Obligatory Amino Acid Exchange via Systems $b^{o,+}$-like and y^+L-like

A TERTIARY ACTIVE TRANSPORT MECHANISM FOR RENAL REABSORPTION OF CYSTINE AND DIBASIC AMINO ACIDS*

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Mutations in the *rBAT* gene cause type I cystinuria, a common inherited aminoaciduria of cystine and dibasic amino acids due to their defective renal and intestinal reabsorption (Calonge, M. J., Gasparini, P., Chillarón, J., Chillón, M., Gallucci, M., Rousaud, F., Zelante, L., Testar, X., Dallapiccola, B., Di Silverio, F., Barceló, P., Estivill, X., Zorzano, A., Nunes, V., and Palacín, M. (1994) *Nat. Genet.* 6, 420–426; Calonge, M. J., Volipini, V., Bisceglia, L., Rousaud, F., De Sanctis, L., Beccia, E., Zelante, L., Testar, X., Zorzano, A., Estivill, X., Gasparini, P., Nunes, V., and Palacín, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 9667–9671). One important question that remains to be clarified is how the apparently non-concentrative system $b^{o,+}$-like, associated with rBAT expression, participates in the active renal reabsorption of these amino acids. Several studies have demonstrated exchange of amino acids induced by rBAT in *Xenopus* oocytes. Here we offer evidence that system $b^{o,+}$-like is an obligatory amino acid exchanger in oocytes and in the "renal proximal tubular" cell line OK. System $b^{o,+}$-like showed a 1:1 stoichiometry of exchange, and the hetero-exchange dibasic (inward) with neutral (outward) amino acids were favored in oocytes. Obligatory exchange of amino acids via system $b^{o,+}$-like fully explained the amino acid-induced current in rBAT-injected oocytes. Exchange via system $b^{o,+}$-like is coupled enough to ensure a specific accumulation of substrates until the complete replacement of the internal oocyte substrates. Due to structural and functional analogies of the cell surface antigen 4F2hc to rBAT, we tested for amino acid exchange via system y^+L-like. 4F2hc-injected oocytes accumulated substrates to a level higher than CAT1-injected oocytes (*i.e.* oocytes expressing system y^+) and showed exchange of amino acids with the substrate specificity of system y^+L and L-leucine-induced outward currents in the absence of extracellular sodium. In contrast to L-arginine, system y^+L-like did not mediate measurable L-leucine efflux from the oocyte. We propose a role of systems $b^{o,+}$-like and y^+L-like in the renal reabsorption of cystine and dibasic amino acids that is based on their active tertiary transport mechanism and on the apical and basolateral localization of rBAT and 4F2hc, respectively, in the epithelial cells of the proximal tubule of the nephron.

Mutations in the human *rBAT* gene are responsible for classic cystinuria (1–3). This is a common inherited aminoaciduria due to the defective transport of cystine and dibasic amino acids through the epithelial cells of the renal tubule and intestinal tract (4). Patients show urinary hyperexcretion of dibasic amino acids and cystine but not of other neutral amino acids; the low solubility of cystine leads to its precipitation and the consequent formation of renal calculi (4). Three types of classic cystinuria have been described on the basis of the amino acid hyperexcretion of heterozygotes and the degree of the intestinal transport defect (5–6). It has been demonstrated that rBAT is only responsible for type I, where heterozygotes are silent (7–8).

The rBAT protein is located in the brush border plasma membrane of the proximal straight tubules of the nephron and of the small intestine (9–10). Due to the role of rBAT in cystinuria, it is considered to be responsible for the reabsorption of cystine and dibasic amino acids in the proximal straight tubule. Human rBAT expressed in *Xenopus* oocytes elicits high affinity sodium-independent transport of cystine, dibasic amino acids, and some neutral amino acids via a $b^{o,+}$-like transport system (11–12). Very recently, the responsibility of rBAT for this amino acid transport activity has also been demonstrated in the "renal proximal tubular" cell line OK (13). We refer to this as system $b^{o,+}$-like, since this activity is very similar to system $b^{o,+}$ described in mouse blastocysts (14); these transport activities are not identical, since the latter does not transport L-cystine. An electrogenic exchange diffusion mechanism for dibasic and neutral amino acids has been reported for the transport activity expressed by rabbit (15–16) and rat (17) rBAT cRNA in oocytes. Coady and collaborators (16) showed that neutral amino acids in the trans-side are needed to observe the currents associated with the transport of L-arginine in oocytes expressing rabbit rBAT. Rennie and collaborators (17) suggested that this hetero-exchange could not fully explain the electric activity associated with the induced neutral amino acid transport due to rat rBAT expression in oocytes. In addi-

tion, it has been suggested that an exchange mechanism of transport does not fit the proposed role for system $b^{o,+}$-like/rBAT in the active renal and intestinal amino acid reabsorption (15, 16, 18). Therefore, the concentrative mechanism of amino acid transport that explains the role of system $b^{o,+}$-like in the active renal reabsorption of dibasic amino acids and cystine remains to be clarified. A further objective is the identification of the transport mechanisms linking the structurally related rBAT and 4F2hc proteins (19–20).

Here we offer evidence that system $b^{o,+}$-like, associated with rBAT expression in oocytes, is an obligatory amino acid exchanger that accumulates substrates as a tertiary amino acid transporter. Hetero-exchange between dibasic and neutral amino acids fully explains the electric activity associated with the induced amino acid transport due to human rBAT expression in oocytes, and the exchange of dibasic amino acids (inward) with neutral (outward) amino acids is favored. This amino acid transport activity explains the role of rBAT/system $b^{o,+}$-like in cystinuria. In addition, we offer evidence that the human cell surface antigen 4F2hc also induces, in oocytes, an asymmetric obligatory amino acid exchanger (system y^+L-like) between dibasic (outward) and neutral amino acids (inward). The participation of these two amino acid transport systems in renal reabsorption is discussed.

MATERIALS AND METHODS

Oocytes, Injections, and cRNA Synthesis—Oocyte origin, management, and injections were as described elsewhere (11). Defolliculated stage VI *Xenopus laevis* oocytes were injected with saturating concentrations (1–5 ng/oocyte) of human rBAT, human 4F2hc, or mouse CAT1 cRNA. Except where indicated, noninjected oocytes were used as controls; amino acid transport rates obtained with oocytes injected with water (50 nl) were similar to those of uninjected oocytes (data not shown). Synthesis of human rBAT, human 4F2hc (cDNA cloned in *Eco*RI-*Hin*dIII pSPORT-1, from the original cDNA cloned in pSP65 by Teixeira and collaborators (21)), and mouse CAT1 cRNAs is described elsewhere (11, 22).

Oocyte Uptake Studies—Influx rate measurements of L-[³H]arginine, L-[³H]leucine, and L-[³⁵S]cystine (NEN Radiochemicals) were measured in 100 mM NaCl or 100 mM choline Cl medium at the indicated days after injection and in linear conditions as described elsewhere (11, 19, 23). When presented, cRNA (rBAT, 4F2hc, or CAT1)-induced uptake was calculated by subtracting uptake values in uninjected oocytes from those of the corresponding cRNA-injected oocytes.

For efflux rates measurements, 3 or 4 days after injection of the corresponding cRNA, groups of 5–7 oocytes were incubated, at 25 °C, for 30 min (rBAT and CAT1 experiments) or 60 min (4F2hc experiments) in medium containing 50 μM L-[³H]arginine or L-[³H]leucine (3–10 μCi/90 μl). More than 95% of the oocyte-soluble radioactivity corresponded to the original labeled amino acid (see below). In five independent rBAT experiments, this loading ranged between 145,000 and 295,000 cpm/rBAT-injected oocyte and 139,000 and 315,000 cpm/rBAT-injected oocyte for L-[³H]arginine and L-[³H]leucine uptakes, respectively. In the 4F2hc experiments (*n* = 4), this loading ranged between 50,000 and 121,000 cpm/4F2hc-injected oocyte and 35,000 and 133,000 cpm/4F2hc-injected oocyte for L-[³H]arginine and L-[³H]leucine uptakes, respectively. For those experiments, loading of uninjected oocytes ranged between 8,000 and 21,000 cpm/oocyte and 27,000 and 88,000 cpm/oocyte for L-[³H] arginine and L-[³H]leucine uptakes, respectively. After this loading, the radioactive medium was washed 4 times in choline medium at 25 °C. Then, efflux was measured as the appearance of tritium in unlabeled incubation medium (0.6–1 ml of sodium or choline medium as indicated) containing no amino acids (none) or different L-amino acids at the indicated concentrations. When L-cystine was used, efflux was always measured in the presence of 10 mM diamide to prevent L-cystine reduction. In these conditions, diamide did not affect efflux by uninjected oocytes (data not shown). Efflux was measured taking aliquots (200 μl) from the medium at zero time and at different times. Efflux rates were calculated by subtracting the radioactivity present at zero time. Previous studies demonstrated that after subtracting the zero value the best fit line passed through the origin, and it was linear for 1, 2, or 5 min for CAT1-injected, rBAT-injected, or 4F2hc-injected oocytes, respectively (data not shown). Efflux rates are expressed either as the radioactivity (cpm × 1,000) appearing in the

medium per unit time (2 or 5 min) per group of 5–7 oocytes, when representative experiments are shown, or as the percent of the total radioactivity loaded into the oocyte appearing in the medium per unit time (2 or 5 min), when combined experiments are shown. Statistical comparisons were performed using the Student's *t* test.

