Biochem. J. (1992) 281, 717–723 (Printed in Great Britain)

Expression of Na^+-independent amino acid transport in *Xenopus laevis* oocytes by injection of rabbit kidney cortex mRNA

Joan BERTRAN,*† Andreas WERNER,* Gerti STANGE,* Daniel MARKOVICH,* Jürg BIBER,* Xavier TESTAR,† Antonio ZORZANO,† Manuel PALACIN† and Heini MURER*‡

*Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, and †University of Barcelona, Biochemical and Physiological Department, E-08028 Barcelona, Spain

Poly(A)$^+$ mRNA was isolated from rabbit kidney cortex and injected into *Xenopus laevis* oocytes. Injection of mRNA resulted in a time- and dose-dependent increase in Na^+-independent uptake of L-[^{3}H]alanine and L-[^{3}H]arginine. L-Alanine uptake was stimulated about 3-fold and L-arginine uptake was stimulated about 8-fold after injection of mRNA (25–50 ng, after 3–6 days) as compared with water-injected oocytes. T.l.c. of oocyte extracts suggested that the increased uptake actually represented an increase in the oocyte content of labelled L-alanine and L-arginine. The expressed L-alanine uptake, obtained by subtracting the uptake in water-injected oocytes from that in mRNA-injected oocytes, showed saturability and was inhibited completely by 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH) and L-arginine. The expressed L-arginine uptake in mRNA-injected oocytes also showed saturability, being completely inhibited by L-(dibasic amino acids) and partially inhibited by BCH. Expression of both L-alanine and L-arginine uptake showed clear *cis*-inhibition by cationic (e.g. L-arginine) and neutral (e.g. L-leucine) amino acids. In all, this points to the expression of a Na^+-independent transport system with broad specificity (i.e. $b^{0,+}$-like). In addition, part of the expressed uptake of L-arginine could be due to a system y^+-like transporter. After size fractionation through a sucrose density gradient, the mRNA species encoding these increased transport activities (Na^+-independent transport of L-alanine and of L-arginine) were found in fractions of an average mRNA chain-length of 1.8–2.4 kb. On the basis of these results, we conclude that Na^+-independent transport system(s) for L-alanine and L-arginine from rabbit renal cortical tissues, most likely proximal tubules, are expressed in *Xenopus laevis* oocytes. These observations may represent the first steps towards expression and cloning of these transport pathways.

INTRODUCTION

Renal proximal tubular and small intestinal epithelial cells are involved in vectorial transcellular fluxes of different groups of amino acids (re-absorption). Specific transport pathways in the apical membrane (brush border) are involved in the uptake of amino acids from the luminal compartment; basolateral transport systems may mediate uptake from the interstitium or efflux into the interstitium to complete the re-absorptive movements (for reviews see Stevens *et al.*, 1984; Silbernagl, 1988). In general, amino acids cross the plasma membrane of mammalian cells by both Na^+-independent and Na^+-dependent transport processes (Kilberg, 1982; Collarini & Oxender, 1987; Saier *et al.*, 1988; Van Winkle, 1988; Christensen, 1990). The basolateral membranes of proximal tubular and small intestinal epithelial cells are believed to contain amino acid carriers which are similar in specificity to those described for other mammalian (non-polarized) cells, whereas in the brush border membrane additional transport systems seem to be present (Stevens *et al.*, 1984; Silbernagl, 1988).

Intestinal malabsorption and renal hyperexcretion of amino acids seem to be related to specific genetic defects in amino acid transport systems (Scriver *et al.*, 1976). For example, in Hartnup's disease, a defect in Na^+-independent systems (e.g. system L) and/or in the Na^+-dependent systems NBB (Neutral Brush Border) and Phe (Phenylalanine) has been hypothesized (Stevens *et al.*, 1984). Similarly, hyperdibasicaminaciduria and cystinuria could involve a genetic disorder in system y^+ (Stevens *et al.*, 1984; Silbernagl, 1988).

Our knowledge about the structural identity of amino acid transport systems is very limited. Recently, an estimate of the molecular sizes of the functional units responsible for the Na^+-dependent transport of L-glutamic acid, L-proline, L-leucine and L-alanine (between 220 and 300 kDa) and for the Na^+-independent transport of L-lysine (around 90 kDa) has been reported, based on radiation-inactivation studies using kidney cortex brush border vesicles (Béliveau *et al.*, 1990). Wright & Peerce (1984) provided biochemical evidence for the involvement of a 100 kDa protein in Na^+-dependent L-proline transport in intestinal brush border membranes.

The lack of high-affinity inhibitors for mammalian amino acid carriers complicates their structural identification and isolation. A possible strategy to identify membrane transport mechanisms is by their functional expression in *Xenopus laevis* oocytes, as demonstrated for intestinal Na^+/D-glucose transport (Hediger *et al.*, 1987). Recently, expression of hepatic amino acid transport, probably related to the activity of systems A and ASC, has been reported (Palacin *et al.*, 1990; Tarnuzzer *et al.*, 1990). Activity related to system A has also been expressed in *Xenopus* oocytes by injection of mRNA extracted from either CHO cells or human placenta (Tarnuzzer *et al.*, 1990); the average chain length of the mRNA species encoding these activities was found to be between 1.9 and 2.5 kb (Palacin *et al.*, 1990; Tarnuzzer *et al.*, 1990). Similarly, expression of the Na^+-dependent uptake of several amino acids after injection of rat small intestinal mRNA (Aoshima *et al.*, 1988) and rabbit renal cortical mRNA (Coady *et al.*, 1990) has been reported; Na^+-independent uptake of L-alanine, L-phenylalanine and L-lysine was obtained by injection

Abbreviations used: BCH, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid; MeAIB, 2-(methylamino)isobutyric acid; AIB, 2-aminoisobutyric acid.

