Multiple components of arginine and phenylalanine transport induced in neutral and basic amino acid transporter-cRNA-injected *Xenopus* oocytes

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The induced uptakes of L-[^{3}H]phenylalanine and L-[^{3}H]arginine in oocytes injected with clonal NBAT (neutral and basic amino acid transporter) cRNA show differential inactivation by pre-treatment with *N*-ethylmaleimide (NEM), revealing at least two distinct transport processes. NEM-resistant arginine transport is inhibited by leucine and phenylalanine but not by alanine or valine; mutual competitive inhibition of NEM-resistant uptake of arginine and phenylalanine indicates that the two amino acids share a single transporter. NEM-senstive arginine transport is inhibited by leucine, phenylalanine, alanine and valine. At least two NEM-sensitive transporters may be expressed because we have been unable to confirm mutual competitive inhibition between arginine and phenylalanine transport. The NEM-resistant transport mechanism appears to involve distinct but overlapping binding sites for cationic and zwitterionic substrates. NBAT is known to form oligomeric protein complexes in cell membranes, and its functional roles when expressed in *Xenopus* oocytes may include interaction with oocyte proteins, leading to increased native amino acid transport activities; these resemble NBAT-expressed activities in terms of NEM-sensitivity and apparent substrate range (including an unusual inhibition by β-phenylalanine).

INTRODUCTION

The protein product of neutral and basic amino acid transporter (NBAT) [1], a cDNA clone from rat kidney [2], is localized primarily to the brush-border membranes of renal and jejunal epithelia [3] . *Xenopus* oocytes injected with cRNA transcribed from NBAT (known alternatively as D2 [4]) or an homologous rabbit kidney cDNA clone rBAT [5], exhibit markedly increased Na^+-independent uptake of neutral and cationic amino acids and cystine by a transport mechanism resembling the activity of the functionally-characterized Na^+-independent amino acid transport system $b^{0,+}$. NBAT is clearly involved in amino acid transport (indeed mutations in the human NBAT gene are associated with the transport defects of cystinuria [6,7]), but the molecular structure of the NBAT protein is atypical of membrane transporters, leading to considerable speculation that NBAT/rBAT are subunits of a larger transporter [4,5,8]. NBAT is now known [9] to be attached by disulphide bonds to proteins of approx. 50 kDa in the cell membranes of oocytes and certain epithelia, and these heteromeric protein complexes are suggested to be the 'minimum functional units' of NBAT-induced amino acid transport. Oocyte expression studies with NBAT mutants associated with cystinuria are also consistent with the idea that NBAT functions as a 'regulatory subunit' of multimeric protein complexes [7].

A reassessment of the original data on amino acid transport in rBAT-expressing oocytes [8] has concluded that, rather than the single system $b^{0,+}$-like activity originally reported [5], two kinetically-distinct pathways for neutral amino acid (L-leucine) transport were 'activated'. Furthermore, neutral and cationic amino acids evoke opposite Na^+-independent currents in NBAT-expressing oocytes [10,11], leading us to speculate [11] that NBAT induces more than one type of native oocyte transport protein, which together, in parallel, exhibit system $b^{0,+}$-like activity; such 'activation' may result if NBAT forms complexes with more than one type of native catalytic transporter subunit. The present study was therefore aimed specifically to clarify both the number and type of amino acid transport activities induced by NBAT expression in oocytes, by application of experimental methods [involving the thiol group reagent *N*-ethylmaleimide (NEM) and the novel amino acid analogue 2-trifluoromethylhistidine (TFMH)] designed to distinguish between transport activities for neutral and cationic amino acids. These methods were also applied in parallel to native oocytes in order to facilitate comparison of NBAT-induced and native transport activities. We report that the NBAT-stimulated activities of both neutral and cationic amino acid transport appear to consist of (at least) two distinct pathways, and that these activities bear functional similarities to transporters in native oocytes which might be 'activated' by formation of complexes with NBAT protein.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from Sigma (U.K.) with the exception of collagenase A (Boehringer, U.K.) and Ultraspec water (Ambion, U.K.). Radiotracers were purchased from New England Nuclear (U.K.).

Isolation and maintenance of oocytes

Female toads (*Xenopus laevis*) were supplied by a South African *Xenopus* facility (Noordhoek, South Africa) and maintained in

Abbreviations used: NEM, *N*-ethylmaleimide; TFMH, 2-trifluoromethylhistidine; BCH, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid; MBM, modified Barth's medium; NBAT, neutral and basic amino acid transporter; TMA, tetramethylammonium.

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freshwater aquaria at 22 °C. Ovarian tissue was surgically removed from toads (under 0.1 % aminoethylbenzoate anaesthesia), rinsed rapidly and incubated for 3 h at 20 °C in Ca^{+}-free modified Barth's medium (MBM) containing 2 mg/ml of collagenase A. The composition of MBM is (in mM): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4.7H_2O$, 0.66 $NaNO_3$, 0.75 $CaCl_2.2H_2O$, 5.0 Hepes, pH 7.6 with Tris base, and 10 mg/l gentamycin sulphate. Defolliculated, stage V–VI (prophase-arrested) oocytes were selected and maintained at 18 °C (cooled incubator; BDH) in MBM.