In the accumulation studies, the radioactivity content of rBAT cRNA-injected oocytes after 3 h incubation with L-[³H]arginine, L-[³H]leucine, or L-[³⁵S]cystine (in the presence of 10 mM diamide) was tested for metabolization. Oocyte homogenates (choline medium) were precipitated with 5% trichloroacetic acid. More than 97% of the radioactivity from L-[³H]arginine- and L-[³⁵S]cystine-incubated oocytes remained in the soluble phase; for L-[³H]leucine-incubated oocytes 15% of the radioactivity was trichloroacetic acid-precipitated, suggesting incorporation into proteins. In the L-[³⁵S]cystine experiments, oocytes were homogenized in the presence of 20 mM *N*-ethylmaleimide (NEM)[1] to analyze L-[³⁵S]cystine metabolites-NEM derivatives, as described elsewhere (25). The soluble phase of trichloroacetic acid precipitation was analyzed by thin layer chromatography as described elsewhere (Ref. 26 for L-[³H]arginine and L-[³H]leucine experiments and Ref. 25 for L-[³⁵S]cystine experiments). In all cases, >95% of the radioactivity showed the same chromatographic mobility as the original incubated amino acids, visualized with 0.2% ninhydrin in acetone (L-[³H]arginine and L-[³H]leucine experiments) or by autoradiography (L-[³⁵S]cystine experiments) (data not shown). In the absence of diamide, almost all L-[³⁵S]cystine radioactivity was recovered, in both the oocytes and the medium, as a product with identical chromatographic mobility to the L-[³⁵S]cysteine-NEM derivative.

As a reference value for the space distribution of amino acids in the oocyte in the accumulation studies, the space distribution of water was measured by incubating groups of 7–8 oocytes with [³H]water (2 μCi; ~2.4 × 10⁶ cpm/90 μl; Amersham Corp.) for up to 30 min. After incubation, oocytes were washed 3 times in ice-cold choline medium, and the radioactivity of SDS-dissolved single oocytes was counted with scintillation fluid in a β-radioactivity counter, as described elsewhere for uptake studies in oocytes (23). The uptake of [³H]water increased from 30 s to 2 min and then reached a plateau (4,000–5,000 cpm), which was maintained for the next 30 min. From this we estimated a space distribution of water in stage VI oocytes of 176 ± 14 nl (mean ± S.E. from 10 groups of oocytes in 2 independent experiments).

Oocyte Electric Measurements—Dissection of *X. laevis*, collection, and handling of the oocytes was described in detail elsewhere (27). Oocytes were injected with cRNA (1 ng/oocyte) or water, and two-electrode voltage and current clamp recordings were performed 3–8 days later in single oocytes in a perfused chamber, as described elsewhere (15). The external control solution (ND96 medium) contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, and 5 mM HEPES at pH 7.5. In some experiments, Na⁺ was replaced by choline. The amino acids were added to the solution at the indicated concentrations, and the tested oocyte was perfused at 20 ml/min, which guaranteed a complete solution exchange in the recording chamber within 10–15 s. The maximal current amplitude induced during amino acid superfusion was measured. Inward currents are shown with the prefix -. All data are given as means (± S.E.), where *n* gives the number of oocyte measurements. Statistical comparisons were performed using the Student's *t* test. Experiments were repeated with 2 batches of oocytes; in all repetitions, qualitatively similar data were obtained.

OK Cell Studies—The OK cell line clone 3B/2 (28), derived by selection from the original OK cells (29), between passages 16 and 21, was used in this study. Selected OK cell clones (13) that express human rBAT antisense (AS1) and sense (S1) sequences after permanent transfection of a 669-base pair *Eco*RI/*Cla*I fragment from the 5′-end of the full-length human rBAT cDNA (11) were also used. Cell culture conditions were as described (13, 30).

Efflux rates measurements of L-[³H]arginine into MGA medium (137 mM *N*-methyl-D-glucamine, 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgSO₄, 10 mM HEPES, pH 7.4) containing or not containing unlabeled L-amino acids were as described elsewhere (13). Results are expressed as cpm, corrected per 150,000 cpm loaded/mg of protein·min. Statistical comparisons were done using the Student's *t* test. Thin layer chromatography analysis (26) of the efflux medium revealed that ~95% of the efflux radioactivity corresponded to L-arginine (data not shown).

Simulation of the b^{o,+}-like Amino Acid Exchanger—To simulate the L-³H-amino-acid, accumulation experiments were performed in oocytes expressing the human rBAT cRNA; we constructed a model based on

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[1] The abbreviations used are: NEM, *N*-ethylmaleimide; CAT1, cationic amino acid transporter 1.

the following premises. 1) The induced amino acid transport activity is an obligatory exchanger of dibasic and neutral amino acids with a 1:1 stoichiometry. 2) An endogenous, independent, and equilibrative transport system is necessary to explain the amino acid transport of uninjected oocytes. Fig. 1 shows the obligatory exchange "ping-pong" mechanism considered here for system $b^{o,+}$-like. An alternative concerted mechanism of obligatory exchange, tested in parallel, gave similar results for the accumulation behavior to the ping-pong mechanism. The computer program written to simulate the amino acid transport activity in oocytes expressing rBAT is available upon request.

The simulation was prepared according to the following steps.

(i) *Characteristics of the simulated system.* The experimental system of L-[³H]arginine accumulation studies (Fig. 5) was reproduced as two separate compartments of 90 μl (the outside, uptake medium) and 180 nl per oocyte (the inside, the space distribution for [³H]water, see above). Initial inner concentrations of the amino acid substrates of system $b^{o,+}$-like for stage VI *Xenopus* oocytes were set according to Taylor and Smith (31). The concentrations of L-arginine (and L-[³H]arginine), L-leucine, and L-cystine were treated individually, whereas the rest of the amino acids were considered in two groups, neutral and dibasic. Transport rates were evaluated from the relative concentrations of transporter binary and empty complexes at either side, according to the formalism of Cha (32), which allows us to combine equilibrium and steady-state steps in a single mechanism. Binding steps were considered as equilibria (defined as dissociation constants, K_o, outside and K_i, inside), whereas translocation steps were considered as steady-state (defined as two translocation rate constants, k_{in}, k_{out}) (Fig. 1). During the simulation, amino acid concentrations at either side were modified by numerical integration of the transport rates. The equilibrium assumptions do not significantly alter the calculated rates with respect to a full steady-state mechanism. For the experiment shown in Fig. 6, superfusion experimental conditions were simulated by fixing the external concentrations to the initial values, and only internal concentrations were changed. This is reasonable as the external solution is exchanged in 10–15 s (see above).

The transport rates of dibasic amino acids are markedly influenced by the membrane potential (Φ). The effect of this parameter was introduced by modification of dissociation and translocation constants according to the following equation:

$$P_i = P_i^0 exp\{\partial Z_i F\Phi/nRT\} \tag{Eq. 1}$$

where P_i is the given parameter, P_i^0, its value at zero potential, Z_i, the amino acid charge, F, 96,500 coulomb/mol, R, 8.31 J/mol·K, and T, the absolute temperature (fixed to 298 K), n was set to 1 for equilibrium constants, and 2 for rate constants. ∂ represents the effective fraction of the membrane potential that influences the step indicated by P. This fraction was arbitrarily set to 0.2 for binding and to 0.6 for translocation steps. This parameter (∂) has little influence on the accumulation curves (data not shown). Membrane potential was fixed to −50 mV for resting oocytes and to −35 mV for 50 μM L-arginine uptake (see legend to Fig. 5). Initial potentials for other concentrations were varied according to the expected initial rates. The membrane potential was returned to the resting value after some time (see legend to Fig. 5). This effect was also included in the simulation by using a time constant of 100 min, which is a good representation of the experimental evolution.