‡ To whom correspondence should be addressed.

of rabbit renal cortex mRNA of 1.5–3 kb in length (Coady *et al.*, 1990). Finally, Na^+-independent uptake of L-leucine and L-phenylalanine has been expressed after injection of mRNA from rat kidney and human lymphoid cells with an average chain length of 3–4 kb (Tate *et al.*, 1989).

The above-mentioned reports on the expression of Na^+-independent amino acid transport in *Xenopus laevis* oocytes after injection of (kidney cortex) mRNA do not describe attempts to clarify the involvement of 'specific' carriers responsible for the expressed uptake activities. In the present paper, we provide evidence for the involvement of a transport system with broad specificity for neutral and cationic amino acids (i.e. $b^{0,+}$-like) in the increased Na^+-independent amino transport in *Xenopus laevis* oocytes after injection of rabbit kidney cortex mRNA of average chain length 1.8–2.4 kb. In addition, part of the expressed uptake activity of L-arginine could be due to the expression of system y^+.

MATERIALS AND METHODS

Oocytes and injections

Xenopus laevis females were obtained from H. Kähler (Institut für Entwicklungsbiologie, Hamburg, Germany). Small clumps of oocytes were treated for 2 × 90 min with collagenase (Boehringer, Mannheim, Germany) at 2 mg/ml in a Ca^{2+}-free solution (ORII: 82.5 mM-NaCl, 2 mM-KCl, 1 mM-$MgCl_2$, 10 mM-Hepes/Tris, pH 7.5) to remove the follicular layer. After thorough washing with ORII solution and with modified Barth's solution [88 mM-NaCl, 1 mM-KCl, 0.82 mM-$MgSO_4$, 0.4 mM-$CaCl_2$, 0.33 mM-$Ca(NO_3)_2$, 2.4 mM-$NaHCO_3$, 10 mM-Hepes/Tris, pH 7.5], the oocytes were kept in modified Barth's solution overnight at 18 °C. After this incubation period, healthy-looking stage VI oocytes were injected with mRNA or water. mRNA samples were dissolved in water at concentrations varying from 0.03 to 1 mg/ml. mRNA was injected into oocytes by using a semi-automatic injector (Inject + Matic-system; J. A. Gabay, Geneva, Switzerland). The volume injected was 50 nl. The oocytes were then incubated at 18 °C for 1–6 days in modified Barth's solution containing gentamycin sulphate (10 mg/l).

Isolation and fractionation of poly(A)$^+$ RNA

RNA from rabbit kidney cortex was extracted using the caesium trifluoroacetate/guanidinium thiocyanate centrifugation method according to the protocol given by Pharmacia AB (Uppsala, Sweden). Oligo(dT)–cellulose for selection of poly(A)$^+$ RNA (mRNA) was purchased from Beohringer and was used according to the manufacturer's protocol. For size fractionation of mRNA, a 6–20% (w/w) sucrose gradient in a solution containing EDTA (1 mM) and Tris (10 mM, pH 7.5) was used. mRNA (60 μg in 100 μl of water) was denatured for 3 min at 65 °C and rapidly chilled on ice. Then the samples were loaded on to the gradient, followed by centrifugation for 16 h at 130000 $g_{av.}$ and 4 °C in a TST 41 rotor (Kontron, Zürich, Switzerland). Samples (0.4 ml) were collected, precipitated with ethanol, washed with 70% ethanol, dried and resuspended in 10 μl of water. A sample was used to measure absorbance at 260 nm and for analysis on a denaturing 1% agarose minigel to determine the average chain length of the mRNA in each fraction collected from the sucrose density gradient.

Uptake measurements

To measure the uptake of L-[^{3}H]arginine or L-[^{3}H]alanine (both purchased from NEN Radiochemicals), 7–10 oocytes were used per individual time point. Oocytes were first washed for 2 min in solution A (100 mM-choline chloride, 2 mM-KCl, 1 mM-$CaCl_2$, 1 mM-$MgCl_2$, 10 mM-Hepes/Tris, pH 7.5). To initiate uptake, this solution was replaced by 100 μl of solution A supplemented with the desired concentration of the substrate, typically 50 μM-L-[^{3}H]arginine at 12 μCi/ml or 1 mM-L-[^{3}H]alanine at 50 μCi/ml. The uptake was performed at 25 °C for 1 h. To measure Na^+-dependent uptake, choline chloride was replaced by NaCl in solution A and the value in the absence of Na^+ was subtracted from that in its presence. After incubation, the uptake solution was removed and the oocytes were washed with 3 × 4 ml of solution A supplemented with 0.1 mM-L-arginine or 2 mM-L-alanine. Each single oocyte was subsequently placed into a scintillation vial, dissolved in 200 μl of 10% SDS and the radioactivity counted after adding 3 ml of scintillation fluid.

T.l.c.

Oocytes (four for each condition) were incubated for 60 min in uptake solution as described for the flux measurements. After washing (analogous to flux measurements), oocytes were placed in 100 μl of solution A and passed several times through a yellow pipette tip until rupture of the oocyte occurred. The 'homogenates' were spun for 5 min in an Eppendorf microcentrifuge, and trichloroacetic acid was added to the supernatant to a final concentration of 5% (v/v). Precipitates were separated by a 10 min centrifugation (Eppendorf) and 10 μl samples of the resulting supernatants were used for t.l.c. on silica gel 60 thin layer plates using butanol/acetic acid/water (4:1:1, by vol.) as a solvent; 20 μg of unlabelled amino acid was added to the 10 μl of extract. The chromatography was performed for 6 h and plates were then dried in air, and placed again into the solvent until the same migration of the solvent front was obtained as in the first run. Amino acids were visualized with ninhydrin, the silica gel was scraped off from specific regions (according to the R_F values given in the figure) and placed into 500 μl of water. After addition of 8 ml of scintillation fluid, radioactivity was counted.