Synthesis and injection of NBAT cRNA

The cDNA clone for NBAT (in pSPORT 1 plasmid vector [2]) was a gift from Dr. S. Udenfriend, Roche Research Center, NJ, U.S.A. Plasmid DNA was linearized with *Not*1 and transcribed *in vitro* with T7 RNA polymerase in the presence of the GpppG cap analogue, using the mMessage mMachine transcription system (Ambion, Austin, TX, U.S.A.). NBAT cRNA was resuspended in Ultraspec water at 0.1 mg/ml, and approx. 50 nl was injected into oocytes (5 ng of cRNA/oocyte) using a glass micropipette attached to a pressure-controlled delivery system (PV830 Pneumatic PicoPump, World Precision Instruments, New Haven, CT, U.S.A.). Control (native) oocytes were injected with water only. All oocytes were injected the day after isolation.

Measurement of amino acid uptake in oocytes

[^{3}H]-Labelled amino acid uptake into oocytes was measured 3–4 days post-injection by a method described previously [12], using experimental medium containing NaCl or tetramethylammonium (TMA) chloride each at 100 mM, along with 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes, pH 7.5 (unless otherwise indicated) with Tris base. Uptake was measured over a period of 30 min (within the time scale of linear tracer-uptake into oocytes [11]. In certain experiments, tracer uptake was studied with a variety of unlabelled amino acids in the transport medium at specified concentrations. In studies using the thiol-group reagent NEM, oocytes were pre-incubated with NEM for periods of up to 30 min, rinsed and assayed for amino acid uptake in the absence of NEM as described above.

Synthesis of TFMH

L-2-(Trifluoromethyl)histidine (TFMH) was prepared from protected L-histidine by imidazole ring opening (ring-3-decarboxylation) with benzoyl chloride [13], followed by reclosure with trifluoroacetic anhydride, deprotection under dilute acid conditions and recrystallization [14]. The pK_a values of TFMH are assumed to be equal to those of histidine (2-trifluoromethyl derivitization of histidine is unlikely to significantly affect pK_a values).

Data analysis and presentation

Amino acid uptake data are expressed as mean values ± S.E.M. for experiments performed on a denoted number of oocyte preparations (9–11 individual oocytes per preparation) unless otherwise stated. Differences between mean values were assessed using Student's *t*-test with significance assigned at $P < 0.05$. Lines were fitted to transformed transport data using commercial software (SlideWrite Plus V 3.00; Advanced Graphics Software, U.S.A.).

RESULTS

Amino acid uptakes in NBAT-cRNA-injected oocytes: inhibition by cationic and neutral amino acids

NBAT-cRNA-injected oocytes showed markedly increased Na^{+}-independent uptakes of L-arginine, L-lysine, L-phenylalanine and (to a lesser extent) alanine 3–4 days post-injection, as reported previously [2,11]. The NBAT-induced arginine uptake was inhibited to a greater extent by the cationic amino acid lysine than by neutral amino acids (including phenylalanine, alanine, leucine and valine) at 0.2–5 mM (Figure 1a); indeed a significant component (~ 40 %) of induced arginine uptake was resistant to inhibition by 5 mM phenylalanine, alanine (Figure 1a), L-β-phenylalanine and valine (results not shown). In contrast, phenylalanine uptake was markedly inhibited by both cationic and neutral amino acids (arginine and alanine/leucine/valine/β-phenylalanine respectively) at 1 mM (Figure 1b and results not shown). 2-Aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH), a synthetic substrate of the Na^{+}-independent transport system L,

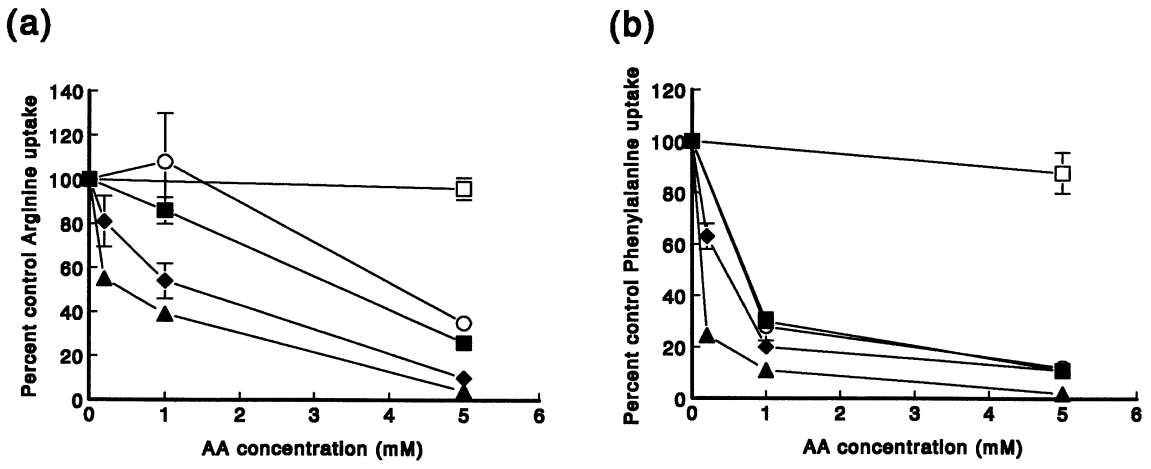

Figure 1 Effect of amino acids (AA) on uptake of (a) arginine and (b) phenylalanine (both at 50 μM) in NBAT-cRNA-injected oocytes