(ii) *Amino acid kinetic parameters.* The simulation system prepared as indicated above permits us to follow the evolution of amino acid concentrations and, therefore, to simulate experimental influx and efflux rates in any experimental condition. However, before using the system, values are required for the kinetic parameters (see Fig. 1) for every amino acid and the concentration of transporters. No direct experimental determination of transporter concentration is available; therefore, V_{max} values as reported in Table I represent inseparable combinations of the transporter concentration and the translocation constants. For this reason, the translocation constants (k_{in} and k_{out}) for L-arginine were set arbitrarily to 1, and used as reference. The translocation constants for the remaining amino acids were then used as relative values. In this way, changes in the transporter concentration are represented by a single parameter (adjusted as indicated below), which affects every amino acid in the right proportion.

Relative values of translocation constants were estimated as follows. The efflux rates determined according to the experiment of Fig. 2 were simulated using several combinations of parameters. An analytic dependence of the calculated rates and the parameters was then deduced, and the best estimates of the parameters were obtained. Apparent parameters for the pools of neutral or dibasic amino acid were obtained by averaging the individual values of each amino acid at its relative
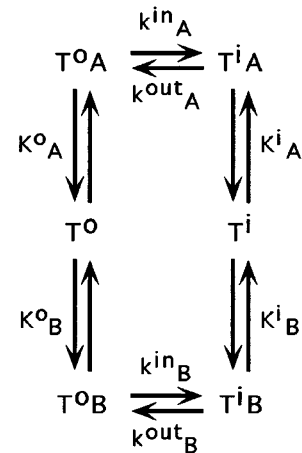

FIG. 1. **Reaction mechanism for the amino acid exchanger model.** A ping-pong mechanism (only one amino acid, either A or B, is transported at once) of obligatory exchange is shown. Binding steps were considered as equilibria (defined as dissociation constants K_o and K_i), whereas translocation steps were considered as steady state (defined as two translocation rate constants k^{in}, k^{out}). The system has been assumed to be symmetric (*i.e.* $k^{in} = k^{out}$, $K_o = K_i$). The equilibrium assumptions do not significantly alter the calculated rates with respect to a full steady-state mechanism. Translocation of the empty transporter (T^o, facing outside; T^i, facing inside) is not considered, since system $b^{o,+}$-like is assumed to be an obligatory exchanger. The same mechanism of transport was used for the endogenous transporter but allowing free translocation of the empty transporter, as this is considered an equilibrative system.

concentration. The whole simulation and fitting cycle was repeated until self-consistence. The final values of the relative translocation constants ($k_{in} = k_{out}$) at zero membrane potential were 2.1 for L-leucine, 1.05 for the pool of neutral amino acids, and 0.78 for the pool of dibasic amino acids. Dissociation constants (K_o, K_i) were set to 90 μM for all the amino acids, according to the external apparent K_m values obtained (Table I). Due to the high internal concentrations of substrates, the $b^{o,+}$-like transporter remained saturated during the simulation. Therefore, the precise values of the internal dissociation constants have little influence on the results obtained.

(iii) *Simulation of the accumulation experiments.* To reproduce accumulation studies shown in Fig. 5, first the concentration and kinetic parameters of the endogenous transporter were manually adjusted to reproduce the accumulation curves obtained in uninjected oocytes (see legend to Fig. 5). Second, in the presence of this endogenous activity, the V_{max} of L-arginine influx, which includes the concentration of $b^{o,+}$-like transporter and the actual value of the L-arginine translocation constant, was set to fit the initial influx rates of rBAT-injected oocytes (see legend to Fig. 5). Finally, with the concentrations chosen and the parameters indicated above the system was simulated for the desired time.

RESULTS

Substrate Specificity and Kinetic Parameters of the $b^{o,+}$-like Amino Acid Exchanger Expressed in Oocytes—Several studies have suggested that the amino acid transport system $b^{o,+}$-like, associated with rBAT expression in oocytes, is an amino acid exchanger (15–17). If this is correct, the substrate specificity and the apparent kinetic parameters should be identical when influx or the amino acid-dependent stimulation of efflux are measured. The efflux of L-[³H]arginine via system $b^{o,+}$-like in rBAT-injected oocytes was stimulated by amino acids in the external medium with the same substrate specificity shown in uptake studies (influx) through this transport activity (Fig. 2A). Thus, 1 mM dibasic and neutral L-amino acids (or 200 μM L-cystine) in the external medium, which are substrates for system $b^{o,+}$-like (23, 33–35), increased efflux of L-arginine in rBAT-injected but not in uninjected oocytes. L-Proline and L-glutamate, which are not substrates for system $b^{o,+}$-like (23, 33–34), did not increase efflux of L-[³H]arginine in rBAT-injected oocytes (Fig. 2A). Similar data of substrate specificity
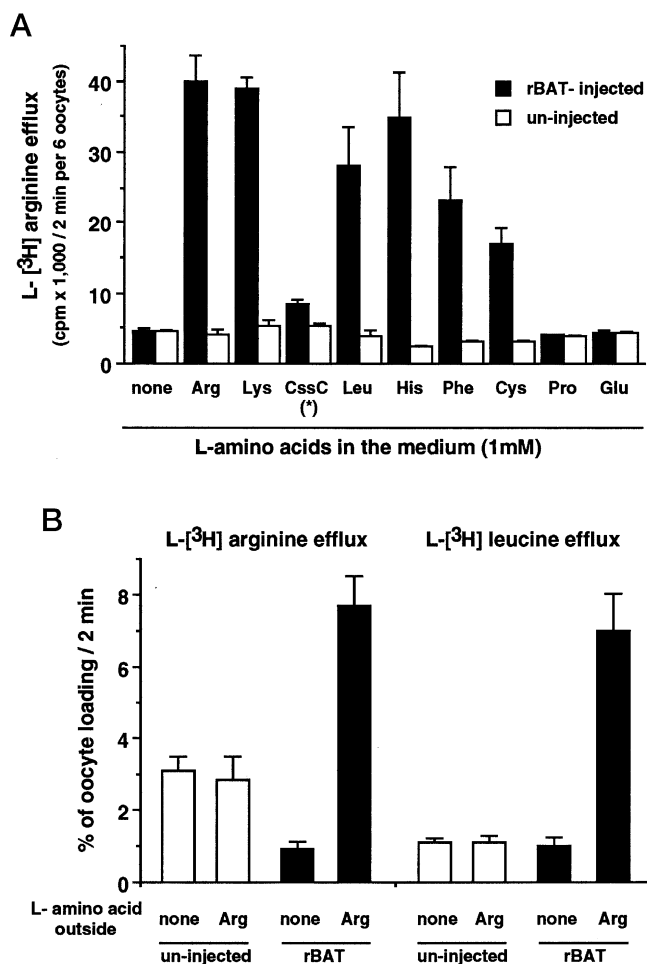
A

B

FIG. 2. L- [³H]Arginine and L- [³H]leucine efflux via amino acid transport system $b^{o,+}$-like in oocytes. Oocytes were injected with 5 ng of rBAT cRNA (*black bars*) or uninjected oocytes (*open bars*). Four days later, amino acid efflux rates were measured in choline medium containing no amino acids (none) or the indicated 1 mM L-amino acids. 200 μM L-cystine (*CssC*) was used in the presence of 10 mM diamide. *A*, L- [³H]arginine efflux rates (expressed as the radioactivity (cpm × 1,000) appearing into the medium per 2 min per group of 6 oocytes) correspond to the mean ± S.E. of triplicates from a representative experiment. All the L-amino acids, but L-proline and L-glutamate, in the medium significantly increased efflux from rBAT cRNA-injected, but not from uninjected oocytes ($p \leq 0.01$). *B*, L-[³H]Arginine and L-[³H]leucine efflux rates into unlabeled medium containing no amino acids (none) or 1 mM L-arginine (*Arg*) from rBAT cRNA-injected or uninjected oocytes. Efflux rates (*i.e.* radioactivity appearing in the medium/2 min) are expressed as percent of the total radioactivity loaded into the oocyte. Data (mean ± S.E.) are from 9–12 determinations (5 independent experiments, L-arginine efflux) and from 9 determinations (3 independent experiments, L-leucine efflux). L-Arginine significantly induced L-[³H]arginine and L-[³H]leucine efflux from rBAT cRNA-injected ($p \leq 0.001$) but not from uninjected oocytes. L-[³H]Arginine efflux into medium containing no amino acids (*none*) was lower from rBAT cRNA-injected than from uninjected oocytes ($p \leq 0.001$), whereas L-[³H]leucine efflux into medium containing no amino acids (none) was similar in the two groups.