RESULTS

Basic observations

Time course of Na^+-independent uptake of L-alanine and L-arginine by water-injected oocytes. As illustrated in Fig. 1(*a*), uptake of L-[^{3}H]alanine by water-injected oocytes was linear and very slow during the first 2 h of the assay. The extrapolation to time zero of the time course of uptake does not cross the zero uptake value. This suggests that the inward transport of the amino acid may be preceded by some rapid event or binding to the outer surface of the oocyte. The uptake value that was reached after 2 h of incubation (approx. 50 pmol/oocyte) is far from equilibrium with the medium, since it represents a distribution space of less than 5% of the total oocyte volume (approx. 900 nl). Na^+-independent uptake of L-alanine was not saturable by increasing the substrate concentration in the range 10 μM–10 mM (e.g. 10 μM, 1.3 ± 0.03; 50 μM, 3.2 ± 0.07; 100 μM, 5 ± 0.17; 200 μM, 9.1 ± 0.2; 500 μM, 16.1 ± 0.9; 700 μM, 25.1 ± 1.1; 1 mM, 37.3 ± 1.2; 2 mM, 58.6 ± 2.2; 5 mM, 133.3 ± 6.2; 10 mM, 257 ± 18.6 pmol/h per oocyte). These findings are in agreement with previous reports (Jung *et al.*, 1984; Palacin *et al.*, 1990). Furthermore, the presence of different amino acids at a concentration of 10 mM in the incubation medium did not inhibit the uptake of 1 mM-L-alanine by the oocytes [109 + 7% of control for 2-aminoisobutyric acid (AIB), 113 ± 10% for 2-(methylamino)isobutyric acid (MeAIB), 106 ± 5% for 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH), 92 ± 6% for L-leucine, 99 + 13% for L-glutamine, 95 + 4% for L-phenylalanine and 96 + 5% for L-arginine; n = 3–5 independent batches of oocytes]. Thus we have no evidence for the involvement of a carrier in L-alanine uptake by water-injected oocytes.

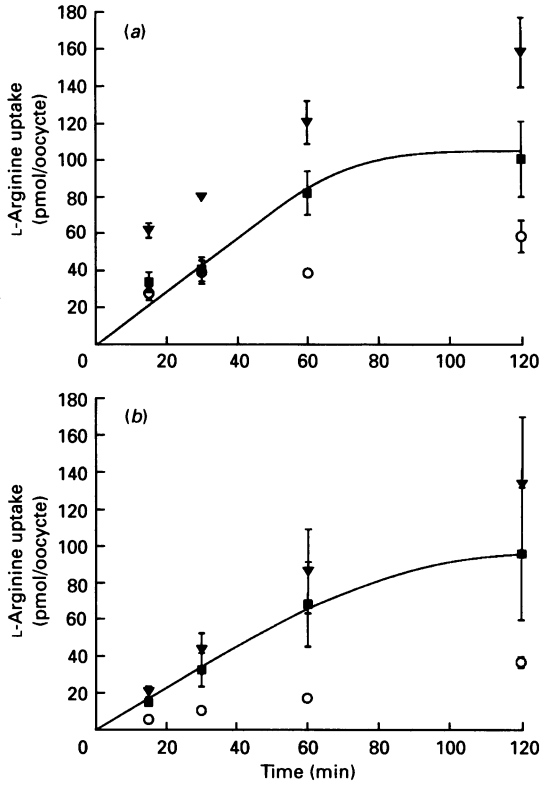

Fig. 1. Time courses of uptake of L-[^{3}H]alanine and L-[^{3}H]arginine by water- and mRNA-injected oocytes

At 4 days after injection of 50 nl of water containing 0 (○) or 25 (▼) ng of mRNA, the uptake of 1 mM-L-alanine (*a*) or 50 μM-L-arginine (*b*) was measured after various periods of incubation in Na^+-free uptake media. Net expressed uptake (■) was calculated as the difference between the uptake in mRNA-injected oocytes and that in water-injected oocytes. Data represent means ± S.E.M. of eight oocytes in a representative experiment. Error bars smaller than the symbols are not included.

As illustrated in Fig. 1(*b*), Na^+-independent uptake of L-[^{3}H]arginine increased during the first 120 min of incubation. As will be shown below, saturation as well as inhibition by dibasic amino acids of L-arginine uptake in water-injected oocytes suggests that it is occurring via a y^+-like system (see also Campa & Kilberg, 1989).

Time course of Na^+-independent uptake of L-alanine and L-arginine by mRNA-injected oocytes. The uptake of L-[^{3}H]alanine in mRNA-injected oocytes (25 ng/50 nl) is also shown in Fig. 1(*a*). The uptake of 1 mM-L-alanine increased continuously during the first 2 h of the incubation assay, and reached a value (approx. 160 pmol/oocyte) that represents a distribution space of 17% of the total oocyte volume. The difference between the values in mRNA-injected and water-injected oocytes eliminates the endogenous components of L-alanine uptake and represents the expressed uptake. As shown in Fig. 1(*a*), this expressed L-alanine uptake was linear during the first 60 min. Thus in subsequent experiments L-alanine uptake was routinely analysed during a 60 min incubation period in both the water-injected and mRNA-injected oocytes, and the expressed uptake was calculated.

The analogous experiments is shown in Fig. 1(*b*) for L-[^{3}H]arginine uptake in mRNA-injected oocytes (25 ng/50 nl). In mRNA-injected oocytes, as in water-injected oocytes, L-arginine uptake was linearly related to incubation time up to 60 min. Furthermore, within this time period, L-[^{3}H]arginine uptake in mRNA-injected oocytes was mostly (~ 80%) due to the mRNA injection, i.e. expression of the transport function. Thus we have also selected an uptake period of 60 min for all subsequent experiments on L-arginine uptake.

