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Peptide aminonitrogen transport by the lactating rat mammary gland

D.B. Shennan [a,*], D.T. Calvert [a], F.R.C. Backwell [b], C.A.R. Boyd [c]

[a] *Hannah Research Institute, Ayr KA6 5HL, UK*
[b] *Rowett Research Institute, Aberdeen AB21 9SB, UK*
[c] *Department of Human Anatomy, South Parks Road, Oxford OX1 3 QX, UK*

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Abstract

Recent studies have shown that the lactating mammary gland is able to utilize plasma-derived dipeptides for milk protein synthesis. However, it was not clear whether the peptides were hydrolysed followed by uptake of the constituent amino acids or were taken up intact. In view of this, we have designed experiments to investigate (a) whether the lactating rat mammary gland is capable of transporting hydrolysis-resistant dipeptides and (b) whether or not mammary cells are able to hydrolyse peptides, including glutathione, extracellularly. The uptake of the hydrolysis-resistant dipeptides D-[^{3}H]Phe-L-Gln and D-[^{3}H]Phe-L-Glu by the perfused rat mammary gland was low. Concomitant addition of L-Leu-L-Ala (50 mM) had no effect on the clearance of either labelled dipeptide suggesting that the small, albeit significant, uptake of the dipeptides is not via a high affinity peptide transporter (PepT1/PepT2). All anionic dipeptides tested (L-Glu-L-Ala, L-Asp-L-Ala, L-Ala-L-Asp, L-Asp-Gly, Gly-L-Asp and Gly-L-Glu) with the exception of D-Phe-L-Glu were able to *trans*-accelerate the efflux of labelled D-aspartate from preloaded rat mammary tissue (explants and perfused mammary gland). It appears that these peptides were being hydrolysed extracellularly followed by the uptake of free anionic amino acids via the mammary tissue high affinity, Na^+-dependent anionic amino acid carrier operating in the exchange mode. Glutathione was able to *trans*-accelerate D-aspartate efflux from lactating rat mammary tissue in a fashion which was sensitive to the peptidase inhibitor acivicin. This suggests that γ-glutamyltranspeptidase hydrolyses glutathione to produce L-glutamate which is subsequently transported via the high-affinity anionic amino acid carrier. Hydrolysis of peptides followed by uptake of the constituent amino acids may provide an important source of amino acids for milk protein synthesis.

Keywords: Mammary gland; Peptide; Transport

1. Introduction

Free amino acids are the major form in which amino-N is taken up by lactating mammary tissue. Accordingly, transport systems for neutral [1–4], anionic [5,6] and cationic amino acids [7,8] have been identified in mammary tissue from a variety of species. A number of studies, however, have shown that the uptake of some essential amino acids, i.e. those which cannot be synthesized within the gland, is insufficient to account for their output in milk protein [9,10]. In the goat, for example, it is apparent that the uptake of L-phenylalanine and L-histidine falls short of the gland's requirements for milk protein production [11] and it has been suggested that the utilization of circulating peptides, as a source of amino acids for mammary protein synthesis, may account for the shortfall [12]. It appears

* Corresponding author. Fax: +44 (1292) 674003; E-mail: shennand@main.hri.sari.ac.uk

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that the mouse mammary gland is also capable of utilizing peptides as peptide-bound methionine can be used as a source of methionine for the synthesis of secreted proteins [13]. However, the mechanism by which the mammary gland utilizes peptides is not clear but may involve (a) extracellular hydrolysis followed by uptake of the constituent free amino acids, (b) intact peptide transport followed by intracellular hydrolysis to free amino acids, or (c) a combination of both hydrolysis and transport of peptides.