Data refer to uptake (mean per cent of control uptake ± S.E.M. of three oocyte preparations) in the absence (control) or presence of amino acid for (**a**): lysine (▲); BCH (□); alanine (○); leucine (◆); phenylalanine (■); and for (**b**): arginine (▲); BCH (□); alanine (○); leucine (◆); valine (■) at the concentrations indicated.

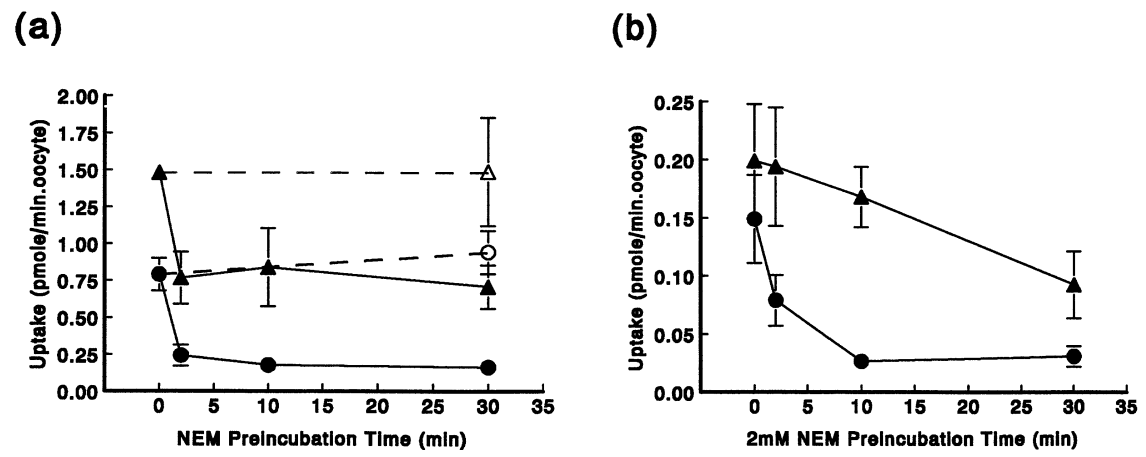

Figure 2 Time course of the effects of NEM preincubation on uptake of 10 μM arginine (▲, at 2 mM NEM; △, at 0.2 mM NEM) and 10 μM phenylalanine (●, at 2 mM NEM; ○, at 0.2 mM NEM) in oocytes injected with (a) NBAT-cRNA and (b) water

Data are mean uptake ± S.E.M. for ten oocytes from a single preparation; similar results were obtained with two other oocyte preparations.

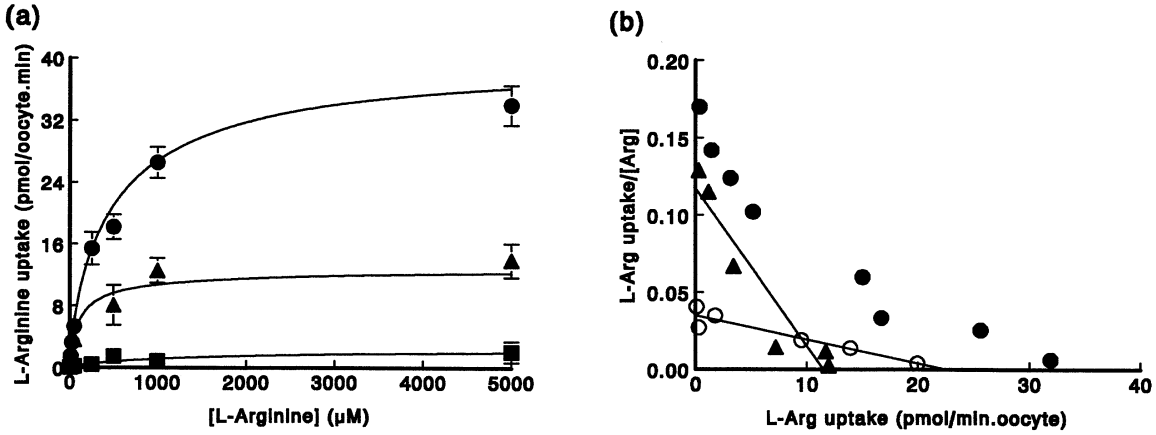

Figure 3 Effect of NEM-preincubation (2 mM; 30 min) on transport kinetics for arginine in NBAT-cRNA-injected oocytes

(**a**) Transport data presented as mean uptake ± S.E.M. from three batches of oocytes: (●), NBAT injected, (▲) NEM preincubated, NBAT injected, (■) water-injected (native). Curves represent the summation of influxes calculated from the derived K_m and V_{max} values shown in Table 1. (**b**) Eadie–Hofstee linear transformation of mean NBAT-induced uptake, [total (●), NEM-resistant (▲), and by difference, NEM-sensitive (○)], corrected by subtraction of uptake in native oocytes. Line-fits to transformed data were performed by an iterative procedure using commercial software.

had no effect on NBAT-induced uptake of either arginine or phenylalanine (Figure 1).