were obtained when the efflux rates of L-[³H]leucine were measured (data not shown). The efflux rates of L-[³H]arginine and L-[³H]leucine from several experiments, and expressed as percent of the previous loading, are shown in Fig. 2*B*. The efflux rates of L-[³H]leucine into amino acid-free medium were identical in rBAT-injected and uninjected oocytes, whereas they were increased ≤7-fold by 1 mM L-arginine only in oocytes expressing rBAT (Fig. 2*B*). This supports the idea that system $b^{o,+}$-like is an obligatory amino acid exchanger; transport via system $b^{o,+}$-like occurs only when substrates are present on the

TABLE I
Kinetic parameters of system $b^{o,+}$-like amino acid exchanger activity associated with rBAT expression in oocytes

rBAT-induced influx rates for L-cystine, L-arginine, and L-leucine were measured 3–4 days after injection of rBAT cRNA (5 ng/oocyte) at 7–8 different substrate concentrations (10–250 μM) and calculated as described under "Materials and Methods." The amino acid-elicited L-[³H]arginine efflux rates were assayed at 6-different concentrations (10–1,000 μM) of the external substrate and calculated by subtracting the rates of efflux into medium containing no amino acids. K_m for efflux represents the concentration of external amino acid needed for the semi-maximal amino acid-elicited efflux. Influx data (mean ± S.E.) correspond to representative kinetic experiments run in triplicate. Efflux data (mean ± S.E.) correspond to 3–6 determinations from 3 independent experiments. ND, not determined.

Substrate	L-Cystine	L-Arginine	L-Leucine
Influx			
K_m (μM)	41 ± 7	85 ± 7	90 ± 12
V_{max} (pmol/min/oocyte)	9 ± 1	211 ± 11	59 ± 4
Efflux (L-arginine)			
K_m (μM)	ND	65 ± 5	67 ± 26
V_{max} (% oocyte loading/2 min)	ND	6.1 ± 0.2	2.9 ± 0.6

trans-side. A similar interpretation of the efflux rates of L-[³H]arginine is difficult to postulate. The efflux rates of L-[³H]arginine, expressed as percent of the previous loading, into medium containing no amino acids in rBAT-injected (~1% in 2 min) are lower than in uninjected oocytes (>3% in 2 min) (Fig. 2*B*). This suggests that either rBAT expression results in the retention of L-arginine inside the oocyte or L-[³H]arginine uptake reaches two different pools in the oocyte, one of these pools being quantitatively important for the uptake measured in uninjected oocytes but not in rBAT-injected oocytes. In any case, L-[³H]arginine efflux was increased 8-fold by 1 mM L-arginine only in oocytes expressing rBAT (Fig. 2*B*). This demonstrates that L- [³H]arginine efflux via system $b^{o,+}$-like is also dependent on the presence of amino acid substrates on the trans-side.

Next, the kinetic parameters of the amino acid transport activity (efflux and influx) induced by rBAT in oocytes were measured. L-Arginine- and L-leucine-elicited efflux of L-[³H]arginine showed saturability (data not shown) with similar apparent K_m values for the external amino acids (Table I). Interestingly, the apparent K_m values for L-arginine and L-leucine either acting as substrates for influx or stimulating efflux of L-[³H]arginine were similar (μM range, Table I). This is expected for an obligatory exchanger, and it is inconsistent with an allosteric mechanism of trans-stimulation of efflux. For all the substrates, when efflux or influx was measured, the Hill coefficient was never significantly different from 1 (data not shown), suggesting interaction of one molecule of external substrate per functional molecular unit of transporter. The V_{max} values for influx via system $b^{o,+}$-like in oocytes ordered the three substrates considered as follows, L-arginine > L-leucine ≫ L-cystine. Comparison of this with the potency of these amino acids to elicit L-arginine efflux (Fig. 2*A* and Table I) suggested that in the hetero-exchange events via system $b^{o,+}$-like the "slowest" substrate (*i.e.* L-cystine) limits the transport activity.

The System $b^{o,+}$-like Associated with rBAT Expression in OK Cells also Behaves as an Amino Acid Exchanger—We have shown in a previous study that the *rBAT* gene is necessary for the amino acid transport system $b^{o,+}$-like activity in the apical pole of the renal proximal tubular cell line OK; permanent transfection of antisense rBAT sequences results in a specific decrease (60% inhibition in the antisense clone AS1 but not in the sense clone S1) in this transport activity (13). The substrate specificity of the stimulation of L-[³H]arginine efflux in OK cells
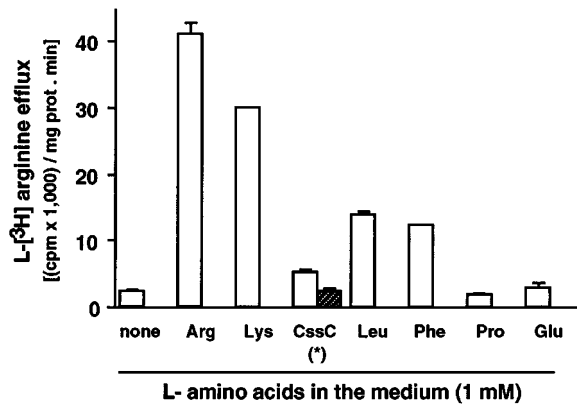
FIG. 3. L- **[³H]Arginine efflux in OK cells.** L-[³H]Arginine efflux rates were measured in media containing no amino acids (*none*) or the indicated L-amino acids at 1 mM, except for L-cystine (*CssC*), which was present at 200 μM plus 5 mM diamide (as controls, cells were incubated in amino acid-free medium plus 5 mM diamide, *hatched bar*). Efflux rates (mean ± S.E.) are cpm × 1,000 measured in the medium (corrected for 150,000 cpm loaded into the cells)/mg of protein per min from 3 to 12 determinations (5 independent experiments). Data for L-lysine and L-phenylalanine groups are the mean from two determinations in a representative experiment. Efflux rates in the L-cystine, L-leucine, and L-arginine groups were significantly different ($p \leq 0.05$) from those of the none, L-proline, and L-glutamate groups.

was similar to that of system $b^{o,+}$-like expressed in oocytes (Figs. 2*A* and 3). L-Leucine-elicited L-[³H]arginine efflux decreased by 60% in the rBAT antisense-transfected clone (16,800 ± 2,400 and 6,500 ± 700 cpm/mg protein·min in the rBAT-sense S1 and rBAT-antisense AS1 clones respectively, n = 6). In contrast, L-[³H]arginine efflux into medium containing 1 mM L-glutamate, which is not a system $b^{o,+}$-like substrate (Figs. 2*A* and 3), was not affected by antisense expression (L-[³H]arginine efflux was 1,490 ± 840 and 1,480 ± 580 cpm/mg protein·min in the rBAT-sense S1 and rBAT-antisense AS1 clones, respectively, n = 6). This indicates that L-[³H]arginine efflux via system $b^{o,+}$-like does not occur in the absence of substrates in the medium and demonstrates that L-leucine-elicited L- [³H]arginine efflux occurs via system $b^{o,+}$-like (associated with rBAT expression). Kinetic analysis of this efflux showed saturability via a single component with an apparent K_m value of 295 ± 64 μM (data not shown). This value fits reasonably well with the estimated apparent K_m value for the influx of L-leucine through the component inhibited by L-arginine (175 ± 56 μM; Ref. 13). Then, as already shown in the oocyte studies, system $b^{o,+}$-like associated with rBAT behaves as an obligatory exchanger in OK cells.

