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A study of L-leucine, L-phenylalanine and L-alanine transport in the perfused rat mammary gland: possible involvement of LAT1 and LAT2

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Abstract

The transport of L-leucine, L-phenylalanine and L-alanine by the perfused lactating rat mammary gland has been examined using a rapid, paired-tracer dilution technique. The clearances of all three amino acids by the mammary gland consisted of a rising phase followed by a rapid fall-off, respectively, reflecting influx and efflux of the radiotracers. The peak clearance of L-leucine was inhibited by BCH (65%) and D-leucine (58%) but not by L-proline. The inhibition of L-leucine clearance by BCH and D-leucine was not additive. L-leucine inhibited the peak clearance of radiolabelled L-leucine by 78%. BCH also inhibited the peak clearance of L-phenylalanine (66%) and L-alanine (33%) by the perfused mammary gland. Lactating rat mammary tissue was found to express both LAT1 and LAT2 mRNA. The results suggest that system L is situated in the basolateral aspect of the lactating rat mammary epithelium and thus probably plays a central role in neutral amino acid uptake from blood. The finding that L-alanine uptake by the gland was inhibited by BCH suggests that LAT2 may make a significant contribution to neutral amino acid uptake by the mammary epithelium.

Keywords: Mammary gland; L-leucine; L-phenylalanine; L-alanine

1. Introduction

The lactating mammary gland requires a considerable supply of amino acids to support protein synthesis. In this connection, a number of amino acid transport mechanisms have been identified in lactating mammary tissue. Na^+-dependent mechanisms which have been identified include system A [1–6], system X_{AG}^- [7–9] and system β [10,11]. Indeed, mRNA for two Na^+-dependent anionic amino acid transporters, namely GLAST and GLT-1, is expressed in the rat mammary gland [12]. Other Na^+-dependent systems which have been identified in lactating mammary tissue include two mechanisms which operate with a broad-substrate specificity, one of which may be system $B^{0,+}$ [5,13]. In relation to this, mRNA encoding a Na^+- and Cl^--dependent amino acid transport system with a broad-substrate specificity is expressed in the human mammary gland [14]. In addition, a Na^+-dependent system which accepts neutral amino acids (but not methylaminoisobutyric acid) as substrates has been identified in pig mammary tissue [15]. The lactating rat mammary gland also expresses Na^+-independent amino acid transport mechanisms. There is evidence for cationic amino acid transport in bovine, rat, mouse and porcine mammary tissue: systems resembling y^+ and y^+L have been described [16–19].

Mammary tissue also appears to express system L, thus, neutral amino acid transport via a Na^+-independent, BCH-sensitive pathway has been found in rat and mouse mammary tissue explants [1,4,5]. It is predicted that system L mediates the uptake of neutral amino acids across the blood-facing aspect of the mammary epithelium. To test this prediction, we have investigated the transport of neutral amino acids (L-leucine, L-phenylalanine and L-alanine) using the perfused lactating rat mammary gland in conjunction with the rapid, paired-tracer dilution technique. It is reasonable to assume that the perfused mammary gland gives a measure of transport across the basolateral pole of the mammary epithelium [20]. In particular, we have looked for the presence of BCH-sensitive neutral amino acid transport. In addition, we have tested for the presence of LAT1 and LAT2 mRNA in lactating rat mammary tissue. LAT1 and LAT2, induce system L-like activity when co-expressed with CD98 (formerly known as

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4F2hc). LAT1 transports neutral amino acids with branched or aromatic side chains in a manner not dependent upon Na^+ [21]. LAT2 also transports neutral amino acids in a fashion which does not require Na^+ but appears to have a wider substrate specificity than LAT1 [22–25].

2. Methods

2.1. Animals

Lactating Wistar rats 10 days postpartum which were suckling 8–10 pups were used in this study. The animals were maintained on a 12 h light–12 h dark cycle and allowed free access to water and chow.