Effect of NEM on NBAT-induced and native amino acid transport

The thiol-group reagent NEM has been used in previous studies (e.g. [15]) to selectively block individual components of cationic amino acid uptake in mammalian cells. In the present study, NEM (2 mM) appeared to have differential effects on uptake of cationic and neutral amino acids in NBAT-expressing oocytes, inactivating phenylalanine uptake about twice as much as arginine uptake (10 μM amino acid uptake after 30 min of NEM preincubation; Figure 2a). NEM at 0.2 mM was not an effective inactivator of these transport processes within a 30 min pre-incubation period (Figure 2a). The existence of an NEM-resistant component of arginine uptake in NBAT-expressing oocytes is direct evidence that multiple transport pathways for cationic amino acids are being induced by NBAT. In native oocytes, phenylalanine uptake was also more sensitive to inactivation by 2 mM NEM than arginine uptake (Figure 2b) and could be inactivated by 0.2 mM NEM (30 min preincubation; results not shown).

NEM-resistant and NEM-sensitive pathways of NBAT-induced amino acid transport have distinct functional characteristics

We next examined the kinetics of arginine and phenylalanine uptake in NBAT-injected oocytes (over a relatively broad range of substrate concentrations) with or without NEM pretreatment. Linear transformation of the transport data enabled us to distinguish two major transport components for each amino acid (Figures 3 and 4 and Table 1). For NBAT-expressing oocytes, the NEM-resistant component of arginine transport exhibited K_m and V_{max} values corresponding to the higher-affinity transport component derived from total arginine uptake. In contrast, NEM-resistant phenylalanine uptake appeared to correspond to

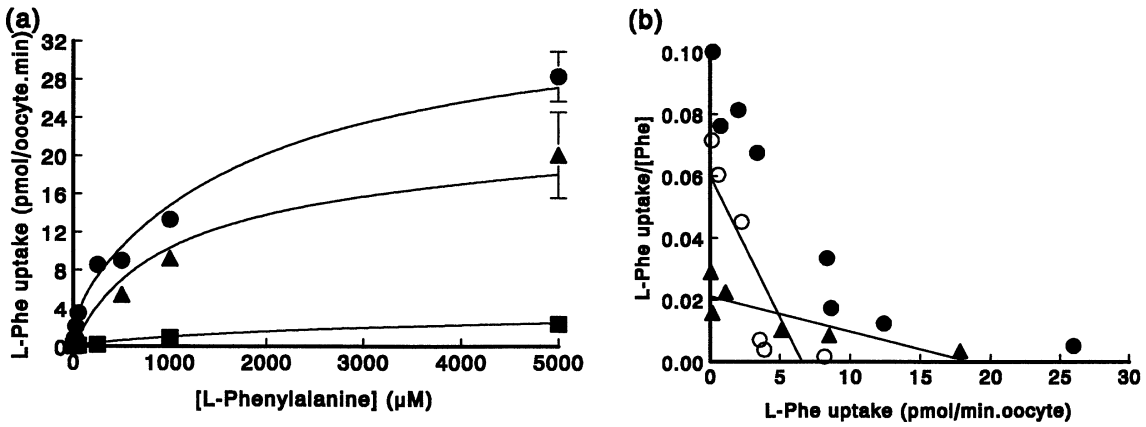

Figure 4 Effect of NEM-preincubation (2 mM; 30 min) on transport kinetics for phenylalanine in NBAT-cRNA-injected oocytes

(**a**) Transport data presented as mean uptake ± S.E.M. from three batches of oocytes: (●), NBAT injected; (▲) NEM preincubated, NBAT injected; and (■) water-injected (native). Curves represent the summation of influxes calculated from the derived K_m and V_{max} values shown in Table 1. (**b**) Eadie–Hofstee linear transformation of mean NBAT-induced uptake [total (●), NEM-resistant (▲) and, by difference, NEM-sensitive (○)], corrected by subtraction of uptake in native oocytes. Line-fits to transformed data were performed by an iterative procedure using commercial software.

Table 1 Kinetic characteristics of arginine and phenylalanine uptake in NBAT-cRNA- and water-injected oocytes

All measurements were made in TMACl transport medium. Results are expressed as mean ± S.E.M. derived by Eadie–Hofstee linear transformation (see Figures 3b and 4b) from data at seven different substrate concentrations each averaged from uptake measurements in 3–5 oocyte preparations.

