substrate to either site removes a barrier; binding of substrates at both sites removes both barriers (State IV).

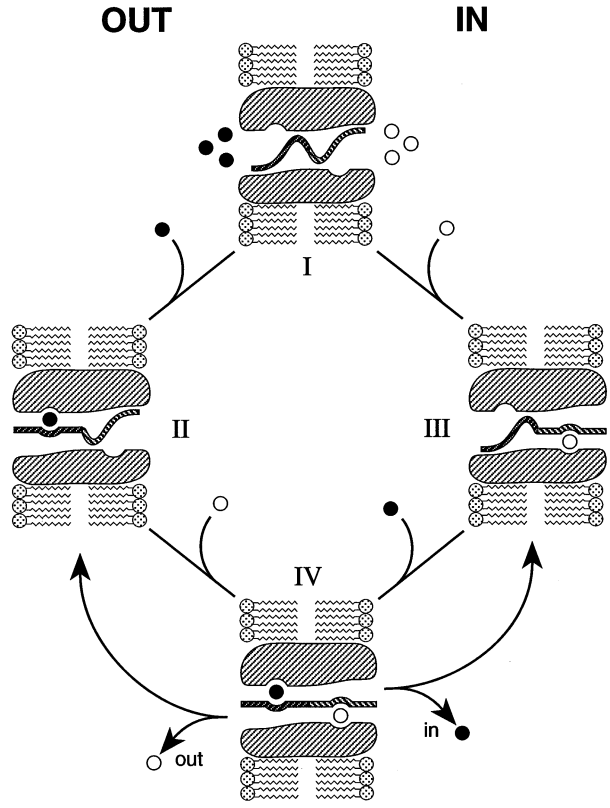

Fig. 8. Suggested model for rBAT transport. A schematic representation of rBAT amino acid exchange is illustrated which incorporates the salient features of AIB-induced transport. The black and white circles represent substrates which are initially (state I) present at the external and internal faces.

Once both barriers are removed, either of the two substrates can be released from their binding sites and diffuse to the other side of the membrane bilayer. Following this release of one substrate, the carrier is able to return to State II or State III, depending upon which substrate was released, and the corresponding barrier to diffusion is resumed. With this model, the ratio between amino acid influx and efflux is not constant and depends on the relative debinding rate constants of each substrate. Note also that the diffusion of a given substrate across the membrane must occur in the presence of the second substrate. Thus, external AIB would bind to the rBAT protein and permit a conformational change but would be relatively slow to debind; the other substrate, bound to the other site, would stand a better chance of debinding first (and generating a current if it carried a charge). AIB debinding is sufficiently slow that the protein, still bound to AIB, returns to the previous conformation (State IV to State II). While other models are certainly possible, the double-gated pore represents, in our view, the simplest model able to account for the requirement of substrate binding on both sides of the membrane and an apparent independence in amino acid influx and efflux. The ca-

pacity of the model to convert energy from one substrate concentration gradient into a secondary active flux of a second substrate is presently under theoretical and experimental investigation.

In summary, rBAT protein was originally presumed to simply facilitate amino acid transport but was recently shown to be involved in amino acid exchange. While the present study confirms that rBAT, like classical exchangers, does require substrate on both sides of the membrane to mediate transport, the efflux of amino acids is not stoichiometrically coupled to the influx. This is clearly revealed by comparing the effects of external alanine and external AIB. The proposed model of a double-gated pore is consistent with these experimental observations which, to our knowledge, have never been reported for another transporter/exchanger protein.

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