2.2. Perfusion of the lactating rat mammary gland

The lactating rat mammary gland was perfused according to the method described in detail by Clegg and Calvert [26]. Anaesthesia was induced and maintained throughout the experiment by an intraperitoneal injection of sodium pentabarbitone (40 mg/kg of body weight). A skin incision was made from pubis to sternum. The left inguinal-abdominal mammary tissue was freed from the abdominal musculature and a ligature was tied around the vein communicating with the pectoral mammary tissue. In addition, ligatures were tied around the iliac branches of the iliolumbar artery and vein, the external pudendal arteries and veins and the femoral artery and vein. A second incision was made on the medial surface of the left thigh to expose the superficial epigastric artery and veins. Both blood vessels were cleared of fat and connective tissue and catheterized. The body temperature of the rat was maintained by the use of a lamp positioned above the animal. The mammary gland was perfused via the superficial epigastric blood vessels with a solution containing (in mM) 118 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose. and 1 amino(oxy)acetic acid. The perfusates also contained bovine serum albumin (5% w/v) and were gassed to pH 7.4 with 95% O_2/5% CO_2. Amino(oxy)acetic acid was included in the perfusates because it has been shown that amino acids are transaminated within mammary tissue (e.g. see Refs. 27,28). Indeed, experiments showed that the peak clearance of radiolabelled L-leucine was slightly enhanced by amino(oxy)acetic acid, suggesting that L-leucine can be rapidly metabolised in lactating rat mammary tissue (Fig. 1). At the end of the experiment, the perfused mammary tissue was removed from the animal, lightly blotted and weighed.

2.3. Measurement of L-leucine, L-phenylalanine and L-alanine transport by the perfused mammary gland

Leucine, phenylalanine and alanine transport by the perfused rat mammary gland was measured at 37 °C using [^{3}H]leucine, [^{3}H]phenylalanine and [^{3}H]alanine, respectively. The transport of the radiolabelled amino acids was measured by a rapid, paired-tracer dilution technique as described by Millar et al. [7]. Radiolabelled sucrose was used as an extracellular marker. A bolus containing the radiolabelled amino acid (21.4 μCi/ml) and [^{14}C]sucrose (7.1 μCi/ml) was injected close arterially (~ 60 μl in <2 s) and venous outflow samples were collected at 6-s intervals. The fractional uptake (U) for each collection period was calculated according to Eq. (1)

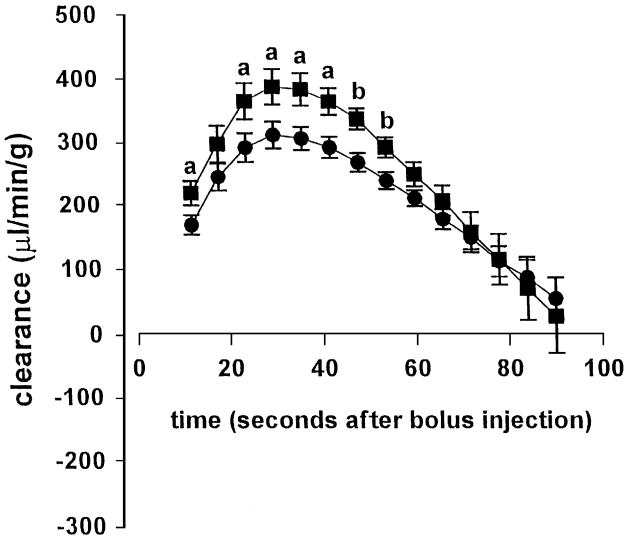

Fig. 1. The clearance of L-[^{3}H]leucine by the perfused lactating rat mammary gland in the absence (●) and presence (■) of amino(oxy)acetic acid. Mammary tissue was perfused with a medium containing (in mM) 118 NaCl, 4 KCl, 2 $CaCl_2$, 1 KH_2PO_4, 1 $MgSO_4$, 25 $NaHCO_3$ and 10 glucose. The perfusate also contained 5% BSA and was gassed to pH 7.4 with 95% O_2/5% CO_2. Amino(oxy)acetic acid was used at a concentration of 1 mM. Each point is the mean ± SE of 11 and 16 experiments, respectively, in the absence and presence of amino(oxy)acetic acid. $^aP<0.05$; $^bP<0.01$.

$$U = 1 - (R_t/R_i) \quad (1)$$

where R_t is the ratio of radiolabelled amino acid/radiolabelled sucrose in the venous outflow samples at time t and R_i is the ratio of radiolabelled amino acid/radiolabelled sucrose in the injectate. Radiolabelled amino acid clearance (i.e. the volume of perfusate notionally cleared of isotope) was calculated by multiplying the perfusate flow rate (μl/min per g tissue wet weight) by the uptake (U) for each collection period.