Identification of L-[^{3}H]amino acids in oocytes. To confirm that increased uptake of L-[^{3}H]amino acids after mRNA injection was not related to increased metabolic consumption, but rather to increased influx capacity, we analysed by t.l.c. the tracer content in L-alanine and L-arginine extracted from oocytes injected with 25 ng of mRNA. Three days after mRNA injection, the oocytes were exposed to 1 mM-L-[^{3}H]alanine or 50 μM-L-[^{3}H]arginine for 60 min and homogenized, and the trichloroacetic acid supernatants were applied to t.l.c., as described in the Materials and methods section. The R_F values for labelled substances extracted from the mRNA-injected oocytes were identical to those for tracer L-alanine and L-arginine which were not exposed to the oocytes (results not shown). Since in most cells arginine is rapidly metabolized to ornithine by the enzyme arginase, we have also analysed by t.l.c. the tracer content of ornithine in the extracts of mRNA-injected oocytes. The contribution of the metabolism of L-arginine to L-ornithine to the total L-arginine uptake in the oocytes can be estimated as less than 5% (results not shown). The total radioactivity recovered in the trichloroacetic acid supernatants of oocyte extracts was about 3-fold higher for L-alanine uptake after mRNA injection and about 6-fold higher for L-arginine uptake after mRNA injection (results not shown). Thus increased uptake of tracer after mRNA injection is not related to increased conversion of amino acids, but rather to increased influx capacity.

Time- and dose-dependence of the effect of mRNA injection on Na^+-independent uptake of L-alanine and L-arginine. Fig. 2(*a*) depicts the time course of expression of L-alanine uptake after mRNA injection into oocytes (25 ng/50 nl). Marked expression of L-alanine uptake was observed after 2 days, and this increased further to reach a plateau after 3–4 days. L-Alanine uptake in water-injected oocytes also increased slightly with time; the maximum difference between water-injected and mRNA-injected oocytes was always observed between 3 and 4 days, and was between 1.7- and 4.7-fold (using four different mRNA preparations; results not shown).

The increase in L-arginine uptake was also related to the time after injection of oocytes with mRNA. However, there was no apparent plateau reached after 3–4 days, and uptake at 5 or 6 days of incubation (optimal possible period for routine oocyte survival) was higher than uptake measured after shorter incubation periods (results not shown).

As demonstrated in Fig. 2(*b*), expression of L-alanine uptake showed a clear dose–response relationship between increased transport and the amount of mRNA injected. The expression was almost linearly proportional to the amount injected up to 12 ng of mRNA, and flattened at 25 ng. Therefore injections of 25 ng or more of mRNA and an incubation period of 3–5 days seem to be optimal conditions for routine work on expression of L-alanine transport.

Fig. 2(*b*) shows that the expression of L-arginine uptake could already be detected after injection of 3 ng of mRNA, increased up to 12 ng of mRNA and flattened at 25 ng. Therefore injections of 25 ng or more and transport analysis after 3–6 days seem to be the most favourable conditions for the expression of L-arginine transport; under these conditions, the expression was between 3.3- and 16.7-fold higher than the endogenous activity (for three independent mRNA preparations; results not shown).

Size fractionation of mRNA encoding increased transport activity

mRNA was applied on to a linear 6–20% (w/w) sucrose density gradient and fractionated into 22 fractions. As shown in

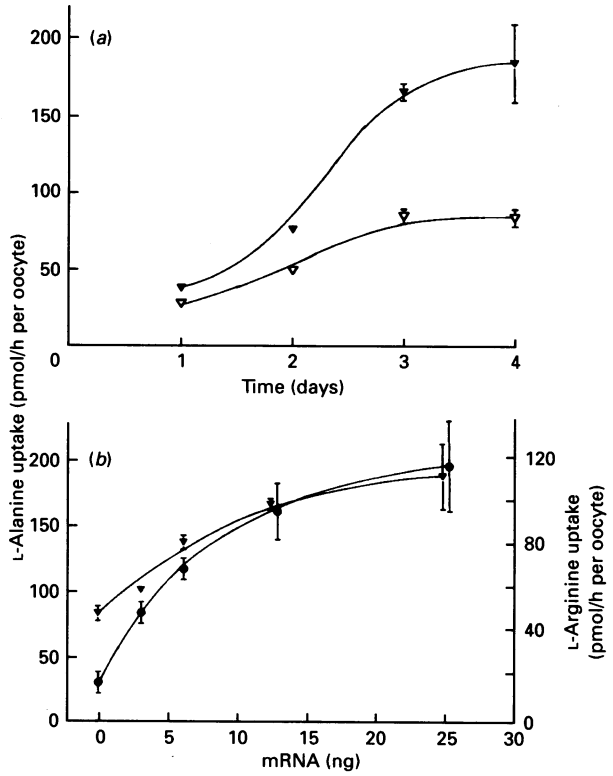

Fig. 2. Dose- and time-dependent expression of L-alanine and L-arginine uptake

Oocytes injected with 50 nl of water (▽) or 50 nl of water containing 25 ng of mRNA (▼) were assayed for the Na^+-independent uptake of L-alanine on different days after injection (*a*). For measurement of dose-dependency (*b*), water (50 nl) containing different amounts of mRNA was injected into oocytes and the Na^+-independent uptake of 1 mM-L-alanine (▼) and 50 μM-L-arginine (●) was measured after 4 days. Each data point represents the mean ± S.E.M. of eight oocytes from a representative experiment. Error bars smaller than symbols are not included.

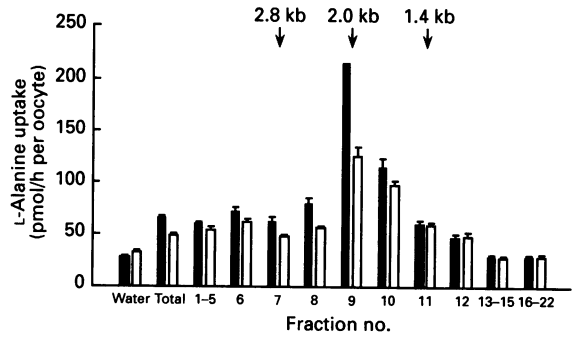

Fig. 3. Expression of uptake of L-alanine in response to size-fractionated mRNA

Oocytes were injected with water, total mRNA (30 ng) or different mRNA fractions (15–20 ng each) separated by size on a sucrose gradient. The average length in kilobases (kb) of the mRNA in the fractions is indicated by the arrows. The Na^+-independent uptake of 1 mM-L-alanine was determined 5 days later. The bars represent means ± S.E.M. obtained from 7–10 oocytes per group in a representative experiment. The total Na^+-independent uptake of L-alanine in the absence (■) and presence (□) of 10 mM-BCH is represented.