Using the rat as a model, the present study was designed to examine the mechanism by which the lactating mammary gland utilizes peptides for milk protein synthesis. The first step in the investigation was to measure the transport of hydrolysis-resistant peptides by the lactating rat mammary gland. Thus, we have examined the transport of dipeptides which have a D-isomer amino acid at the N terminus: this configuration confers resistance to hydrolysis [14]. Secondly, we have sought evidence for the extracellular hydrolysis of peptides followed by uptake of the free amino acids. To do this we have exploited the characteristics of a high-affinity, Na^+-dependent anionic amino acid transporter which has been shown to be present in the basolateral aspect of the rat mammary epithelium [5,6]. It is apparent that the mammary tissue high affinity anionic amino acid transporter, in common with that expressed in neuronal cells [15], is capable of operating as an exchanger as well as a cotransport system [6]; L-glutamate, L-aspartate and D-aspartate are able to *trans*-accelerate D-aspartate efflux (a poorly metabolized substrate of the transporter) from rat mammary tissue. We have examined the effect of anionic dipeptides on the efflux of D-aspartate from mammary tissue. The rationale is that if mammary tissue is able to hydrolyse the peptides extracellularly (but not if the peptide remains intact) then the liberated anionic amino acid should subsequently *trans*-stimulate D-aspartate efflux from mammary tissue via the high-affinity anionic amino acid carrier.

2. Methods and materials

2.1. Animals

Lactating Wistar rats suckling between eight and ten pups and between 9 and 15 days of lactation were used in this study. The rats were fed on standard rat chow (Special Diet Services, Cambridge) and allowed free access to water. The animals were housed at constant temperature of 17°C and under conditions of 12 h light-dark cycles.

2.2. Measurement of dipeptide and amino acid uptake by the perfused mammary gland

Mammary tissue was perfused according to the method described in detail by Clegg and Calvert [16]. Anaesthesia was induced and maintained throughout the experiment by an intraperitoneal injection of sodium pentobarbitone (40 mg/kg of body weight). Abdominal/inguinal mammary tissue was perfused via the superficial epigastric vessels. The perfusates contained (mM) 118 NaCl, 4 KCl, 2 $CaCl_2$, 1 NaH_2PO_4, 1 $MgSO_4$, 25 $NaHCO_3$ and 10 glucose and were gassed to pH 7.4 with 95% O_2/5% CO_2. In addition, all perfusates were supplemented with bovine serum albumin (5%).

The transport of dipeptides (D-[^{3}H]Phe-L-Glu and D-[^{3}H]Phe-L-Gln) and amino acids (L-[^{3}H]Glu and L-[^{3}H]Gln) by the perfused rat mammary gland was measured using the rapid, paired-tracer dilution technique originally described by Yudilevich et al. [17] as modified by Millar et al. [5]. A mixture containing a radiolabelled dipeptide or amino acid and [^{14}C]sucrose was injected close arterially (approx. 60 μl in <2 s) and 20 venous outflow samples were collected at 6 s intervals. Venous outflow samples (200 μl) were mixed with 200 μl of trichloroacetic acid and centrifuged at $13\,000\times g$ for 2 min. Two hundred microlitres of the resultant supernatants were prepared for counting by adding 7 ml of Ultimagold liquid scintillation cocktail. The fractional uptake (U) of the dipeptides and amino acids for each collection period was calculated according to Eq. 1:

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

where R_t is the ratio of radiolabelled dipeptide (or amino acid)/radiolabelled sucrose in the venous outflow samples at time t and R_i is the ratio of radiolabelled dipeptide (or amino acid)/radiolabelled sucrose in the injectate. The clearance (i.e. the volume of perfusate notionally cleared of radiolabelled di-

peptide or amino acid) was calculated by multiplying the perfusate flow rate (μl/min/g tissue) by the uptake for each collection period.