2.3.1. RNA isolation and RT-PCR

Total cellular RNA was isolated from lactating rat mammary gland using guanidinium thiocyanate and CsCl centrifugation [29]. RNA was resuspended in water and the concentration determined by the A260, where 1 A260 corresponds to 40 μg of RNA. Samples of RNA were treated with DNase I before RT-PCR to remove contaminating DNA. The primers used in the subsequent RT-PCR were as follows: CD98 [21], forward, 5′-AGCGCTCTCTGTTACACGGT-3′ and reverse, 5′-CTCAGGTTTTCCAGGCTCAG-3′; LAT1 [21], forward, 5′-CTGGCCATCATCATCTCCTT-3′ and re-

verse 5′-GGACGTGAACAGAGACCCAT-3′; LAT2 [22], forward 5′-GCTGGAAGAAGCCTGACATC-3′ and reverse, 5′-ATACAGGAACACCCGTCAGC-3′; GAPDH [30], forward, 5′-GGTGATGCTGGTGCTGAGTA-3′ and reverse, 5′-CCACAGTCTTCTGAGTGGCA-3′. The expected size of the PCR products were 256 bp for CD98, 213 bp for LAT1, 159 bp for LAT2 and 304 bp for GAPDH. One microgram of total RNA was reverse transcribed into cDNA using oligo(dT)$_{12-18}$ primer. The reverse transcription reaction, containing 500 μM dNTP, 25 μg ml^{-1} oligo(dT)$_{12-18}$ primer, 10 units μl^{-1} M-MLV reverse transcriptase, 3 mM $MgCl_2$, 75 mM KCl, 10 mM dithiothreitol and 50 mM Tris–HCl (pH 8.3) was sequentially incubated at 25 °C for 10 min, at 42 °C for 50 min and at 70 °C for 15 min and cooled on ice. To control for DNA contamination, reactions were run without RNA or with RNA in the absence of the reverse transcriptase (no amplified product was detected, results not shown). The synthesised cDNA (0.05 μg equivalent to RNA) was used for PCR amplification in a reaction mixture containing 200 μM dNTP, 1 μM forward and reverse primers, 0.05 units μl^{-1} Taq DNA polymerase, 1.5 mM $MgCl_2$, 50 mM KCl, and 20 mM Tris–HCl (pH 8.4). The PCR conditions were; 94 °C for 3 min, 60 °C for 1 min and 72 °C for 2 min; then 30 cycles (for CD98, LAT1 and LAT2) and 27 cycles (for GAPDH) at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min followed by a 10-min final extension at 72 °C. PCR products were separated on a 2% agarose gel which was subsequently stained with ethidium bromide.

2.4. Materials

L-[^{3}H]leucine (specific activity = 61 Ci/mmol), L-[^{3}H]phenylalanine (specific activity = 26 Ci/mmol), L-[^{3}H]alanine (specific activity = 47 Ci/mmol) and [^{14}C]sucrose (specific activity 56 mCi/mmol) were purchased from Amersham International PLC, Amersham, Bucks, UK. Moloney murine leukemia virus (M-MLV) reverse transcriptase, oligo(dT)$_{12-18}$ primer, deoxynucleotide 5′-triphosphate (dNTP) and Taq DNA polymerase were purchased from Gibco, Paisley, UK. Deoxyribonuclease 1 (DNase 1) was from Promega, Southampton, Hampshire, UK). All other chemicals including BCH were obtained from Sigma, Poole, Dorset, UK.

2.5. Statistics

Differences were assessed by Student's paired or unpaired *t*-test as appropriate and were considered significant when $P < 0.05$.

3. Results

3.1. L-leucine transport by the perfused rat mammary gland

Figs. 2 and 3 illustrate the clearance profile of radiolabelled L-leucine by the perfused mammary gland in the absence and presence of unlabelled L-leucine, BCH, D-leucine and L-proline. The unlabelled compounds, when required, were co-injected with the radiotracers. Unlabelled amino acids were added to the bolus to give a final concentration of 25 or 50 mM. Although this appears to be a high concentration to use, it must be borne in mind that the concentration of each unlabelled amino acid at the site of transport would have been less owing to the diluting effect of the perfusate. For example, Fig. 2b shows the dilution profile for L-leucine constructed from the percentage recovery of the extracellular marker. Under control conditions (i.e. no addi-