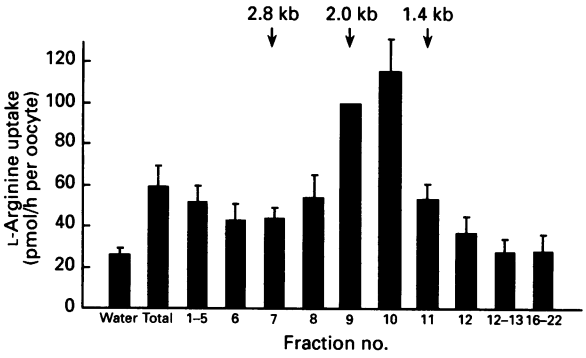

Fig. 4. Expression of uptake of L-arginine in response to size-fractionated mRNA

Oocytes were injected with water, total mRNA (30 ng) or different mRNA fractions (15–20 ng each) separated by size on a sucrose gradient. The average length in kilobases (kb) of the mRNA in the fractions is indicated by the arrows. The Na^+-independent uptake of 50 μM-L-arginine was determined 5 days later. The bars represent means ± S.E.M. obtained from 7–10 oocytes per group in a representative experiment.

Figs. 3 and 4, maximal expression of L-alanine and L-arginine uptake (3-fold and 2-fold higher than total mRNA respectively) was observed in fractions 8–10, containing mRNA species with an average chain length of 1.8–2.4 kb. Similar results were obtained with two other independent mRNA preparations/gradient centrifugations (results not shown). The addition of the bicyclic amino acid BCH at a concentration of 10 mM to the uptake medium inhibited transport of L-alanine in fractions 7–10 (Fig. 3).

Characterization of expressed Na^+-independent uptake of L-alanine

Saturation. Expressed L-alanine uptake, obtained by subtracting the uptake in water-injected oocytes from that in mRNA-injected oocytes, showed Michaelis–Menten kinetics (Fig. 5); Eadie–Hofstee transformation resulted in apparent K_m values of 0.6 mM (Fig. 5, insert) and 0.68 mM (in a second independent experiment; results not shown).

Inhibition by other L-amino acids. To analyse the specificity of Na^+-independent transport systems involved in transport after mRNA injection, L-alanine (1 mM) uptake was measured in the presence of other L-amino acids (10 mM). Total uptake of L-alanine in mRNA-injected oocytes (165.3 ± 22 pmol of L-alanine/h per oocyte) was inhibited by L-leucine (to 41 ± 10 % of control), BCH (76 ± 2 %), L-phenylalanine (29 ± 1 %), L-glutamine (59 ± 10 %) and L-arginine (40 ± 7 %). On the contrary, AIB and MeAIB (93 ± 9 and 91 ± 4 % respectively) were unable to significantly inhibit L-alanine uptake in mRNA-injected oocytes (n = 3–5 independent batches of oocytes and two different mRNA preparations). Table 1 shows inhibition of expressed activity only, i.e. after subtraction of values obtained in water-injected oocytes (with the exception of MeAIB and AIB, all the amino acids tested inhibited largely the expressed activity. BCH, at a concentration of 10 mM, inhibited the expressed uptake of 1 mM-L-alanine by 50 %, whereas the other neutral amino acids tested (L-leucine, L-phenylalanine and L-glutamine) produced a greater inhibition (> 80 %). BCH, at a 1000-fold excess over the L-alanine concentration, abolished the expressed uptake of L-alanine. Thus the expressed uptake of 50 μM-L-alanine (control 8.3 ± 0.8 pmol/h per oocyte) in the presence of BCH was: at 500 μM, 8.0 ± 1.0; at 5 mM, 5.9 ± 0.3; at 50 mM, 0.9 ± 0.1 pmol/h per oocyte, (n = 7–8 oocytes in a representative

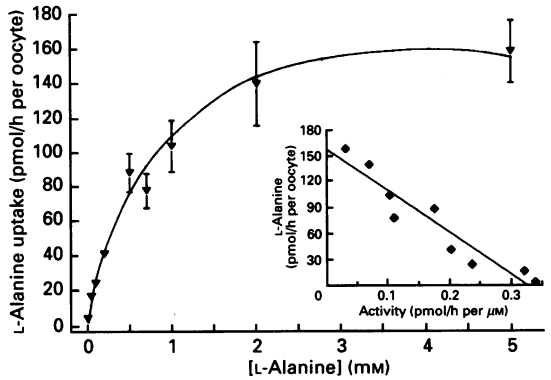

Fig. 5. Kinetic analysis of the expressed Na^+-independent uptake of L-alanine in mRNA-injected oocytes

Oocytes were injected with 25 ng of mRNA dissolved in 50 nl of water or with water alone (50 nl), and the uptake of L-alanine was measured, in the absence of Na^+, at different L-alanine concentrations 4 days after the injection. The presented data (expressed uptake) were obtained by subtracting the uptake in water-injected oocytes from that in mRNA-injected oocytes. Data represent means ± S.E.M. of 8 oocytes per condition in a representative experiment. Eadie–Hofstee transformation (insert) was calculated with the mean values of the expressed uptake. Error bars smaller than symbols are not included.

Table 1. Inhibition of the expressed uptake of L-alanine by different amino acids or analogues in mRNA-injected oocytes

Oocytes were injected with water containing 0 or 25 ng of mRNA and the uptake of 1 mM-L-alanine was determined 5 days later, in the presence (10 mM) or absence of different amino acids or analogues. Data (means ± S.E.M. of 3–5 independent experiments, in which 8 oocytes were used for each single determination) represent the percentages of residual transport in the presence of the tested inhibitors. The basal expression (100 %), in the absence of inhibitors, was calculated as the difference between the uptake in mRNA-injected oocytes and that in water-injected oocytes (100 ± 22 pmol of L-alanine/h per oocyte).