	Arginine uptake		Phenylalanine uptake	
Oocytes	K_m (μM)	V_{max} (pmol/oocyte per min)	K_m (μM)	V_{max} (pmol/oocyte per min)
(I) NBAT-cRNA injected				
(a) NEM-resistant	98 ± 18	11.5 ± 1.7	876 ± 330	18.5 ± 5.0
(b) NEM-sensitive	643 ± 114	22.5 ± 2.9	109 ± 37	6.6 ± 1.8
(II) Water-injected				
(a) NEM-resistant*	1200 ± 500	2.3 ± 0.9	3000 ± 1000	2.8 ± 1.3
(b) NEM-sensitive	9 ± 6	0.22 ± 0.07	15 ± 10	0.12 ± 0.04

* These values are estimates derived from low uptake values likely to include a significant non-saturable component.

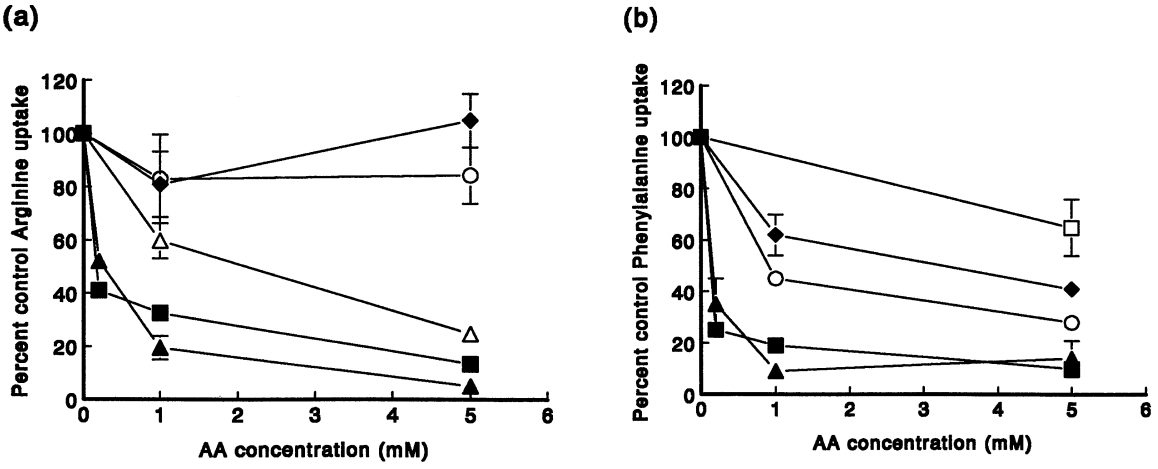

Figure 5 Effect of amino acids (AA) on NEM-resistant uptake of (a) arginine and (b) phenylalanine (both at 50 μM) in NBAT-cRNA-injected oocytes

Oocytes were preincubated with 2 mM NEM for 30 min before uptake measurement. Data refer to uptake (mean per cent of control uptake ± S.E.M. of three oocyte preparations) in the absence (control) or presence of amino acid for (**a**): lysine (▲); leucine (■); alanine (○); valine (◆); phenylalanine (△); and for (**b**): arginine (▲); leucine (■); alanine (○); valine (◆); BCH (□), at the concentrations indicated.

Table 2 Effect of Na^+ on arginine (50 μM) uptake and inhibition by neutral amino acids in NBAT-cRNA-injected oocytes after NEM pretreatment (2 mM, 30 min)

All measurement were made after preincubation of oocytes in NEM (2 mM) for 30 min. Uptakes were performed in the absence (TMACl) or presence of Na^+ (NaCl). Results are expressed as the mean uptake $\pm$ S.E.M. for three oocyte preparations. Numbers in parentheses refer to the percentage of control uptake remaining in the presence of inhibition.

Amino acid inhibitor (1 mM)	Arginine uptake (pmol/oocyte per min)	
	TMACl	NaCl
Control	3.64 $\pm$ 0.6	3.54 $\pm$ 0.6
Phenylalanine	2.29 $\pm$ 0.24 (63%)	1.02 $\pm$ 0.2 (29%)
Leucine	0.81 $\pm$ 0.01 (22%)	0.59 $\pm$ 0.12 (17%)

the lower-affinity transport component of total phenylalanine uptake. NEM-resistant arginine transport was inhibited by phenylalanine with relatively low affinity, giving 50% inhibition of tracer arginine uptake (i.e. the approximate K_i) at about 1.5 mM (Figure 5); this is of similar magnitude to the measured K_m of the NEM-resistant phenylalanine transport (0.9 mM) and is therefore consistent with competitive inhibition between phenylalanine and arginine transport. Furthermore, this phenylalanine transport component was strongly inhibited by arginine in an apparently competitive manner (the approximate K_i from Figure 5 and the arginine transport K_m are both approx. 0.1 mM). This evidence for mutual competitive inhibition is consistent with the proposal that NEM-resistant transport of the two amino acids occurs by a single transport process.