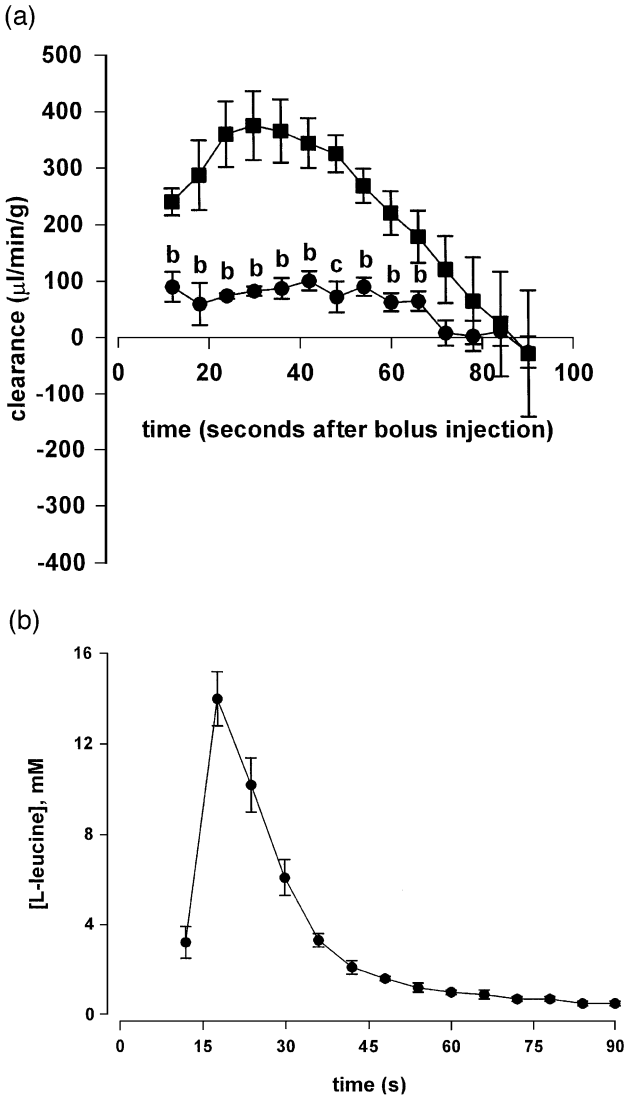

Fig. 2. (a) The clearance of L-[^{3}H]leucine by the perfused rat mammary gland in the absence (■) and presence (●) of unlabelled L-leucine. (b) The dilution profile of unlabelled L-leucine constructed from the percentage recovery of the extracellular marker. Mammary tissue was perfused with a buffer containing (in mM) 118 NaCl, 5 KCl, 2 $CaCl_2$, 1 KH_2PO_4, 1 $MgSO_4$, 25 $NaHCO_3$, 10 glucose and 1 amino(oxy)acetic acid. The perfusate also contained 5% BSA and was gassed to pH 7.4 with 95% O_2/5% CO_2. Unlabelled L-leucine, when required, was added to the bolus at a concentration of 50 mM. The results are the mean ± SE of five experiments. $^bP < 0.01$; $^cP < 0.001$.

tions), the clearance of radiolabelled leucine consisted of a rising phase followed by a rapid fall-off (Figs. 2 and 3). The peak clearance was reached approximately 30 s after the isotope was injected into the superficial epigastric artery and 18 s after the appearance of tracer in the eluent. It is evident from Fig. 2a that the inclusion of unlabelled L-leucine in the bolus markedly changed the clearance profile of radiolabelled L-leucine by the mammary gland: the peak clearance was reduced by 78% from 375 ± 61 to 82 ± 8.5 μl/min/g tissue wet weight at $t = 30$ s (± SE, $n = 5$, $P < 0.01$). Unlabelled D-leucine also reduced the clearance of radiolabelled leucine by the mammary gland but not to the same extent as unlabelled L-leucine (Fig. 3a). Thus, the peak clearance (at $t = 30$ s) was reduced by 58% from 374 ± 49 to 157 ± 22 μl/min/g tissue wet weight (± SE, $n = 5$, $P < 0.002$). Similarly, D-leucine at a concentration of 25 mM reduced the peak