Inhibitor (10 mM)	L-Alanine transport activity (% of basal)
MeAIB	77 ± 11
AIB	83 ± 9
BCH	53 ± 4
L-Leucine	9 ± 3
L-Glutamine	17 ± 10
L-Phenylalanine	10 ± 5
L-Arginine	16 ± 7

experiment). The expressed L-alanine uptake showed a very broad specificity, since the cationic amino acid L-arginine at 10 mM was equally effective in inhibiting the uptake of 1 mM-L-alanine. Indeed, L-arginine, at 5-fold excess over L-alanine concentration, nearly abolished the expressed uptake of L-alanine. Thus 250 μM-L-arginine inhibited the expressed uptake of 50 μM-L-alanine to 12 ± 1 % of the control (n = 7 oocytes from a representative experiment).

Characterization of intrinsic and expressed Na^+-independent uptake of L-arginine

Saturation. Saturation experiments on L-arginine uptake revealed no difference in apparent affinity (K_m) between water- and mRNA-injected oocytes: both components (intrinsic and expressed activity) showed an apparent K_m value of 80 μM (Figs. 6*a* and 6*b*). The $V_{max.}$ value was obviously increased in mRNA-injected oocytes.

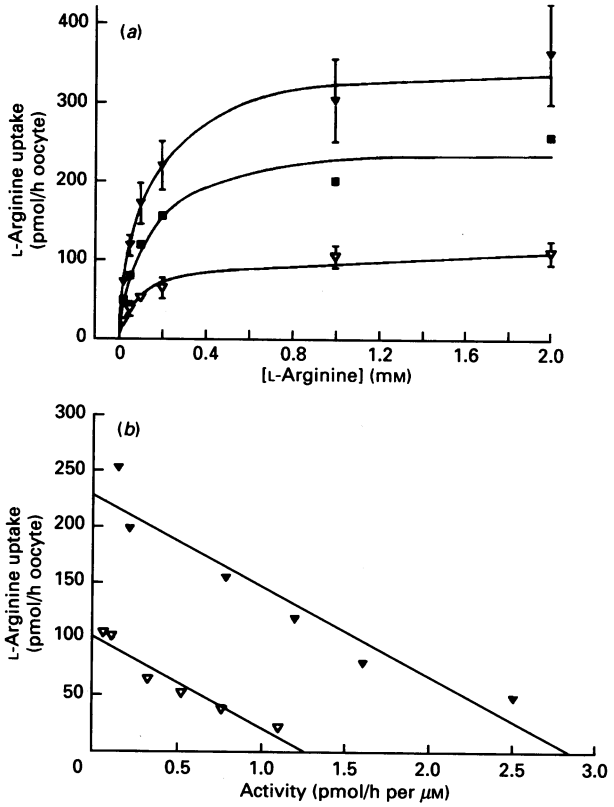

Fig. 6. Kinetic analysis of the Na^+-independent uptake of L-arginine in water- and mRNA-injected oocytes

Oocytes were injected with 25 ng of mRNA dissolved in 50 nl of water (▼) or with water alone (▽) (50 nl), and the uptake of L-arginine was measured, in the absence of Na^+, at different L-arginine concentrations 4 days after the injection. The expressed activity (■) was calculated by subtracting the values in water-injected oocytes from those in mRNA-injected oocytes. Data represent means ± S.E.M. of 8 oocytes for each condition of a representative experiment (*a*). Eadie–Hofstee transformations (*b*) were calculated with the mean values for intrinsic (▽) and expressed (▼) activity. Error bars smaller than symbols are not included.

Inhibition by other amino acids. In water-injected oocytes, L-[^{3}H]arginine uptake measured at 50 μM (3 pmol/h per oocyte) was decreased to 26 ± 3 % of control by a 10-fold excess of unlabelled L-arginine, to 26 ± 3 % by L-ornithine (0.5 mM) and to 90 ± 2 % by D-ornithine (0.5 mM) but was not affected by the same concentrations of L-leucine, L-alanine and the bicyclic amino acid BCH (results not shown). This pattern of inhibition is indicative of uptake of L-arginine via system y^+ in water-injected oocytes (i.e. intrinsic activity); system $b^{o,+}$-like activity seems not to participate in L-arginine uptake under these conditions (no inhibition by L-leucine and L-alanine).

The expressed activity, calculated by the subtraction of the uptake in water-injected oocytes from that in mRNA-injected oocytes, showed a different pattern of inhibition by a 10-fold excess (0.5 mM) of L-amino acids compared with the intrinsic activity. As shown in Table 2, the expressed Na^+-independent uptake of L-arginine (50 μM) was almost abolished by a 10-fold excess of the tested L-dibasic amino acids (L-arginine and L-ornithine, but not D-ornithine), slightly inhibited by L-alanine

Table 2. Inhibition of the expressed uptake of L-arginine by different amino acids or analogues in mRNA-injected oocytes

Oocytes were injected with water containing 0 or 25 ng of rabbit kidney cortex mRNA, and the uptake of 50 μM-L-arginine was determined 5 days after injection in the presence (0.5 mM) or in the absence of different amino acids or analogues. Data (means ± S.E.M. of 3–4 independent experiments, in which 8 oocytes were used for each single determination) represent the percentages of residual transport in the presence of the tested inhibitors. The basal expression (100 %), in the absence of inhibitors, was calculated as the difference between the uptake in mRNA-injected oocytes and that in water-injected oocytes (121 ± 11 pmol of L-arginine/h per oocyte).