2.3. Measurement of D-aspartate efflux from the perfused mammary gland

Mammary tissue was perfused as described above. The perfused mammary tissue was loaded with radiolabelled D-aspartate by perfusing with a buffer containing (mM) 118 NaCl, 4 KCl, 2 $CaCl_2$, 1 NaH_2PO_4, 1 $MgSO_4$, 25 $NaHCO_3$ and 10 glucose plus 0.2–0.4 μCi/ml of D-[^{3}H]aspartate for approx. 20 min. All perfusates were supplemented with bovine serum albumin (5% w/v) and gassed to pH 7.4 with 95% O_2/5% CO_2. Following the loading period, the mammary gland was perfused with solutions free of radioisotopes (see figure legends for details of composition) and venous outflow samples were collected at 2 min intervals. At the end of the perfusion period the gland was removed and homogenized. Samples of the homogenate were incubated in distilled water for at least 16 h in order to leach out the isotope. The amount of radioactivity associated with the homogenates was used to calculate the amount of isotope remaining in the tissue at the end of the perfusion. Venous outflow and homogenate samples (0.75 ml) were mixed with 0.75 ml of 10% trichloroacetic acid and centrifuged at 13 000 × g for 3 min. One millilitre of the resulting supernatants was prepared for counting by adding 14 ml of Ultimagold liquid scintillation cocktail. The fractional efflux of radiolabelled D-aspartate efflux from the perfused mammary gland was calculated according to Eq. 2:

$$\text{Fractional efflux } (\text{min}^{-1}) = \Delta X / \Delta t X_t \quad (2)$$

where ΔX represents the amount of radiolabelled D-aspartate released from the tissue in the time interval Δt and X_t is the tissue D-[^{3}H]aspartate content at time t. The radioactivity associated with the tissue at any point of the time course was obtained by summing the counts left in the tissue at the end of the efflux time course with that of the 2 min samples in reverse order.

2.4. Preparation of mammary tissue explants

Mammary tissue explants were prepared by the method described by Shennan [18]. Rats were killed by cervical dislocation and the abdominal mammary glands were immediately removed and placed in an ice-cold buffer containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4. The mammary tissue was finely dissected to produce explants weighing between 4 and 8 mg. Care was taken to remove as much connective tissue as possible during the preparation of the explants. The tissue explants were repeatedly washed with buffer prior to the start of the efflux experiments.

2.5. Measurement of D-aspartate efflux from mammary tissue explants

D-Aspartate efflux, using D-[^{3}H]aspartate as tracer, was assayed at 37°C according to the method described by Shennan [18]. Mammary tissue explants were loaded with radiolabelled D-aspartate by incubation at 20°C for 60 min in a buffer containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4 plus 3–5 μCi/ml D-[^{3}H]aspartate. Following the loading incubation, the tissue explants were transferred through a series of tubes each containing 2 ml of radioactive-free solutions (see figure legends for precise details of composition) at 2 min intervals. The radioactivity in these samples was measured. At the end of the efflux incubation, the tissue explant was placed in 4 ml of distilled water (for at least 16 h) in order to leach out the remaining isotope. The fractional efflux for each collection period was calculated according to Eq. 2.

2.6. Measurement of taurine efflux from mammary tissue explants

Isolated rat mammary tissue explants, prepared as described above, were loaded with [^{3}H]taurine by incubating for 60 min at 20°C in a medium containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4 to which was added 1–3 μCi ml^{-1} of radiolabelled taurine. The efflux of radiolabelled taurine was assayed at 37°C as described for D-aspartate.

2.7. Materials

D-[^{3}H]Aspartate and [^{3}H]taurine were purchased from Amersham International (UK). D-[^{3}H]Phe-L-Glu and D-[^{3}H]Phe-L-Gln were customer synthesized by Zeneca (Cambridge Research Biochemicals, Northwich Cheshire, UK). All other chemicals, including the peptides, were obtained from Sigma (UK).