The System $b^{o,+}$-like Accumulates Its Substrates in the Oocytes as a Tertiary Active Transporter—An obligatory amino acid exchanger is considered a tertiary active transport mechanism; it accumulates substrates as a result of exchange with amino acids on the trans-side. It has been reported that stage VI *Xenopus* oocytes contain a high amount of free amino acids that are substrates of system $b^{o,+}$-like (31). If the degree of coupling of exchange of system $b^{o,+}$-like expressed in oocytes is high enough, uptake via this system should result in the accumulation of these substrates in the oocyte. Uptake studies of 50 μM L-[³H]arginine, L-[³H]leucine, or L-[³⁵S]cystine during long incubation periods (3–6 h) showed a higher plateau of accumulation of these substrates in oocytes expressing rBAT than in uninjected or CAT1-injected oocytes (*i.e.* oocytes expressing the dibasic amino acid transport y^+; Ref. 22) (data not shown and Fig. 4). Similarly, L- [³H]arginine accumulation reached higher levels in 4F2hc-injected oocytes than in uninjected or CAT1-injected oocytes (data not shown); these data are discussed below. Similar uptake values by rBAT-injected oocytes were
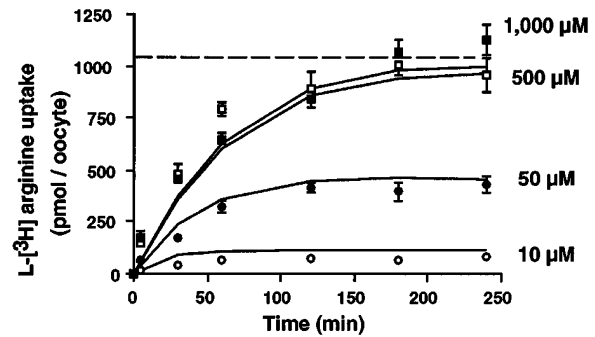

FIG. 4. **Concentration dependence of L-arginine accumulation in rBAT-injected oocytes.** Oocytes were injected with 5 ng of rBAT cRNA or uninjected (not shown). Three days later the uptake of L-[³H]arginine (3–5 μCi/90 μl) was measured at 10 (*open circles*), 50 (*closed circles*), 500 (*open squares*), and 1,000 (*closed squares*) μM concentration of substrate for the time indicated and in sodium uptake medium. Simulations (*curved lines*) of the accumulation progress for the rBAT-injected oocytes were estimated with the obligatory exchange mechanism described under "Materials and Methods." The V_{max} for $b^{o,+}$-like transport activity was adjusted to fit the influx velocity of L-[³H]arginine by rBAT-injected oocytes measured in this experiment. The endogenous transport activity was adjusted to reproduce the influx velocity of L-arginine (3.8 ± 0.4, 4.9 ± 0.7, 7.0 ± 0.8, and 16.5 ± 1.3 pmol/5 min per oocyte at 10, 50, 500, and 1,000 μM L-[³H]arginine), the evolution of the accumulation curves, and the plateau of accumulation (~30, ~125, ~160, and ~230 pmol in 4 h per oocyte at 10, 50, 500, and 1,000 μM L-[³H]arginine) for the uninjected oocytes measured in this experiment; at 500 and 1,000 μM concentrations, the uptake in uninjected oocytes increased over the time (4 h) assayed without reaching a plateau. The total content of internal substrates for system $b^{o,+}$-like (31) is shown by a *horizontal dashed line*. The uptake data (pmol/oocyte) are the mean ± S.E. from 7 oocytes in a representative experiment. When not visible the errors are smaller than symbols. Similar data were obtained in another two independent experiments.

obtained in the absence and in the presence of sodium (data not shown). With an oocyte space distribution for polar substrates of ~180 nl (see "Material and Methods"), the plateau of uptake values of 50 μM L-[³H]arginine uptake represents a 55-fold accumulation (~40-fold for L-[³H]leucine or L-[³⁵S]cystine uptake) of the substrate in rBAT-injected oocytes (Table II). In contrast, the level of L-[³H]arginine accumulation in CAT1-injected and in uninjected oocytes was 14–20-fold (Table II). This demonstrates that under these conditions accumulation of substrates via system $b^{o,+}$-like is clearly higher than via system y^+.

If the accumulation of substrates via system $b^{o,+}$-like is due to exchange with the intracellular oocyte substrates, their total oocyte content (~1,000 pmol/oocyte; Ref. 31) would limit this accumulation. The increase in L-[³H]arginine concentration from 10 to 1,000 μM resulted in a nonlinear increase in L-arginine accumulation, which reached a maximum of ≤1,000 pmol/oocyte at ~500 μM L-arginine (Fig. 4). This is at odds with the uptake via system y^+, associated with CAT1 expression, which increased from ~140 pmol/oocyte at equilibrium with 50 μM L-[³H]arginine to ~750 pmol/oocyte, without reaching equilibrium after 4 h, with 500 μM L-[³H]arginine (data not shown). This level of L-arginine uptake is nearly 4 times the reported dibasic amino acid content of stage VI oocytes (31). To test whether a tertiary active transport mechanism could explain the accumulation of substrates in rBAT-injected oocytes, we simulated the L- [³H]arginine accumulation curves shown in Fig. 4 with a model that considers system $b^{o,+}$-like as an obligatory exchanger with 1:1 stoichiometry (see "Material and Methods"). Interestingly, this modeling reproduces the experimental results (see *lines* in Fig. 4). At the highest L-[³H]arginine concentration used (*i.e.* 1 mM), the model predicts that at equilibrium nearly 98% of the internal substrates of system $b^{o,+}$-like have been replaced by L-arginine with the initial spe-

TABLE II
Accumulated gradient of amino acids at equilibrium

Oocytes were injected (1–5 ng/oocyte) with cRNA (rBAT, 4F2hc, or CAT1) or noninjected, 3–4 days later the uptake of 50 μM L-[^{35}S]cystine. L-[^{3}H]leucine, or L-[^{3}H]arginine was measured in groups of 6–8 oocytes at equilibrium (*i.e.* 3–6 h of uptake incubations). The accumulated gradient of substrates into the oocyte expressed as times the initial concentration of substrate in the medium, was calculated assuming a space distribution of [^{3}H]water of 176 nl (see "Materials and Methods"). Data for rBAT-, CAT1-, and uninjected oocytes are the mean ± S.E. corresponding to 6–15 determinations (3–7 independent experiments). 4F2hc and L cystine (rBAT) data are the mean values (3–6 determinations) in two independent experiments.

Substrate	Un-injected	rBAT	4F2hc	CAT1
L-Arginine	14 ± 1	55 ± 5	34, 39	19 ± 2
L-Leucine	19 ± 3	48 ± 11	27, 37	
L-Cystine	1.1 ± 0.6		40, 42	

cific activity of the substrate and with hardly any change in the total substrate content of the oocyte (Table III). Similarly, the model also predicts that superfusion (20 ml/min) of rBAT-injected oocytes with 50 μM L-arginine or L-leucine for 3 h results in 95 and 79% replacement of the internal oocyte system b$^{o,+}$-like substrates, respectively (data not shown). The main conclusion of this theoretical study is that accumulation of L-[^{3}H]arginine in rBAT-injected oocytes can be fully explained by an obligatory exchanger of 1:1 stoichiometry, in which the driving force of the accumulation is the high internal concentration of amino acids. An interesting consequence of this is that we can almost completely exchange the internal content of substrates of system b$^{o,+}$-like and then estimate the influx and efflux rates through the transporter under conditions of homogeneous exchange of substrates.

This was tested experimentally. Indeed, continuous superfusion of rBAT-injected oocytes with 50 μM L-arginine or L-leucine for 3 h resulted in a dramatic decrease in the inward positive current elicited by L-arginine and the outward positive current elicited by L-leucine, respectively (Fig. 5). In these conditions, L-arginine- and L-leucine-induced currents tended to be zero. This indicates that all the electric activity of system b$^{o,+}$-like is due to the hetero-exchange between neutral and dibasic amino acids and that the stoichiometry of the amino acid homo-exchange is *n:n*. In contrast, superfusion with L-arginine increased the L-leucine-induced currents, and superfusion with L-leucine increased L-arginine-induced currents (Fig. 5). In these conditions, at −50 mV membrane potential, the maximal L-arginine-induced currents (*i.e.* by exchange with the internal L-leucine) are approximately twice as strong as the maximal L-leucine-induced currents (*i.e.* by exchange with the internal L-arginine). This demonstrates that the exchange via system b$^{o,+}$-like of L-arginine inward:L-leucine outward is favored *versus* the reverse direction.