Inhibitor (0.5 mM)	L-Arginine transport activity (% of basal)
BCH	110 ± 8
L-Alanine	75 ± 10
L-Leucine	31 ± 7
L-Arginine	7 ± 3
L-Ornithine	9 ± 2
D-Ornithine	80 ± 5

and greatly inhibited (approx. 70 %) by L-leucine. On the contrary, a 10-fold excess of BCH (0.5 mM) did not inhibit the expressed uptake of L-arginine (50 μM), whereas a 1000-fold excess inhibited this uptake to approx. 40 % (112.9 ± 19.0 pmol/h per oocyte and 46.9 ± 6.6 pmol/h per oocyte in the absence and in the presence respectively of 50 mM-BCH; $n = 6$ oocytes in a representative experiment). Therefore, as illustrated for the expressed L-alanine uptake, the expressed uptake of L-arginine shows a very broad specificity for neutral and cationic amino acids.

DISCUSSION

Injection of rabbit renal cortex mRNA into *Xenopus laevis* oocytes resulted in expression of Na^+-independent uptake of L-alanine and L-arginine. Increased L-amino acid uptake was related to increased transport capacity at the oocyte membrane level, rather than to increased metabolic conversion. This expressed uptake of L-alanine and L-arginine in the mRNA-injected oocytes shows characteristics of transport systems operating in renal and intestinal tissues.

The results obtained from the size-fractionation experiments indicate that above-mentioned Na^+-independent transport systems for L-alanine and L-arginine are encoded by mRNA species of average chain length 1.8–2.4 kb. This information is in agreement with the values published by Coady *et al.* (1990) for the expression of Na^+-independent uptake of L-phenylalanine and L-lysine by rabbit kidney mRNA. However, an apparent length of 3–4 kb for rat kidney mRNA which leads to expression of Na^+-independent uptake of L-leucine and L-phenylalanine has also been reported (Tate *et al.*, 1989).

The expressed Na^+-independent uptake of L-alanine shows two characteristics that are different from those of endogenous uptake. (i) The expressed uptake is saturable and shows an apparent K_m of around 0.6 mM; this value is in good agreement with the K_m described for the saturating component of Na^+-independent L-alanine uptake in basal-lateral (0.73 mM) and brush-border vesicles (0.37 mM) isolated from small intestinal epithelial cells (Stevens *et al.*, 1982; Mircheff *et al.*, 1982). Unfortunately, similar data are not available for renal membrane preparations. (ii) The expressed uptake is *cis*-inhibited by a 10-fold excess concentration of several amino acids and analogues (i.e. BCH, L-leucine, L-phenylalanine, L-glutamine and L-arginine).

In the present study, one striking difference between intrinsic uptake of L-arginine and that observed after mRNA injection is the different inhibition by neutral amino acids: 0.5 mM-L-dibasic amino acids largely inhibited the uptake of 50 μM-L-arginine in water-injected oocytes, while neutral amino acids (i.e. L-alanine and L-leucine) did not affect the uptake of L-arginine. This observation is indicative of system y^+ transport, and at variance with the results of Campa & Kilberg (1989), who concluded that system $b^{o,+}$ also participates in intrinsic L-arginine uptake by non-injected oocytes, based on a 30 % inhibition of L-arginine uptake by a 100-fold excess of L-leucine. In contrast, after injection of mRNA, the expressed uptake of 50 μM-L-arginine was strongly inhibited by a 10-fold excess of L-dibasic amino acids, and also clearly decreased (approx. 70 %) by the addition of a 10-fold excess of L-leucine.

The Na^+-independent transport of L-alanine and L-arginine in the plasma membrane of mammalian cells has been mostly attributed to the activity of systems L and y^+ respectively (White *et al.*, 1982; White & Christensen, 1982; Christensen, 1990). Cationic amino acids show very weak, if any, interaction with L-type systems (Christensen & Antonioli, 1969; Weissbach *et al.*, 1982; Christensen, 1984). On the other hand, interaction of zwitterionic amino acids with system y^+ is very weak in the absence of sodium (Christensen & Antonioli, 1969; White *et al.*, 1982). In addition to systems L and y^+, in renal and intestinal brush borders the participation of a system ($b^{o,+}$) for the Na^+-independent transport of zwitterionic and cationic amino acids has been suggested (Van Winkle *et al.*, 1988). Indeed, neutral and dibasic amino acids do show clear cross-inhibition for Na^+-independent uptake in renal and intestinal brush border vesicles (Stevens *et al.*, 1982, 1984; Mircheff *et al.*, 1982). In the present study, the expressed uptake of both L-alanine and L-arginine in the mRNA-injected oocytes showed a maximal activity of the same order (approx. 150–200 pmol/h per oocyte). This is either compatible with the expression of at least two different transport systems, one for neutral amino acids and another for cationic amino acids, or, alternatively, with the expression of a system with very broad specificity, such as system $b^{o,+}$. The almost complete inhibition of the expressed L-alanine uptake by L-arginine and the large inhibition of expressed L-arginine uptake by L-leucine points to the expression of a $b^{o,+}$-like system. Indeed, the large inhibition (approx. 70 %) by 500 μM-L-leucine of the expressed uptake of 50 μM-L-arginine is in good agreement with the expression of a $b^{o,+}$-like system, since its activity in mouse blastocysts shows a low apparent K_m value for L-leucine (approx. 100 μM) (Van Winkle *et al.*, 1988). On the contrary, the K_i value of leucine for system y^+ in non-epithelial mammalian cells (25–90 mM) (Christensen & Antonioli, 1969) is two orders of magnitude higher than the tested concentration of L-leucine in our *cis*-inhibition studies. Therefore it is very unlikely that the total expressed uptake of L-arginine is mediated by the system y^+ activity described in those cells.

The contribution of system L to the expressed uptake of L-alanine, if any, is not very conspicuous. BCH interacted with the expressed uptake of L-alanine only at high concentrations (in the millimolar range). Indeed, the expressed uptake of L-alanine was not inhibited by a 10-fold excess of BCH (500 μM), whereas it was almost abolished by a 1000-fold excess of BCH (50 mM). This concentration of BCH is at least 200 times higher than its K_m for L-type system(s) in non-epithelial mammalian cells (Weissbach *et al.*, 1982). On the other hand, the expressed L-alanine uptake was nearly abolished by a 5-fold excess of L-arginine (250 μM).