2.8. Statistics

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

3. Results

3.1. The clearance of D-[^{3}H]Phe-L-Glu and L-[^{3}H]Glu by the perfused rat mammary gland

Fig. 1 shows the clearance of D-[^{3}H]Phe-L-Glu by the perfused lactating rat mammary gland measured using a rapid, paired-tracer dilution technique. For comparison we also measured the clearance of L-[^{3}H]Glu. In this set of experiments (and those described below for D-[^{3}H]Phe-L-Gln and L-[^{3}H]Gln) the substrates were present only in the radiolabelled form: this was to ensure maximal extraction of the radiotracers by the perfused tissue. It is evident that the clearance of D-[^{3}H]Phe-L-Glu was markedly lower than that of L-[^{3}H]Glu. The magnitude and profile of L-[^{3}H]Glu clearance, which is via a high affinity anionic amino acid transport system, is similar to that reported by Millar et al. [5]. To test whether or not the small clearance of D-[^{3}H]Phe-L-Glu was via a high affinity peptide transport system we examined the effect of adding excess unlabelled L-Leu-L-Ala to the injectate (at 50 mM). It is evident from Fig. 1 that L-Leu-L-Ala had no effect on the clearance of D-[^{3}H]Phe-L-Glu by the perfused rat mammary epithelium.

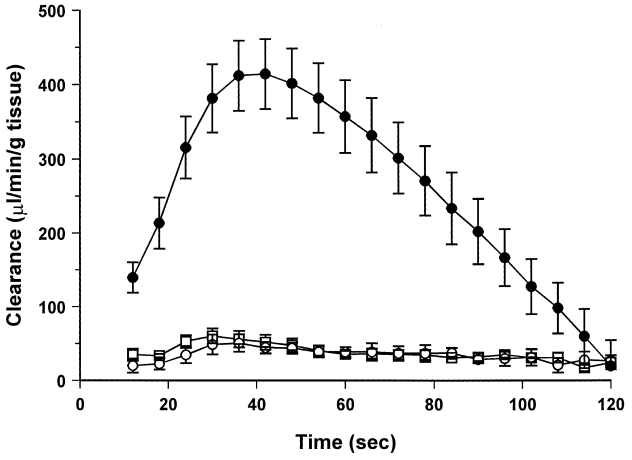

Fig. 1. The clearance of D-[^{3}H]Phe-L-Glu and L-[^{3}H]Glu by the perfused lactating rat mammary gland. The perfusates contained (mM) 118 NaCl, 4 KCl, 2 $CaCl_2$, 1 NaH_2PO_4, 1 $MgSO_4$, 10 glucose and 25 $NaHCO_3$ and were gassed to pH 7.4 with 95% O_2/5% CO_2. The injectates were of similar composition except that they contained 16.79–33.58 μCi/ml of D-[^{3}H]Phe-L-Glu (final concentration 1.66–3.32 μM) or 7.14 μCi/ml of L-[^{3}H]Glu (final concentration approx. 0.16 μM). When required, L-Leu-L-Ala was added to the injectate to give a final concentration of 50 mM. □ and ○ respectively represent D-[^{3}H]Phe-L-Glu uptake without any additions (i.e. control) and with L-Leu-L-Ala present ($n = 3 \pm$ S.E. for each condition). L-[^{3}H]Glu uptake (●) is the mean ± S.E. of six experiments.

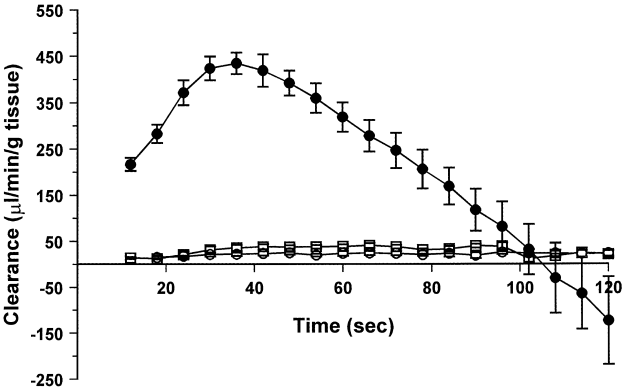

Fig. 2. The clearance of D-[^{3}H]Phe-L-Gln and L-[^{3}H]Gln by the perfused lactating rat mammary gland. The experimental conditions were essentially the same as those described in the legend of Fig. 1 except that the injectates contained 8.14 μCi/ml of D-[^{3}H]Phe-L-Gln (final concentration 0.81 μM) or 7.14 μCi/ml L-[^{3}H]Gln. When required L-Leu-L-Ala was added to the injectate to give a final concentration of 50 mM. □ and ○ respectively represent D-[^{3}H]Phe-L-Gln uptake without any additions (i.e. control), with 50 mM L-Leu-L-Ala present ($n = 4 \pm$ S.E. for each condition). L-[^{3}H]Gln uptake (●) is the mean ± S.E. of five experiments.