To determine the stoichiometry of the exchange of amino acids via system b$^{o,+}$-like, rBAT-injected oocytes were incubated for 4 h with 1 mM labeled (L-[^{3}H]arginine or L-[^{3}H]leucine) or the corresponding unlabeled substrates, and influx and L-amino acid-elicited efflux transport rates were measured immediately. Fig. 6 shows that, for every type of homo- and hetero-exchange between L-arginine and L-leucine, the transport rates for influx and for the L-amino acid-elicited efflux were identical. This demonstrates an *n:n* stoichiometry for the obligatory exchange of amino acids via system b$^{o,+}$-like. This stoichiometry is most probably 1:1 since for all the kinetic studies of L-arginine and L-leucine influx and efflux transport rates, the Hill coefficient was never different from 1 (data not shown). Again, the hetero-exchange L-arginine inward:L-leucine outward is favored *versus* the reverse direction of exchange (Fig. 6).

TABLE III
Simulated internal concentrations and specific activities during L-[^{3}H]arginine accumulation in oocytes expressing rBAT

The internal concentration (μM and % of total substrates of system b$^{o,+}$-like) of the analyzed groups of amino acids at time = 0 (values from Ref. 31) and after 4 h of 1,000 μM L-[^{3}H]arginine uptake in rBAT-injected oocytes are shown (upper). The evolution of the external and internal specific activity of L-[^{3}H]arginine is also indicated (lower). The initial specific activity of L-[^{3}H]arginine, defined as (labeled L-arginine)/(total L-arginine), was set to 0.0001. The actual value of the specific activity does not alter the conclusion, provided that the concentration of labeled L-[^{3}H]arginine does not significantly change the total L-arginine concentration.

	Internal concentration (μM)	
	Time = 0	Time = 4 h
L-Arginine	697	5653.0 (97.7%)
L-Leucine	231	3.1 (0.05%)
L-Neutral	4288	64.3 (1.1%)
L-Dibasic	574	63.0 (1.1%)
Total	5790	5783.4

	L-[^{3}H]Arginine specific activity ($\times10^5$)	
	Time = 0	Time = 4 h
External	10	9.90
Internal	0	9.88

The Amino Acid Transport System y$^+$L-like Behaves as an Obligatory Exchanger with Asymmetry—Due to the structural and functional homology between rBAT and 4F2hc, we tested for the accumulation of substrates in 4F2hc-injected oocytes. As indicated above, uptake of 50 μM L-[^{3}H]arginine or L-[^{3}H]leucine reached levels of accumulation in 4F2hc-injected oocytes higher that those obtained via system y$^+$ or in uninjected oocytes (Table II). These data demonstrated an active mechanism of transport for system y$^+$L-like associated with 4F2hc expression in oocytes. Interestingly, L-arginine and L-leucine, but not L-tryptophan, in the medium stimulated efflux of L-[^{3}H]arginine via system y$^+$L-like, associated with 4F2hc expression in oocytes (Fig. 7A). As already shown (Fig. 2), these amino acids did not stimulate efflux in uninjected oocytes (Fig. 7). L-Leucine-elicited efflux of L-[^{3}H]arginine in 4F2hc-injected oocytes was barely detectable at 0.1 mM in the absence of sodium (choline medium), but it increased dramatically in the presence of sodium; the sodium effect was much less apparent at 10 mM L-leucine (Fig. 7A). This substrate specificity corresponds to that of influx via system y$^+$L-like in oocytes. Thus, the 4F2hc-induced influx of 100 μM L-[^{3}H]leucine was 1 ± 0.3 pmol/5 min per oocyte in the absence of sodium, and 34 ± 3 pmol/5 min per oocyte in the presence of 100 mM sodium (*n* = 6 oocytes). In agreement with this, the y$^+$L amino acid transport activity described in human erythrocytes and placenta carries dibasic amino acids with high affinity, neutral amino acids, like L-leucine, with high affinity only in the presence of sodium, but not L-tryptophan (36–38). All this demonstrated that efflux via the y$^+$L-like transport activity associated with 4F2hc expression in oocytes is highly dependent on the presence of substrates on the trans-side. This, together with the effective accumulation of substrates, such as L-arginine and L-leucine, in oocytes expressing 4F2hc strongly suggests that system y$^+$L-like is a tertiary active amino acid transport system with an obligatory exchanger mechanism.

Next, we studied L-[^{3}H]leucine efflux via system y$^+$L-like associated with 4F2hc expression in oocytes. To our surprise, L-[^{3}H]leucine efflux was not stimulated by external L-arginine (Fig. 7B) or L-leucine (data not shown). Thus, efflux rates, expressed as percent of the radioactivity loaded per 5 min, by 4F2hc-injected (3.3% ± 0.2 in the absence of external amino acids and 3.4% ± 0.3 in the presence of 1 mM L-arginine, *n* = 3
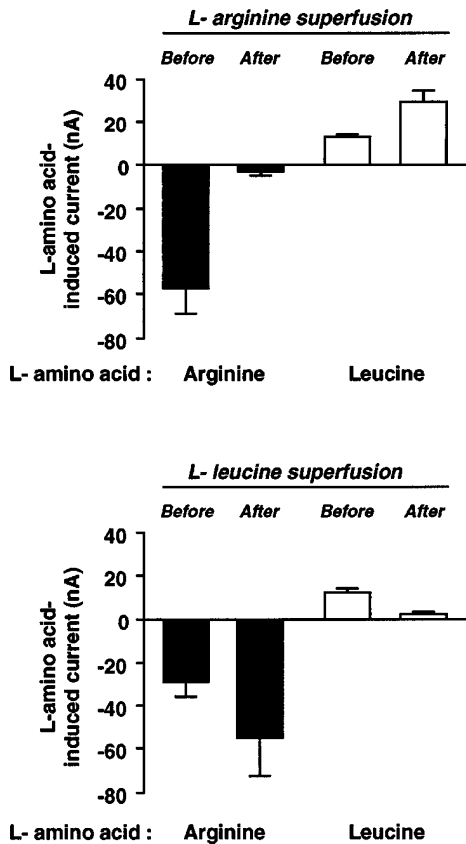
FIG. 5. **Effect of L-arginine and L-leucine superfusion on L-amino acid-induced currents in rBAT-injected oocytes.** Oocytes were injected with 1 ng of rBAT cRNA. 50 μM L-arginine-induced and 50 μM L-leucine-induced currents were measured, at a clamped potential of -50 mV, before and after 3 h superfusion with 50 μM L-arginine (*upper graph*, 8 days after injections) or with 50 μM L-leucine (*lower graph*, 3 days after injections). In current clamped conditions, the membrane resting potential (-54 ± 3 and -60 ± 2 mV in the L-arginine and L-leucine superfusion experiments, respectively) was immediately depolarized to -33 ± 3 mV by L-arginine and repolarized to -66 ± 6 mV by L-leucine. After 3 h of 50 μM L-amino acid superfusion, the membrane potential was almost restored to -46 ± 4 mV (L-arginine superfusion experiment) and to -60 ± 3 mV (L-leucine superfusion experiment). The whole protocol was performed in sodium ND96 medium. Data (nA) are the mean $\pm$ S.E. from 5 oocytes (L-arginine superfusion) and 4 oocytes (L-leucine superfusion) from two different batches.

independent experiments) and uninjected oocytes (3.6% $\pm$ 0.7 in the absence of external amino acids and 3.2% $\pm$ 0.9 in the presence of 1 mM L-arginine) were similar. This is at odds with the $\leq$7-fold increase in L-[³H]leucine efflux due to *trans*-L-arginine in rBAT-injected oocytes (Fig. 2*B*). This demonstrates functional asymmetry of the amino acid exchange via system y^+L-like expressed in oocytes and suggests that hetero-exchange of arginine inward/neutral amino acid outward, if it occurs, is clearly weaker than the reverse hetero-exchange. To provide further evidence for amino acid hetero-exchange (*i.e.* neutral inward/dibasic outward) via system y^+L-like, the electrogenicity of this system was studied in 4F2hc-injected oocytes. These oocytes, but not uninjected oocytes, showed a small but significant and reproducible positive outward current when 10 mM L-leucine was present in the external medium in the absence of sodium (1.2 $\pm$ 0.1 and -0.1 ± 0.1 nA for 4F2hc-injected and uninjected oocytes, respectively, $n = 7$–8 oocytes, $p \leq 0.01$). In the presence of sodium, this current was not detectable (-1.7 ± 0.2 and -1.8 ± 0.3 nA for 4F2hc-injected and uninjected oocytes, respectively, $n = 7$–8 oocytes). This is most probably due to the activity of an endogenous sodium-dependent transporter for leucine and/or co-transport of sodium
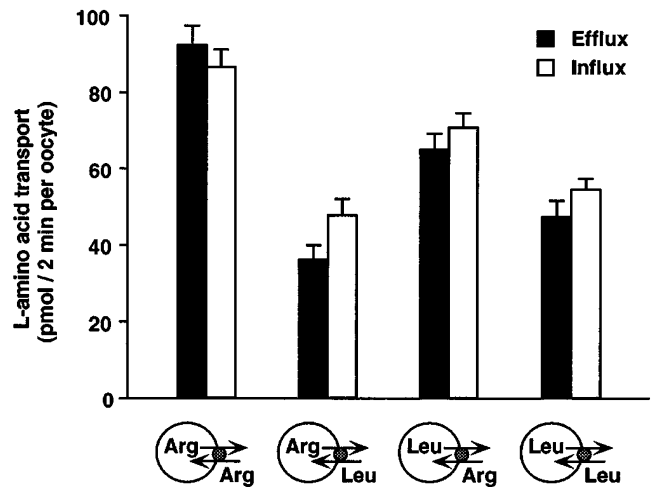