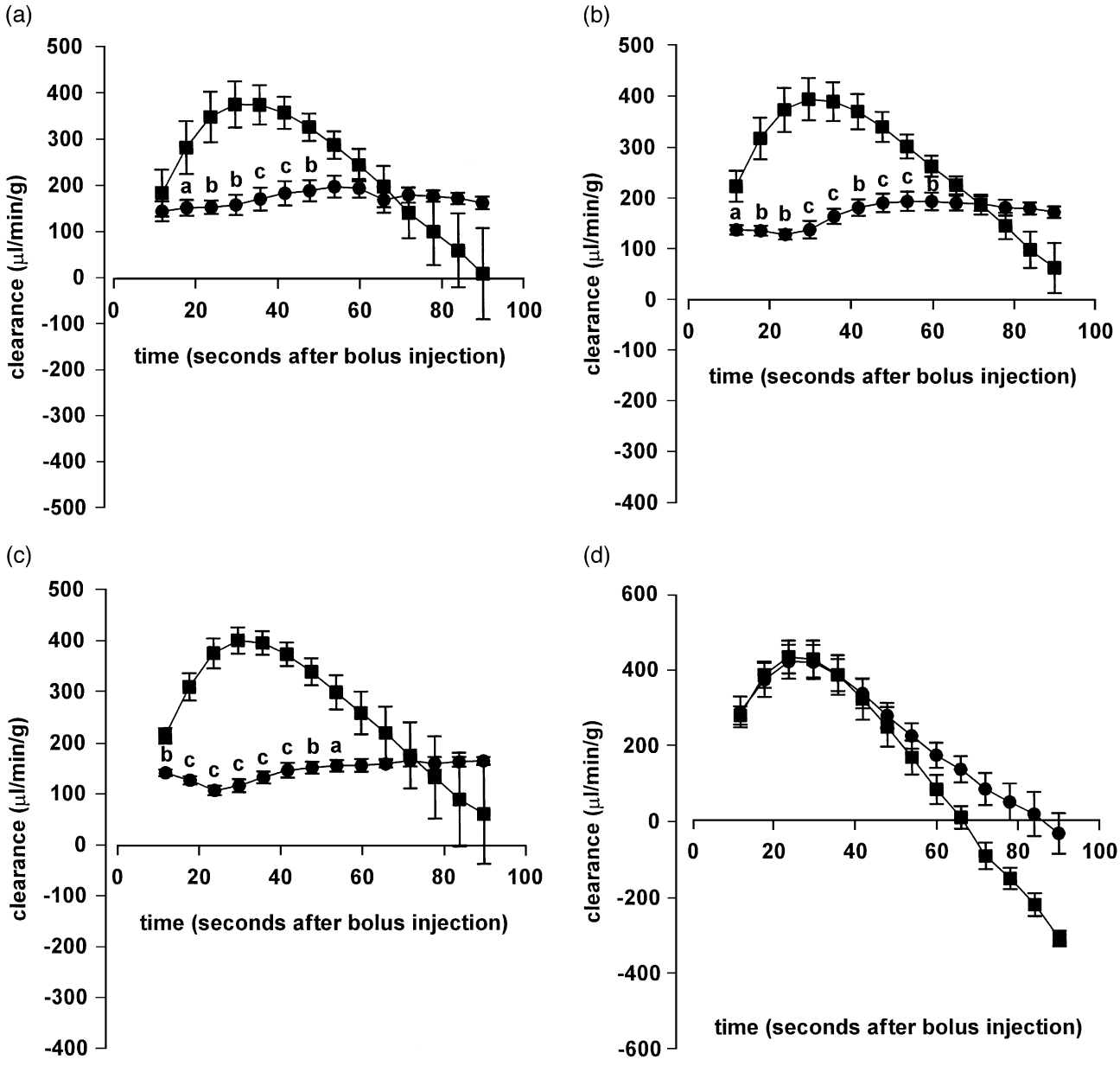

Fig. 3. (a) The clearance of radiolabelled L-leucine by the perfused rat mammary gland in the absence (■) and presence (●) of unlabelled D-leucine. The results are the mean ± SE of five experiments. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. (b) The clearance of radiolabelled L-leucine by the perfused rat mammary gland in the absence (■) and presence (●) of unlabelled BCH. The results are the mean ± SE of five experiments. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. (c) The clearance of radiolabelled L-leucine by the perfused rat mammary gland in the absence (■) and presence (●) of unlabelled D-leucine and BCH. The results are the mean ± SE of five experiments. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. (d) The clearance of radiolabelled L-leucine by the perfused rat mammary gland in the absence (■) and presence (●) of unlabelled L-proline. The results are the mean ± SE of three experiments. The composition of the perfusate was the same as that described in Fig. 2. When required, the unlabelled amino acids were added to the bolus at a concentration of 50 mM (D-leucine, BCH) or 25 mM (L-proline).