3.2. The clearance of D-[^{3}H]Phe-L-Gln and L-[^{3}H]Gln by the perfused mammary gland

The clearance of D-[^{3}H]Phe-L-Gln, like that of D-[^{3}H]Phe-L-Glu, by the perfused mammary gland was

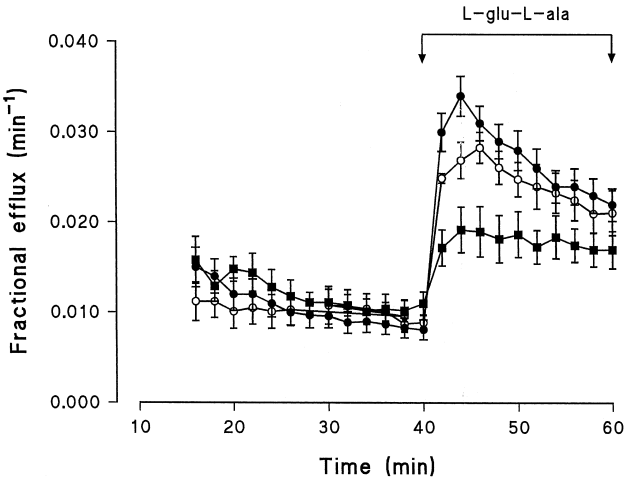

Fig. 3. The dose dependence of L-Glu-L-Ala stimulation of D-aspartate efflux from rat mammary tissue explants. D-Aspartate efflux was first measured (for 40 min) into a medium containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4 followed by one of similar composition (for 20 min) except that it contained 50 μM (■), 200 μM (○) or 500 μM (●) L-Glu-L-Ala. The results shown are the means ± S.E.M. of five experiments when L-Glu-L-Ala was used at 50 μM and 100 μM and seven experiments when L-Glu-L-Ala was used at 500 μM.

low (Fig. 2). It is evident from Fig. 2 that the clearance of D-[^{3}H]Phe-L-Gln was markedly lower than that of L-[^{3}H]Gln. Unlabelled L-Leu-L-Ala added to the injectate at a concentration of 50 mM had no significant effect on the clearance of D-[^{3}H]Phe-L-Gln (Fig. 2).

3.3. The effect of dipeptides on D-aspartate efflux from mammary tissue explants

Previous work has shown that the time course of D-aspartate efflux from mammary tissue explants consists of two components [6]. The fast component which represents D-aspartate efflux from the tissue extracellular space became insignificant by 20 min. Consequently, D-aspartate efflux was measured for 40 min before the effect of external dipeptides were tested. The dipeptides we tested were L-Glu-L-Ala, L-Ala-L-Asp, L-Asp-Gly, Gly-L-Asp, L-Asp-L-Ala, Gly-L-Glu, D-Phe-L-Glu and L-Leu-L-Ala. All of the peptides, with the exception of L-Leu-L-Ala, contained either a glutamate or an aspartate residue. Table 1 shows the effect of these peptides, added to the incubation medium to give a final concentration of 500 μM, on the fractional efflux of D-aspartate. Shown for comparison is the effect of free L-glutamate and L-aspartate (both tested at 500 μM) on D-aspartate efflux. It is evident that L-Glu-L-Ala was the most effective dipeptide tested. Indeed, L-Glu-L-Ala was 75% as effective as free L-glutamate in *trans*-acceler-

Table 1
The effect of dipeptides on the efflux of D-aspartate from lactating rat mammary tissue explants