FIG. 6. **Efflux and influx transport rates via system $b^{o,+}$-like in rBAT-injected oocytes.** Oocytes were injected with 5 ng of rBAT cRNA or uninjected, and 4 days later they were assayed for efflux (*closed bars*) and influx (*open bars*) transport of the indicated amino acids in the oocyte schemes shown at the *foot* of the figure. For efflux studies, groups of 7 oocytes were incubated in choline uptake medium for 4 h in the presence of 1 mM L-[³H]arginine or L-[³H]leucine (3 μCi/90 μl). Then efflux rates were measured in the absence or in the presence of the indicated 250 μM L-amino acids. For influx studies, groups of 7 oocytes were incubated in choline uptake medium (90 μl) for 4 h in the presence of 1 mM cold L-arginine or L-leucine. Then media were removed and the oocytes were washed as indicated for efflux studies. Influx of 250 μM L-[³H]arginine or L-[³H]leucine were immediately assayed for 2 min. Efflux data correspond to the amino acid-elicited efflux rates (*i.e.* efflux into media containing amino acids minus efflux into medium containing no amino acids). To express efflux rates as pmol/2 min per oocyte, the initial specific activity of the tracer during the accumulation phase of the study was used (see Table III). Influx rates correspond to the rBAT-induced transport activity (*i.e.* transport in rBAT-injected minus that in uninjected oocytes). Data (mean $\pm$ S.E.) are from 4 (efflux) and 12–14 (influx) determinations from a representative experiment. The corresponding efflux and influx rates ere not significantly different in the four groups. Efflux and influx rates in the hetero-exchange Leu (outward)/Arg (inward) were significantly higher ($p \leq 0.01$) than those corresponding to the reverse hetero-exchange.

with L-leucine via system y^+L-like. In agreement with this, it has been shown that the placenta system y^+L is largely insensitive to alterations of the membrane potential, suggesting co-transport of sodium and L-neutral amino acids (38).

DISCUSSION

We have shown that amino acid transport systems $b^{o,+}$-like, associated with rBAT expression in oocytes and OK cells, and y^+L-like, associated with 4F2hc expression in oocytes, are highly coupled obligatory exchangers (*i.e.* tertiary active transporters). The exchange via systems $b^{o,+}$-like and y^+L-like is asymmetric, favoring the uptake and the release of dibasic amino acids, respectively. This offers a functional explanation for the role of system $b^{o,+}$-like in type I cystinuria and allows us to propose a role of system y^+L in the active efflux of dibasic amino acids.

Several studies have shown induction of the exchange of amino acids in oocytes expressing rabbit and rat rBAT expression (15–17). Coady and collaborators (16) described an obligatory hetero-exchange mechanism between dibasic and neutral amino acids via system $b^{o,+}$-like to explain the currents associated with the function of this transport system in oocytes. In contrast, Rennie and collaborators (17) suggested that this hetero-exchange could not fully explain the electric activity associated with the induced transport of neutral amino acids due to rat rBAT expression in oocytes. Here we demonstrate that the amino acid transport activity induced by human rBAT in oocytes can be fully explained by system $b^{o,+}$-like activity, as
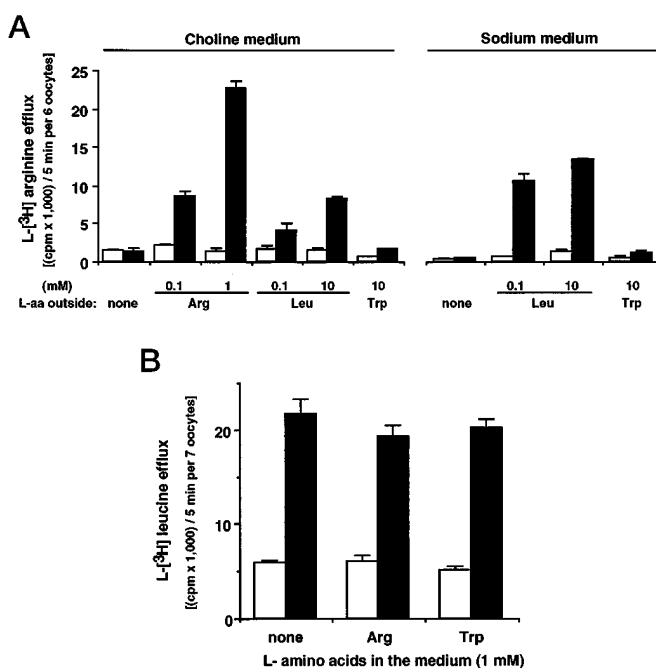
A

B

Fig. 7. L- **[³H]Arginine efflux via amino acid transport system y^+L-like in oocytes.** Oocytes were injected with 1 ng of 4F2hc cRNA (*black bars*). Uninjected oocytes (*open bars*) were used as controls. Four days after, rates of L-[³H]arginine or L-[³H]leucine efflux into sodium or choline medium were measured. In addition, medium contained no amino acids (*none*) or L-amino acids at the indicated concentrations (mM). Efflux rates are expressed as the radioactivity (cpm × 1,000) appearing in the medium/5 min per group of 6 or 7 oocytes. *A*, L-[³H]arginine efflux rates. Data correspond to the mean ± S.E. of triplicates from a representative experiment. The presence of L-arginine (*Arg*) and L-leucine (*Leu*), but not L-tryptophan (*Trp*), in the medium increased significantly efflux by 4F2hc cRNA-injected, but not by uninjected, oocytes ($p \leq 0.05$). *B*, L-[³H]leucine efflux rates. Efflux rates by 4F2hc-injected and uninjected oocytes in the presence of L-arginine (*Arg*) were similar to those in the absence of amino acids or in the presence of tryptophan (*Trp*). Data correspond to the mean ± S.E. from triplicates from a representative experiment. Another 3 independent experiments gave similar results.

an obligatory exchanger, most probably with 1(inward):1(outward) stoichiometry for the homo- and hetero-exchange of its amino acid substrates. In addition, the expression of the *rBAT* gene in the renal proximal tubular cell line OK is also associated with system $b^{o,+}$-like activity, with characteristics of obligatory amino acid exchange.

We have also shown that the amino acid exchange activity of system $b^{o,+}$-like is tightly coupled and allows intracellular concentration of amino acid substrates until the complete replacement of the internal system $b^{o,+}$-like substrates of the oocyte. The maximum level of accumulation of substrates via system $b^{o,+}$-like (~1,000 pmol/oocyte) fits well with the reported content of free amino acid substrates of this system in stage VI oocytes (31). Interestingly, the level of accumulation of substrates at low μM concentration reached in rBAT-injected oocytes exceeds that obtained in uninjected or in CAT1-injected oocytes (*i.e.* expressing system y^+ amino acid transport activity). In contrast to system $b^{o,+}$-like, system y^+ is an equilibrative transport activity that shows a high trans-stimulation effect (Ref. 24; 6-fold in CAT1-injected oocytes, data not shown), but with significant transport activity in the absence of substrates on the trans-side (24, 39), and which leads to a higher accumulation of substrates than that given by the membrane potential in oocytes (present study) and in fibroblasts (40). All this strongly suggests that system $b^{o,+}$-like should be considered as a tertiary active transporter. In contrast, primary and secondary active transport mechanisms could not

explain accumulation of substrates via system $b^{o,+}$-like for the following reasons. (i) The cut-open oocyte model is able to show system $b^{o,+}$-like activity in rBAT-injected oocytes without the addition of triphosphate nucleotides to the external perfusion system (16). (ii) Sodium is not necessary for the accumulation of substrates via system $b^{o,+}$-like (present study), and neither sodium, potassium, nor chloride ions are needed for system $b^{o,+}$-like activity in oocytes (11, 16, 23). Rennie and collaborators (17) suggested hetero-exchange of neutral amino acids (inward) and potassium (outward) in rat rBAT-injected oocytes. In contrast, both for human and for rabbit rBAT-injected oocytes, potassium does not affect the currents induced by L-arginine or L-leucine (15).[2] In conclusion, system $b^{o,+}$-like, associated with rBAT expression, is a tightly coupled exchanger with 1:1 stoichiometry. Whether system $b^{o,+}$-like has a concerted or a ping-pong mechanism of exchange is beyond the scope of the present study and needs further research.