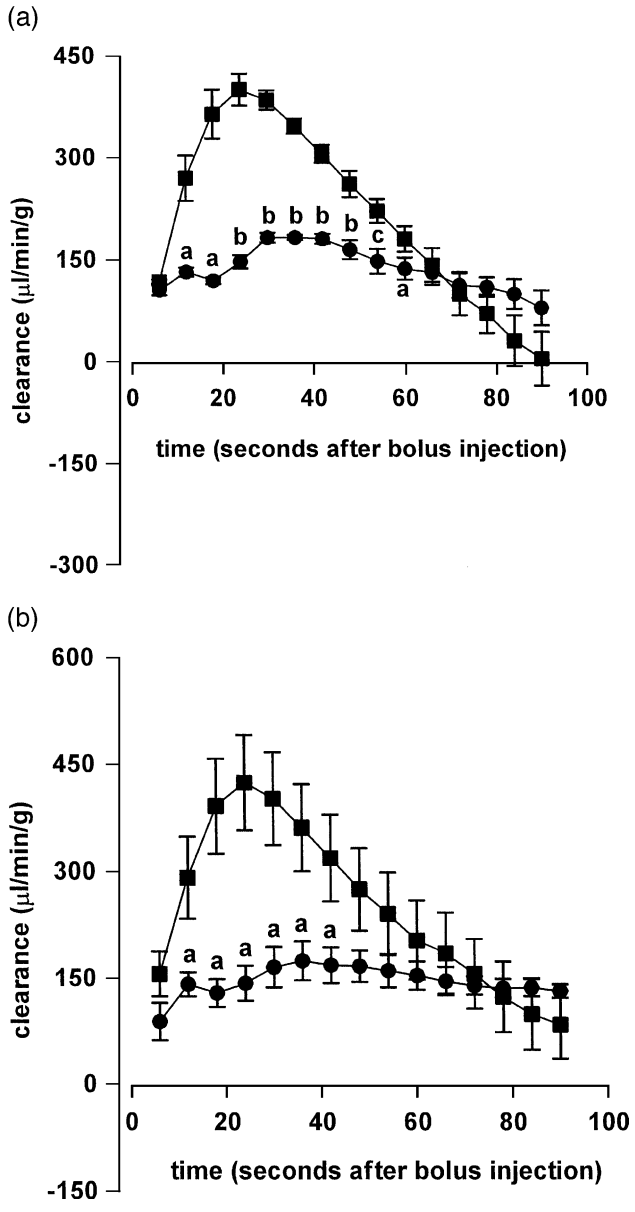

Fig. 4. (a) The clearance of radiolabelled L-phenylalanine by the perfused rat mammary gland in the absence (■) and presence (●) of unlabelled L-leucine. The results are the mean ± SE of three experiments. [a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$. (b) The clearance of radiolabelled L-phenylalanine by the perfused rat mammary gland in the absence (■) and presence (●) of unlabelled BCH. The results are the mean ± SE of four experiments. [a]$P<0.05$. The composition of the perfusate was the same as that described in Fig. 2. When required, L-leucine and BCH were added to the bolus at a concentration of 50 mM.

clearance to the same extent as 50 mM D-leucine (results not shown). The clearance profile of radiolabelled L-leucine by the perfused mammary gland was also altered by the presence of BCH (Fig. 3b). The peak clearance was reduced by 65% from 394 ± 41 to 137 ± 17 µl/min/g tissue wet weight (± SE, $n=5$, $P<0.001$). Fig. 3c shows the effect of adding BCH and D-leucine together to the bolus (each at a concentration of 50 mM) on the clearance of radiolabelled L-leucine. The peak clearance was reduced by 71% from 399 ± 26 to 114 ± 12 µl/min/g tissue wet weight (± SE, $n=5$, $P<0.001$). It is evident, therefore, that the effects of BCH and D-leucine were not additive. Proline (25 mM) had no significant effect on the peak clearance of radiolabelled leucine by the perfused mammary gland (Fig. 3d). However, it appears that L-proline increased the clearance of radiolabelled L-leucine in the latter part of the time course. At present, we have no clear explanation for this observation except for the possibility that L-proline is affecting L-leucine efflux from the gland.

3.2. L-phenylalanine transport by the perfused rat mammary gland

The effect of unlabelled L-leucine and BCH on the clearance of radiolabelled phenylalanine by the perfused gland was examined (Fig. 4). The unlabelled amino acids were added to the injectate at a final concentration of 50 mM. Under control conditions (i.e. no additions), the clearance of radiolabelled phenylalanine rose initially to reach a peak 24 s following the injection of the bolus into the artery and declined thereafter. Unlabelled L-leucine reduced the peak clearance of [^{3}H]phenylalanine by 63% from 400 ± 23 to 147 ± 9 µl/min/g tissue wet weight (± SE, $n=3$, $P<0.01$) at 24 s (Fig. 4a). The peak clearance of radiolabelled L-phenylalanine by the perfused mammary gland was inhibited to a similar extent by BCH (Fig. 4b). Thus, the peak clearance was reduced by 66% from 424 ± 66 to 142 ± 24 µl/min/g tissue wet weight (± SE, $n=4$, $P<0.02$) at $t=24$ s.