Dipeptide	*n*	Fractional efflux (min^{-1})		% increase	*t*	*P*
		−Peptide	+Peptide			
L-Glu-L-Ala	7	0.0082 ± 0.0011	0.0336 ± 0.0022	310	4	< 0.001
L-Ala-L-Asp	6	0.0095 ± 0.0019	0.0333 ± 0.0054	251	6	< 0.01
L-Asp-L-Ala	9	0.0065 ± 0.0009	0.0240 ± 0.0036	269	4	< 0.001
L-Asp-Gly	6	0.0060 ± 0.0011	0.0214 ± 0.0030	257	6	< 0.01
Gly-L-Asp	8	0.0070 ± 0.0008	0.0101 ± 0.0012	44	8	< 0.05
Gly-L-Glu	3	0.0062 ± 0.0020	0.0115 ± 0.0026	86	4	< 0.02
L-Leu-L-Ala	4	0.0101 ± 0.0022	0.0095 ± 0.0018	–	–	–
D-Phe-L-Glu	3	0.0080 ± 0.0004	0.0082 ± 0.0006	–	–	–
L-Glu	4	0.0070 ± 0.0009	0.0351 ± 0.0013	401	4	< 0.001
L-Asp	4	0.0086 ± 0.0008	0.0415 ± 0.0032	383	4	< 0.01

D-Aspartate efflux was first measured (for 40 min) into a buffer containing (mM) 135 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and Tris-MOPS, pH 7.4 followed by one of similar composition (for 20 min) except that it contained a dipeptide or an anionic amino acid (at 500 μM). The fractional effluxes (± S.E.M.) shown are those just prior to the addition of the peptide (−peptide) and those at the peak response (+peptide). '*t*' represents the time (min) taken to reach the peak response following the addition of the peptide or amino acid and '*n*' is the number of experiments performed with each peptide or amino acid. The L-Asp data are taken from Miller et al. [6].

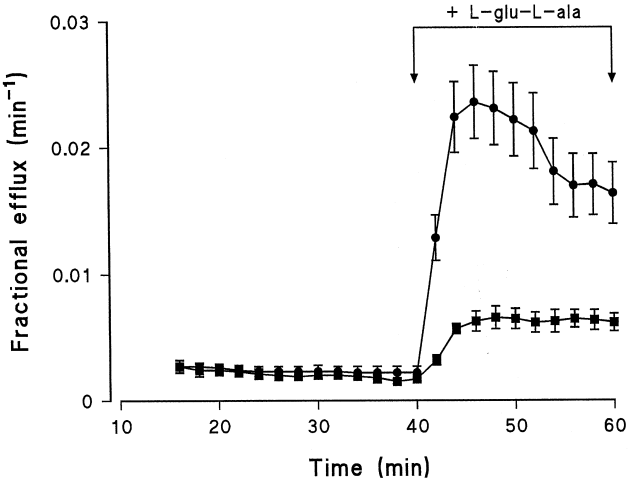

Fig. 4. The effect of L-Glu-L-Ala on the fractional release of D-aspartate from the perfused lactating rat mammary gland. The gland was initially perfused with a buffer containing (mM) 118 NaCl, 4 KCl, 2 $CaCl_2$, 1 KH_2PO_4, 1 $MgSO_4$, 125 $NaHCO_3$, and 10 glucose (supplemented with BSA (5%) and gassed to pH 7.4 with 95% O_2/5% CO_2) followed by one of similar composition except that it contained 100 μM (■) or 500 μM (●) L-Glu-L-Ala. The results shown are means of five and three experiments respectively when 500 μM and 100 μM L-Glu-L-Ala was used. Error bars are shown by the vertical lines unless they fall within the size of the symbols.

ating D-aspartate efflux with respect to both the size and latency of stimulation. Fig. 3 shows that L-Glu-L-Ala stimulated D-aspartate efflux from rat mammary tissue explants in a concentration-dependent manner. The next most potent peptides tested were L-Ala-L-Asp, L-Asp-L-Ala and L-Asp-Gly (Table 1). On the other hand, Gly-L-Asp and Gly-L-Glu were relatively weak stimulators of D-aspartate efflux. In addition, the non-hydrolysable peptide D-Phe-L-Glu was without effect on D-aspartate efflux. Similarly, L-Leu-L-Ala, the only neutral dipeptide tested, had no significant effect on D-aspartate efflux from mammary tissue explants (Table 1).