Here we provide evidence that system y^+L-like, associated with 4F2hc expression in oocytes, is an obligatory amino acid exchanger that mediates efflux of dibasic amino acids (*e.g.* L-arginine) and, in the presence of sodium, influx of neutral amino acids at μM concentration. It has been suggested (41) that the amino acid transport activity associated with human 4F2hc expression in oocytes is identical to the y^+L activity described in human erythrocytes and placenta (36–38, 42). In contrast to this general system, y^+L shows transport activity in the absence of substrates in the trans-side, as an equilibrative transporter with trans-stimulation (36–38). It is also possible that system y^+L may indeed be an obligatory exchanger because the functional isolation of system y^+L from the co-existing system y^+ in erythrocytes has been accomplished by NEM treatment, since the latter system is sensitive to the reagent, whereas the former system is resistant (42). A possible modification of the hypothetical coupled exchange mechanism of transport of system y^+L by NEM treatment has not been ruled out. In fact, mitochondrial exchangers, such as the ATD/ADP carrier, act as equilibrative transport systems after sulfhydryl reagent treatment (43).

To our knowledge this is the first study demonstrating active transport via systems y^+L-like and $b^{o,+}$-like, associated with 4F2hc and rBAT expression in oocytes, respectively. These two amino acid transport systems are very alike. (i) Both are high affinity systems with a broad specificity for dibasic and neutral amino acids. (ii) Both proteins are homologous, with a similar hydrophobicity profile and most probably linked by disulfide bridges to putative "light" subunits of 30–50 kDa (44–45). This fostered the hypothesis that both transporters are heterodimeric, both subunits being essential, but not sufficient, for the transport activity of systems $b^{o,+}$-like and y^+L-like (46). The tertiary active transport mechanism shown here for these two transport systems indicates that they belong to a common family of obligatory amino acid exchangers.

A Role for the Amino Acid Exchanger Systems $b^{o,+}$-like and y^+L-like in Renal Reabsorption—Recent studies have demonstrated that the human rBAT gene is responsible for type I cystinuria (1, 7–8). Patients show hyperexcretion of dibasic amino acids and cystine, but not of neutral amino acids, due to a defect in the active reabsorption of those amino acids in kidney (4). The tertiary active transport mechanism of the high affinity system $b^{o,+}$-like, described here, explains the responsibility of rBAT in cystinuria. We propose a model for the role of system $b^{o,+}$-like (rBAT) in the active renal reabsorption of cystine and dibasic amino acids by obligatory exchange with intracellular neutral amino acids (Fig. 8). The direction of

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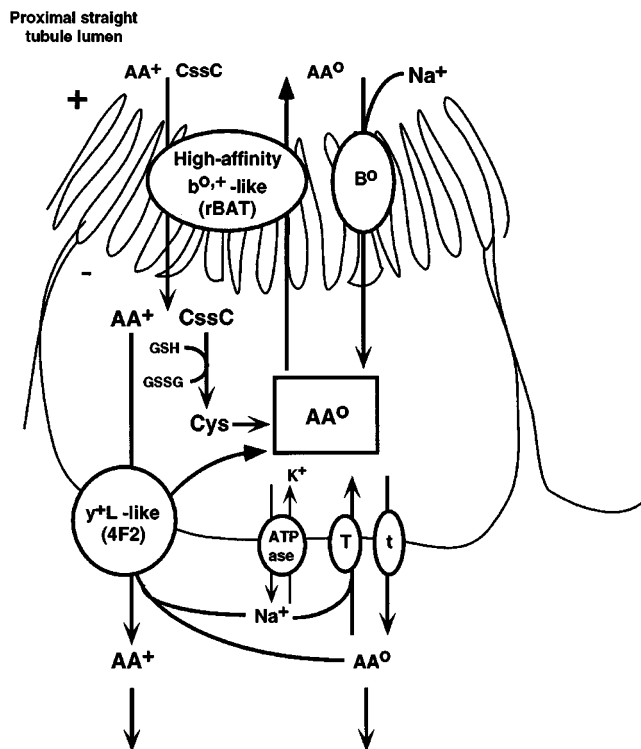[2] A. E. Busch, unpublished results.

Proximal straight tubule lumen

FIG. 8. **Model for the renal reabsorption of dibasic amino acids via systems b$^{o,+}$-like and y$^+$L-like.** Obligatory amino acid exchange with neutral amino acids (*AA^o*) via systems b$^{o,+}$-like (apical) and y$^+$L-like (basolateral) would mediate the active reabsorption of dibasic amino acids (*AA$^+$*) and cystine (*CssC*) in a epithelial cell from the proximal straight tubule. Influx of dibasic amino acids and cystine from the lumen would be favored by the negative membrane potential and by the reduction of cystine to cysteine associated with glutathione oxidation (GSH → GssG), respectively. A high intracellular concentration of neutral amino acid would be ensured by concentrative (Na$^+$ cotransport) neutral amino acid transport activities in the apical pole (system *B^o*, neutral brush border) and the basolateral pole (systems ASC and others, T transporters shown in the scheme). *t*, sodium-independent neutral amino acid transporters (*e.g.* system L). *ATPase,* Na$^+$/K$^+$-ATPase. Co-localization of the cell surface antigen 4F2hc and rBAT in the epithelial cells of the proximal straight tubule is hypothetical.

exchange dibasic-inward/neutral-outward has been shown to be favored in the present study and agrees with the fact that hyperexcretion of neutral amino acids does not occur in cystinuric patients. The negative membrane potential, the intracellular reduction of L-cystine to L-cysteine, and the high intracellular concentration of neutral amino acids, which are substrates for system b$^{o,+}$-like, may be the determinants of the reabsorption of L-dibasic amino acids and L-cystine via system b$^{o,+}$-like. The tightly coupled mechanism of obligatory exchange of system b$^{o,+}$-like would ensure efficient active reabsorption of cystine and dibasic amino acids. In addition this mechanism would prevent the net loss of amino acids via system b$^{o,+}$-like toward the lumen at the end of the proximal straight tubule, where rBAT is expressed (9–10) and a low concentration of amino acids is present. Further studies are needed to examine the factors affecting the activity and the direction of amino acid exchange via system b$^{o,+}$-like.

The obligatory exchange of amino acids via system y$^+$L-like, associated with 4F2hc expression in oocytes, may have important physiological consequences. It has been reported that efflux across the basolateral membrane is the rate-limiting step in the intestinal absorption of dibasic amino acids (48–49). Furthermore, leucine at low μM concentration increases (6–10-fold) the trans-epithelial flux of lysine (49–50). Countertransport between lysine (outward) and leucine (inward) or alloster-

ism were considered to be responsible for this process. System y$^+$L can sustain lysine-leucine exchange with an apparent K_m for leucine of ~10 μM in the presence of sodium (36). If such a system is found in the basolateral membranes of intestinal or renal epithelial cells, system y$^+$L will support the countertransport hypothesis (37). The surface antigen 4F2hc has a basolateral localization in renal epithelial cells from the proximal tubule (51). System y$^+$L-like, associated with 4F2hc expression, could be responsible for the active release of dibasic amino acids through the basolateral membrane of epithelial cells (Fig. 8). The evidence offered here, that the direction of exchange that is favored is L-arginine (outward) with low μM concentration of leucine (inward) in the presence of sodium, strongly supports this hypothesis. Further research is needed to elucidate the mechanism (*e.g.* a weak interaction of neutral amino acids from inside due to the low intracellular concentration of sodium) responsible for this asymmetric exchange.

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