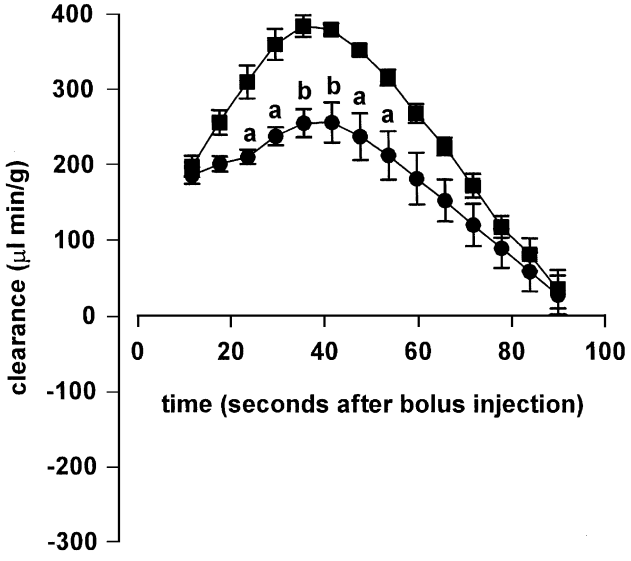

Fig. 5. The clearance of L-[^{3}H]alanine by the perfused rat mammary gland in the absence (■) and presence (●) of unlabelled BCH. The composition of the perfusate was the same as that described in Fig. 2. When required, BCH was added to the bolus at a concentration of 50 mM. The results are the mean ± SE of five experiments. [a]$P<0.05$; [b]$P<0.01$.

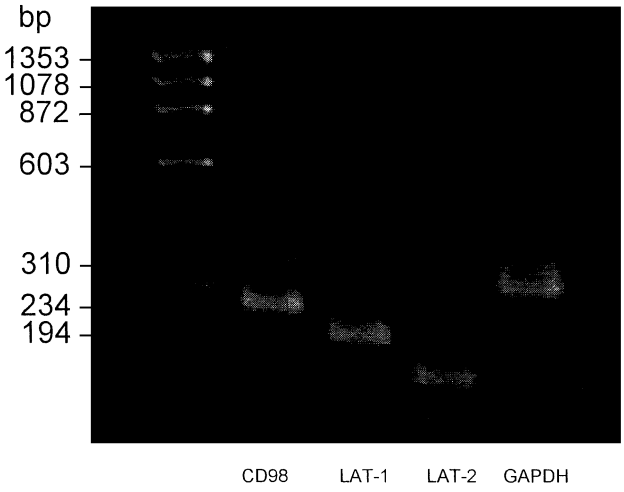

Fig. 6. The detection of CD98, LAT1, LAT2 and GAPDH mRNA in lactating rat mammary tissue using RT-PCR.

3.3. L-alanine transport by the perfused rat mammary gland

Fig. 5 illustrates the clearance of L-alanine by the perfused rat mammary gland in the absence and presence of unlabelled BCH (added to the bolus at 50 mM). L-alanine clearance under control conditions, like that of L-leucine and L-phenylalanine clearance, consisted of a rising phase followed by a rapid fall-off. The peak clearance was attained 36 s after radiolabelled L-alanine was administered into the superficial epigastric artery. It is clear from Fig. 5 that BCH inhibited L-alanine clearance by the rat mammary gland: the peak clearance was reduced by 33% from 384 ± 14 to 255 ± 18 μl/min/g tissue wet weight ($\pm$ SE, $n = 5$, $P < 0.01$).

3.4. RT-PCR analysis of LAT1, LAT2 and CD98 mRNA in lactating mammary tissue

To check for the presence of LAT1, LAT2 and CD98 mRNA in mammary tissue, we subjected total RNA isolated from lactating rat mammary tissue to RT-PCR. Products of the expected size for LAT1, LAT2 and CD98 were amplified from rat mammary tissue (Fig. 6).

4. Discussion

The present study has shown that the perfused rat mammary gland transports L-leucine, L-phenylalanine and L-alanine. Each of the amino acids had a similar clearance profile, i.e. a rising phase followed by a rapid fall-off. The rising phase is believed to be governed by the unidirectional uptake of the radiolabelled amino acids, whereas the fall-off occurs because the tracers exit the cells and return to the circulation (see Ref. 31). In addition, the finding that the clearance profile had an initial rising phase suggests that there is an arterio-venous shunt pathway(s) in the lactating rat mammary gland. It is notable that the time taken to reach the peak clearance differed for each amino acid. Thus, the time taken to reach the peak clearance following the bolus injection for L-phenylalanine, L-leucine and L-alanine was 24, 30 and 36 s, respectively.