NEM-resistant uptakes of arginine (Table 2) and phenylalanine (measured at 2 and 50 μM; results not shown) were similar in the presence of Na^+ or TMA^+ as the major cation, but 1 mM phenylalanine (and possibly leucine also) appeared to exert more potent inhibition of arginine uptake in the presence of Na^+ than in its absence (Table 2). The NEM-resistant components of phenylalanine and arginine uptake in NBAT-expressing oocytes were inhibited by amino acids in the order of potency lysine/arginine = leucine > phenylalanine > alanine/valine (Figure 5); indeed arginine uptake was barely inhibited by valine or alanine even at 5 mM. This pattern of inhibition differs from that observed in oocytes not treated with NEM, and we have deduced from these data that the NEM-sensitive transport components are inhibited by valine and alanine (> 95% inhibition at 5 mM; Table 3) at least as well as by leucine. Exact quantification of NEM-sensitive transport is difficult as it involves indirect estimation by difference of effects seen with or without NEM pretreatment.

Evidence for separate cation and zwitterion binding sites on NBAT-induced transporters

During the preliminary evaluation of intermediate products in the synthesis of amino acid photolabels, we found that TFMH appeared to be a good substrate of NBAT-induced amino acid transport in oocytes because it generated membrane currents of similar magnitude to natural substrates (A. Ahmed, G. J. Peter and P. M. Taylor, unpublished work). Upon further investgation, we discovered that TFMH exhibited unusual properties as an inhibitor of NBAT-induced amino acid transport: TFMH partly inhibited uptake of both phenylalanine and arginine in NBAT-expressing oocytes at pH 7.5 (where TFMH is largely neutral), but at pH 5.5 (where TFMH is predominantly cationic) its inhibitory effect on arginine transport was increased, whereas the effect on phenylalanine uptake was unaltered (Table 4). A broadly similar overall pattern of inhibition by TFMH of arginine or phenylalanine uptake was observed in native oocytes (results not shown). Significant inhibition of NEM-resistant arginine uptake by TFMH was only observed at pH 5.5 (Table 4), although NEM-resistant phenylalanine uptake was partially inhibited by TFMH at pH 7.5 and 5.5; the data in Table 4 allow us to infer that the NEM-sensitive transport of both amino acids undergoes some inhibition by both neutral and cationic forms of TFMH. The unmasking of an NBAT-induced transport component for arginine (but not for phenylalanine), which recognizes TFMH in the cationic form only (at pH 5.5), indicates that separate binding sites for arginine and phenylalanine are involved in NEM-resistant transport.

NEM-resistant and NEM-sensitive pathways of amino acid transport in native oocytes

Water-injected oocytes showed relatively low rates of Na^+-independent arginine and phenylalanine uptake by mechanisms (Table 5) with functional similarities to NBAT-induced transport activities, including (a) separation into saturable, NEM-resistant and NEM-sensitive transport components; (b) inhibition by L-β-phenylalanine; and (c) more effective inhibition by leucine than by alanine or valine. Arginine and phenylalanine uptakes in native oocytes differed from NBAT-induced transport activities in terms of substrate K_m values (Figures 3 and 4; Table 1). NEM-

Table 3 Estimated effect of amino acids on NEM-sensitive transport of arginine and phenylalanine in NBAT-cRNA-injected oocytes

Values estimated as the percentage of total activity remaining in untreated oocytes minus the percentage of total activity remaining in NEM-treated oocytes after appropriate correction for proportion of total uptake inactivated by NEM. Data used in the calculations are shown in Figures 1 and 5. The average error associated with these values was estimated to be $\pm$5%. ND, not determined.

Amino acid inhibitor (5 mM)	Estimated percentage of NEM-sensitive uptake remaining in the presence of inhibitor	
	50 μM arginine uptake	50 μM phenylalanine uptake
Arginine	ND	< 5
Lysine	< 5	ND
Phenylalanine	27	ND
Alanine	< 5	5
Valine	< 5	< 5
Leucine	7	14

Table 4 Effect of TFMH at pH 7.5 and 5.5 on uptake of arginine and phenylalanine in NBAT-cRNA-injected oocytes with or without NEM pretreatment

Data refer to mean uptake ± S.E.M. for ten oocytes from a single preparation (similar results were obtained in a second oocyte preparation). Oocytes were pretreated with NEM (2 mM, 30 min) where indicated. Values in parentheses represent the uptake in the presence of TFMH as a percentage of control uptake.

	Amino acid uptake (pmol/oocyte per min)			
	pH 7.5		pH 5.5	
Experimental conditions	Control	+ 5 mM TFMH	Control	+ 5 mM TFMH
50 μM Arginine				
− NEM	8.21 ± 0.79	6.33 ± 0.63 (77%)	7.47 ± 0.74	2.44 ± 0.10 (33%)
+ NEM	4.42 ± 0.56	3.87 ± 0.54 (88%)	4.58 ± 0.39	0.71 ± 0.06 (16%)
50 μM Phenylalanine				
− NEM	3.93 ± 0.22	2.37 ± 0.20 (60%)	5.4 ± 0.39	3.53 ± 0.40 (65%)
+ NEM	2.44 ± 0.17	1.54 ± 0.08 (63%)	2.86 ± 0.16	1.66 ± 0.13 (58%)

Table 5 Effect of amino acids on arginine and phenylalanine uptake in native (water-injected) oocytes

Values are means ± S.E.M. of 3–4 preparations. ND, not determined.