We wanted to rule out the possibility that the dipeptides were increasing D-aspartate efflux from mammary tissue explants as a consequence of cell lysis. Therefore, we examined the effect of L-Glu-L-Ala on the fractional release of radiolabelled taurine. L-Glu-L-Ala, tested at a concentration of 500 μM, had no effect on the fractional efflux of taurine (results not shown) confirming that the effects of the stimulatory peptides on D-aspartate transport are specific.

3.4. *The effect of L-Glu-L-Ala and L-Glu on D-aspartate efflux from the perfused mammary gland*

We also examined the effect L-Glu-L-Ala on the efflux of D-aspartate from the perfused lactating rat mammary gland. For comparison, we also studied the effect of L-Glu on D-Asp efflux. In this set of experiments the gland was initially perfused with control buffer until the fractional efflux of D-aspartate had reached a steady state. The release of radiolabelled D-aspartate was relatively large for the initial period of the washout (results not shown): this was a consequence of D-aspartate efflux from the tissue extracellular space. However, this component was minimal by 16 min. D-Aspartate efflux was measured for 40 min prior to testing the effect of L-Glu-L-Ala and L-Glu. It is evident from Fig. 4 that addition of L-Glu-L-Ala to the perfusate *trans*-stimulated D-aspartate release from the perfused rat mammary gland in a concentration-dependent fashion. The fractional release of D-aspartate efflux (min^{-1}) was increased (trough-to-peak) from 0.0022 ± 0.0005 to 0.0236 ± 0.0029 min^{-1} ($n = 5$, $P < 0.002$) by 500 μM L-Glu-L-Ala and from 0.0017 ± 0.003 to 0.0066 ± 0.0009

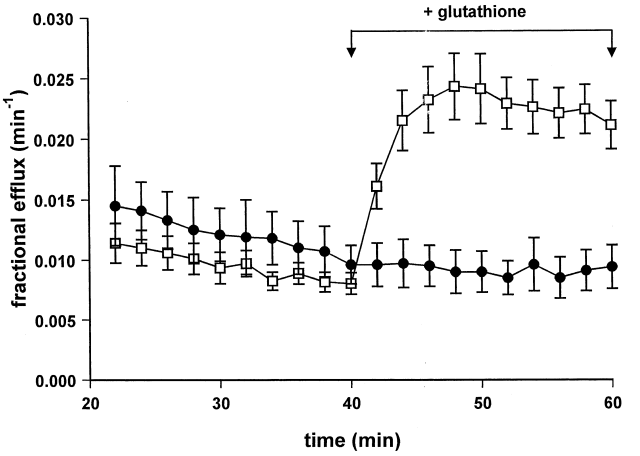

Fig. 5. The effect of glutathione on D-aspartate efflux from mammary tissue explants in the absence (□) and presence (●) of acivicin. D-Aspartate efflux was first measured into a buffer containing (mM) 135 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-MOPS, pH 7.4 followed by one of similar composition except that it contained 500 μM glutathione. When required, acivicin was added to the incubation buffer to give a concentration of 1 mM. The results shown are the means (±S.E.) of eight and four experiments in the absence and presence of acivicin respectively.