Therefore it seems that most of the expressed L-alanine uptake is due to the expression of a carrier ($b^{0,+}$-like, see above) for neutral and cationic amino acids that interacts with high concentrations of BCH. System $b^{0,+}$, as it has been defined in mouse blastocysts, shows no interaction with bicyclic amino acids (e.g. BCH) (Van Winkle *et al.*, 1988). This could be due to variations among $b^{0,+}$ systems in different tissues.

For completeness, it should be mentioned that the brush border membranes of small intestinal and renal proximal tubular epithelial cells contain at least one additional transport pathway which accepts both L-alanine and L-arginine. However, this NBB system (Neutral Brush Border) is Na^+-dependent (Stevens *et al.*, 1984; Silbernagl, 1988). For the observations reported in the present study, system NBB seems not to play a role, as Na^+-dependent transport activity for L-alanine and L-arginine in mRNA-injected oocytes was barely present, and if present it was only weak (results not shown).

We conclude that Na^+-independent transport system(s) for L-alanine and L-arginine in rabbit kidney cortex can be functionally expressed and detected in *Xenopus laevis* oocytes after injection of mRNA with an average chain length of 1.8–2.4 kb. The expressed transport activities show characteristics of a system with broad specificity for neutral and cationic amino acids (i.e. $b^{0,+}$-like). The expression of system y^+ in the mRNA-injected oocytes, as has been described in non-epithelial mammalian cells, cannot be excluded. These observations should allow the isolation and further characterization of cDNAs encoding one or several of these rabbit kidney cortex amino acid carriers. Obviously several questions will remain unanswered until successful expression/cloning of these transport activities is undertaken. Besides substrate specificity and the participation of specific transport pathways, the question of the cellular location within the renal cortical tissue is of major importance. The identification of the proximal tubular location, including brush border and/or basolateral expression of a specific transport activity (identified in such an expression/cloning approach), requires the availability of specific antibodies, since amino acid transport systems are almost ubiquitous.

This work was supported by the Swiss National Science Foundation (Grant 3.854.0.88 to H. M.). J. B. was a recipient of a fellowship from the Spanish Ministry of Science and Education and from the Juvenile Diabetes Foundation International (grant to A. Z.). M. P. was the recipient of a fellowship from CIRIT (Generalitat de Catalunya).

REFERENCES

Aoshima, H., Tomita, K. & Sugio, S. (1988) Arch. Biochem. Biophys. **265**, 73–81
Béliveau, R., Demeule, M., Jetté, M. & Potier, M. (1990) Biochem. J. **268**, 195–200
Campa, M. J. & Kilberg, M. S. (1989) J. Cell. Physiol. **141**, 645–652
Christensen, H. N. (1984) Biochim. Biophys. Acta **779**, 255–269
Christensen, H. N. (1990) Physiol. Rev. **70**, 43–77
Christensen, H. N. & Antonioli, J. A. (1969) J. Biol. Chem. **244**, 1497–1504
Coady, M. J., Pajor, A. M., Toloza, E. M. & Wright, E. M. (1990) Arch. Biochem. Biophys. **283**, 130–134
Collarini, E. J. & Oxender, D. L. (1987) Annu. Rev. Nutr. **7**, 75–90
Hediger, M. A., Coady, M. J., Ikeda, T. S. & Wright, E. M. (1987) Nature (London) **330**, 379–381
Jung, D., Schwarz, W. & Passow, H. (1984) J. Membr. Biol. **78**, 29–34
Kilberg, M. S. (1982) J. Membr. Biol. **69**, 1–12
Mircheff, A. K., van Os, C. H. & Wright, E. M. (1980) J. Membr. Biol. **52**, 83–92
Mircheff, A. K., Kippen, I., Hirayama, B. & Wright, E. M. (1982) J. Membr. Biol. **64**, 113–122
Palacín, M., Werner, A., Dittmer, J., Murer, H. & Biber, J. (1990) Biochem. J. **270**, 189–195
Saier, M. H., Daniels, G. A., Boerner, P. & Lin, J. (1988) J. Membr. Biol. **104**, 1–20
Scriver, C. R., Chesney, R. W. & McInnes, R. R. (1976) Kidney Int. **9**, 149–171
Silbernagl, S. (1988) Physiol. Rev. **68**, 911–1007
Stevens, B. R., Ross, H. J. & Wright, E. M. (1982) J. Membr. Biol. **66**, 213–225
Stevens, B. R., Kaunitz, J. D. & Wright, E. M. (1984) Annu. Rev. Physiol. **46**, 417–433
Tarnuzzer, R. W., Campa, M. J., Nan-Xin, Q., Englesberg, E. & Kilberg, M. S. (1990) J. Biol. Chem. **265**, 13914–13917
Tate, S. S., Urade, R., Getchell, T. V. & Udenfriend, S. (1989) Arch. Biochem. Biophys. **275**, 591–596
Van Winkle, L. J. (1988) Biochim. Biophys. Acta **947**, 173–208
Van Winkle, L. J., Campione, A. L. & Gorman, M. J. (1988) J. Biol. Chem. **263**, 3150–3163
Weissbach, L., Handlogten, M. E., Christensen, H. N. & Kilberg, M. S. (1982) J. Biol. Chem. **257**, 12006–12011
White, M. F. & Christensen, H. N. (1982) J. Biol. Chem. **257**, 4450–4457
White, M. F., Gazzola, G. C. & Christensen, H. N. (1982) J. Biol. Chem. **257**, 4443–4449
Wright, E. M. & Peerce, B. E. (1984) J. Biol. Chem. **259**, 14993–14996

Received 4 April 1991/16 August 1991; accepted 3 September 1991