	Percent amino acid uptake remaining in the presence of inhibitor			
	2 μM Arginine uptake		2 μM Phenylalanine uptake	
Amino acid inhibitor (5 mM)	Total	NEM-resistant	Total	NEM-resistant
None	100	100	100	100
Arginine	ND	ND	124 ± 27	55 ± 14
Lysine	24 ± 17	28 ± 18	ND	ND
Phenylalanine	90 ± 5	59 ± 5	ND	ND
Alanine	104 ± 11	83 ± 5	89 ± 8	52 ± 12
Valine	77 ± 32	64 ± 11	75 ± 8	47 ± 9
Leucine	55 ± 9	57 ± 10	59 ± 6	32 ± 4
β-Phenylalanine	51 ± 10	72 ± 9	53 ± 29	56 ± 7

resistant uptakes of arginine and phenylalanine in native oocytes exhibited mutual inhibition (Table 5), which was not evident from total uptakes.

DISCUSSION

It was originally proposed [2,5] that NBAT/rBAT proteins promote the uptake of amino acids by a single transport activity (system $b^{0,+}$ [5]) in oocytes, but the present studies demonstrate that at least two distinct Na^+-independent amino acid transport mechanisms are overexpressed. (1) A high-affinity ($K_m \sim 0.1$ mM) arginine transport component, which is NEM-resistant, inhibited by neutral amino acids such as leucine and phenylalanine, but resistant to inhibition by alanine, valine and the neutral form of TFMH; there appears to be mutual competitive inhibition between NEM-resistant arginine and phenylalanine uptake, indicating that the two amino acids may share this transport mechanism (which has a relatively low affinity for phenylalanine). (2) A low-affinity arginine transport component which is NEM-sensitive and inhibited by neutral amino acids including alanine and valine. This transport component may include the high-affinity NEM-sensitive phenylalanine transport, but at present we are unable to exclude the possibility that (at least) two NEM-sensitive transporters may be co-expressed.

Recent studies with rBAT [16] have revealed partial non-competitive inactivation of rBAT-induced transport in oocytes by the heavy-metal ions Pb^{2+} and Hg^{2+}, results that are consistent with the effect of NEM observed here and which may reflect induction of multiple transport activities by rBAT (as suggested in [8]). The 4F2hc surface antigen shows significant structural similarity to NBAT and also stimulates amino acid transport when expressed in *Xenopus* oocytes [17–19], apparently by activation of multiple transport activities (with characteristics similar to Systems L [17] and y^+L [18–20]). The multiple transport activities identified in the present study are separable in terms of substrate and thiol-group reagent specificity as well as by transport K_m, and seem unlikely to reflect expression of a dual kinetic phenotype by a single transport system, as suggested [17] for 4F2hc. We conclude that the overall inhibition patterns and kinetic parameters for NBAT/rBAT- (and perhaps 4F2hc-) induced amino acid transport in oocytes presented by ourselves [11] and others (e.g. [2,4,5]) may reflect the combined effects of at least two different transport mechanisms with different substrate specificities.

Induction of multi-component amino acid transport in NBAT-expressing oocytes argues against the idea that NBAT functions by itself as an amino acid transporter, and adds considerable support to the hypothesis that it operates partly or wholly (at least when expressed in oocytes) as an accessory or subunit element 'activating' endogenous transport proteins, possibly including the 50 kDa subunit(s) to which NBAT is attached by disulphide bridges within the oocyte membrane [9]. In this role,

NBAT may show functional similarities to heterologously expressed β-subunits of the Na-pump, which activate native *Xenopus* oocyte 'stores' of catalytic α-subunits by recruiting them to the cell membrane [20,21]. Native amino acid transport activities of oocytes bear similarities to the NBAT-induced activities (although they differ in terms of substrate K_m), and it seems reasonable to speculate that (i) NBAT interacts with at least two distinct oocyte proteins to produce functional heteromeric transporter complexes, and (ii) the formation of such complexes changes the K_m of the native transport activities or 'activates' transporters which are normally silent (e.g. localized in intracellular stores).