4.1. Inhibition of amino acid clearances

The inhibition of radiolabelled amino acid transport by unlabelled amino acids was examined by adding the latter to the bolus at a concentration of 50 mM. The uptake of L-leucine, L-phenylalanine and L-alanine was inhibited by BCH. In this connection, it has been shown that BCH inhibits the transport of Na^+-independent α-aminoisobutyrate uptake in rat mammary tissue explants [4]. The present results appear to confirm the presence of system L in rat mammary tissue. Moreover, the present study places the transporter in the basolateral aspect of the lactating rat mammary epithelium given that the perfused mammary gland preparation allows transport to be measured across the blood-facing aspect of the epithelium. However, the presence of system L in the apical membrane as well as the basolateral membrane of mammary secretory cells cannot, at this stage, be ruled out. We are aware, however, that a portion of the BCH-sensitive amino acid uptake by the perfused gland could be via system $B^{0,+}$. This is based on the finding that system $B^{0,+}$ is inhibited by BCH [32]. In this connection, mRNA encoding a Na^+-dependent amino acid transport system which is inhibited by BCH and thus resembles system $B^{0,+}$ has been detected in the human (presumably non-lactating) mammary gland. However, the finding that L-lysine uptake by lactating rat mammary tissue is not dependent upon Na^+ [17] suggests that system $B^{0,+}$ may only play a relatively minor role in amino acid uptake across the basolateral pole of the lactating rat mammary epithelium. Moreover, system $B^{0,+}$ expressed in the apical membrane of cultured human bronchial epithelial cells appears to accept proline as a substrate [33]. In this study, we found no evidence for an interaction between L-leucine uptake and proline.

It appears that the system which accepts L-leucine as a substrate in the perfused rat mammary gland is not particularly stereospecific. This notion is based on the finding that the BCH-sensitive portion of L-leucine transport by the perfused mammary gland was also inhibited by D-leucine (the effects of D-leucine and BCH were not additive). This observation is in accordance with the earlier finding that BCH-sensitive α-aminoisobutyrate uptake by rat mammary tissue explants can be inhibited by D-leucine [4]. It is notable that neither BCH nor D-leucine inhibited the clearance of radiolabelled L-leucine to the same extent as unlabelled L-leucine. This suggests that L-leucine can also enter mammary cells via a pathway other than system L. The precise identity of this pathway(s) remains to be identified. Similarly, L-alanine and L-phenylalanine uptake across the basolateral aspect of the mammary gland appears to be via more than one pathway given that BCH did not completely inhibit L-alanine

and L-phenylalanine clearance. Uptake via system A could of course account for part of the BCH-insensitive component of L-alanine uptake by the rat mammary gland [4,6].

4.2. Molecular identity of system L in mammary tissue

System L has recently been characterized at the molecular level. Two heterodimeric proteins consisting of a light chain (LAT1 or LAT2) and a heavy chain (CD98) exhibit system L-like activity. The light and heavy chains of the protein are joined by a disulfide bond [34]. LAT1 and LAT2 transport neutral amino acids, however, LAT2 has a much wider substrate specificity than LAT1.We now show, using RT-PCR, that lactating rat mammary tissue expresses mRNA for both LAT1 and LAT2 (and CD98). One major distinguishing feature between LAT1 and LAT2 is that LAT2 transports L-alanine, whereas LAT1 does not [24,25]. On this basis, BCH-sensitive amino acid transport across the basolateral aspect of the mammary epithelium appears to resemble the properties of LAT2. In this connection, Sharma and Kansal [5] have recently shown that Na^+-independent L-alanine uptake by mouse mammary explants is inhibited by BCH. In addition, Na^+-independent AIB uptake by rat mammary tissue explants, which is attributed to system L, is inhibited by alanine [4]. Both of these observations appear to support a functional role for LAT2 in the basolateral aspect of the lactating mammary epithelium. On the other hand, the finding that BCH-sensitive L-leucine uptake by the perfused rat mammary gland can also be inhibited by D-leucine reflects the properties of LAT1 rather than LAT2: D-isomers readily interact with LAT1 but not LAT2 [25]. However, Segawa et al. [22] have shown that L-leucine transport via LAT2 is inhibited by certain D-isomers (i.e. D-serine, D-alanine, D-threonine, D-cysteine). There is the possibility, however, that amino acid uptake into lactating mammary tissue is also facilitated by an L-type transporter distinct from LAT1 and LAT2.

In conclusion, the present study confirms the presence of system L in the lactating rat mammary gland and places it in the blood-facing aspect of the lactating rat mammary epithelium. This transport system may be one of the most important pathways for the uptake of essential amino acids by the mammary gland. The data also provide an insight into the molecular nature of system L in the mammary gland.

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