NEM-resistant NBAT-induced amino acid transport displays several features common to systems $b^{0,+}$ and y^+L, e.g. it is nominally Na^+-independent, interacts with both cationic and neutral amino acids (the affinity for the latter having the order leucine > phenylalanine > alanine) and fails to be inhibited by bicyclic leucine analogues such as BCH [22,23]. Important features of system $b^{0,+}$ [22] include, K_m values for lysine and leucine transport of 48 and 135 μM respectively, weak inhibition by alanine and valine, and no effect of Na^+ on transport activity. In comparison, system y^+L [23] has a higher affinity for cationic amino acids (K_m of 10 μM for lysine) and lysine uptake is inhibited by neutral amino acids with higher affinity in the presence of Na^+ or Li^+ than in the presence of other cations. The NEM-resistant transporter described here has an arginine transport K_m appropriate for system $b^{0,+}$, but the increased potency of phenylalanine as an inhibitor of arginine uptake in NaCl compared with TMACl bears closer resemblance to system y^+L. Furthermore, system y^+L is NEM-resistant [15] whereas system $b^{0,+}$ is at least partially inactivated by NEM in mouse blastocysts [24]. We are therefore unable to definitively identify NEM-resistant, NBAT-induced transport with either of these (or any other) functionally described transport systems of mammalian cells.

The NEM-sensitive component of NBAT-induced arginine uptake is inhibited by valine and alanine; these two amino acids are recognized to be poor substrates of systems $b^{0,+}$ and y^+L [22,23] but are major substrates of system asc [25], which has higher affinity for neutral than for cationic amino acids and has a reaction mechanism entirely independent of Na^+ [26]. It is therefore possible that system asc is overexpressed in NBAT-injected oocytes and is responsible for both NEM-sensitive arginine uptake and the greater part of increased alanine uptake; this would explain the relatively low V_{max} value of NBAT-induced alanine uptake [4,5,8] compared with those of leucine and phenylalanine (which are good substrates of both NEM-resistant and NEM-sensitive transport processes). We have not been able to confirm mutual competitive inhibition between NEM-sensitive arginine and phenylalanine uptakes and, given that phenylalanine is not a substrate of system asc, an additional NEM-sensitive transport pathway for phenylalanine uptake may be expressed in NBAT-injected oocytes (perhaps equivalent to the system L-like activity induced in 4F2hc-expressing oocytes [17]). NEM may inactivate NBAT-induced amino acid transport by direct binding to an essential cysteine residue or by affecting the stability of -S–S-bridges between subunits [9].

Only cationic TFMH inhibits NEM-resistant arginine uptake, whereas both neutral and cationic TFMH have equal (weak) inhibitory effects on phenylalanine uptake. In contrast, arginine is a strong inhibitor of NEM-resistant phenylalanine uptake and we conclude that there are separate binding sites for neutral and cationic amino acids on the NEM-resistant transporter. This appears to conflict with the observation that phenylalanine and arginine exhibit mutual competitive inhibition of NEM-resistant, NBAT-induced transport, but might be explained if the binding sites are in close apposition such that there is a degree of 'overlap', with substrates of one site exhibiting 'pseudo-competitive' inhibition by steric masking of the neighbouring site during the binding process. Only substrate molecules of a particular size and shape might exhibit this type of inhibition, explaining why certain amino acids (e.g. arginine and leucine) are very effective inhibitors of both cationic and neutral amino acid uptakes, whereas others (e.g. alanine, valine and cationic TFMH) only inhibit one type of uptake (they do not mask the neighbouring site when occupying their own binding site). Evidence from previous work indicates that a 'vestigial' Na-binding site in nominally Na-independent transport systems (including $b^{0,+}$ [27] and asc [28]) binds cationic amino acids (and perhaps other cations) and interacts with a separate site for neutral amino acid substrates. We have shown [11] that phenylalanine–arginine exchange accounts for a portion of NBAT-induced phenylalanine uptake in oocytes, but that an additional component is coupled with the outward movement of other ions (including K^+ and possibly small organic ions). This heterogeneity in the exchanging ionic species is consistent both with our identification here of multi-component NBAT-induced phenylalanine uptake and also with the suggestion that a 'loose' cation-binding subsite may be involved in arginine recognition and translocation. It has been speculated [29] that combined binding of Na^+ plus an appropriate neutral amino acid provides necessary recognition features for a cationic amino acid subsite of this type in the system y^+L transporter, and this may account for the increased efficacy of phenylalanine as an inhibitor of NBAT-induced arginine uptake in the presence of Na^+.

We conclude that at least two components of amino acid transport (NEM-resistant and NEM-sensitive) are induced in NBAT-cRNA-injected oocytes; these resemble the functionally characterized systems $b^{0,+}/y^+L$ and asc respectively. Direct comparison between amino acid transport activities associated with NBAT expression in different cell types (e.g. renal or intestinal epithelia and *Xenopus* oocytes) may be inappropriate if NBAT proves to be a subunit of an oligomeric transporter complex, because the actual activities expressed may vary depending upon the complement of other suitable subunits in the host cell and the subcellular targeting of NBAT to specific membranes. Brush-border membranes from epithelial cells exhibit Na^+-independent transport of amino acids and cystine (see [20,30] for review), and native *Xenopus* oocytes express transport activation resembling systems $b^{0,+}$, y^+, y^+L, L and asc [8,12,18,20,31] which could form complexes with NBAT, but we believe there is as yet insufficient information available to attempt 'system-based' comparisons of transport activity between these cell types and thus to absolutely describe NBAT-induced amino acid transport.

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