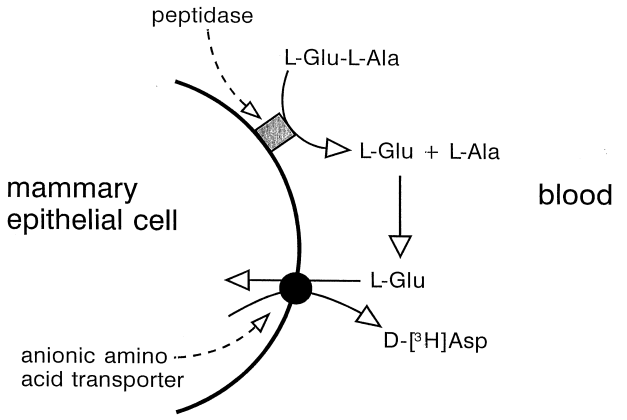

Fig. 6. Proposed scheme of the extracellular hydrolysis of L-Glu-L-Ala with the subsequent uptake of L-glutamate via the high affinity, Na^+-dependent anionic amino acid carrier.

min^{-1} ($n=3$, $P<0.02$) by 100 μM L-Glu-L-Ala. The fractional efflux of D-Asp (min^{-1}) was stimulated from 0.0015 ± 0.0002 to 0.0383 ± 0.0086 min^{-1} ($n=4$, $P<0.05$) by 500 μM L-Glu.

3.5. The effect of glutathione on D-aspartate efflux from mammary tissue

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine), the most abundant oligopeptide in the body, is a potential source of L-glutamate for the lactating mammary gland [19]. Therefore, we tested the effect of glutathione on the efflux of D-aspartate from rat mammary tissue explants and the perfused lactating rat mammary gland. Fig. 5 illustrates that glutathione at a concentration of 500 μM markedly stimulated D-aspartate efflux from mammary tissue explants; the tripeptide increased the fractional efflux (trough-to-peak) from 0.0080 ± 0.0009 min^{-1} to 0.0242 ± 0.0027 min^{-1} (n=8; $P<0.001$). We found that the effect of glutathione on D-aspartate efflux was inhibited by acivicin (1 mM), an inhibitor of γ-glutamyltranspeptidase in lactating rat mammary tissue [20]. In contrast, acivicin (1 mM) did not inhibit the effect of 500 μM L-Glu on D-aspartate efflux from mammary tissue explants suggesting that the effect of acivicin on γ-glutamyltranspeptidase activity is relatively specific (results not shown). Glutathione (at 500 μM) also stimulated D-aspartate release from the perfused mammary gland; the fractional efflux was increased (trough-to-peak) from 0.0017 ± 0.0001 min^{-1} to 0.0046 ± 0.0004 min^{-1} ($n=3$, $P<0.05$).

4. Discussion

4.1. Transport of non-hydrolysable peptides by the perfused mammary gland

The first step in the investigation demonstrated that the clearance of two hydrolysis-resistant dipeptides, namely D-[^{3}H]Phe-L-Glu and D-[^{3}H]Phe-L-Gln which are substrates of epithelial peptide transporters (e.g. see [21,22]), by the perfused lactating mammary gland was very low even under conditions designed to maximize uptake of the radiolabelled compounds. On the other hand, the clearance of two of the constituent amino acids (L-Glu and L-Gln) was high suggesting that the uptake of free amino acids by the lactating mammary gland is quantitatively more important than that of intact dipeptides.

The fact remains, however, that there was a small, albeit significant uptake of the non-hydrolysable peptides by the basolateral membranes of the lactating rat mammary epithelium. The question is what is the nature of the pathway responsible for this measurable uptake of the dipeptides? The clearance of both peptides was not significantly inhibited by excess unlabelled neutral dipeptide suggesting that the small clearances cannot be attributed to uptake via a high affinity peptide transport system such as PepT1 or PepT2 [23,24]. These transporters, which are proton not sodium linked, are expressed in the apical membrane of certain epithelia (e.g. proximal tubule, small intestine); the processes subserving peptide transport across the basolateral membrane of these tissues, as in the mammary gland, remain unknown. In view of these findings it appears that dipeptide uptake by the lactating rat mammary gland is the result of transport via a low affinity pathway (see [25]); experimentally this will be difficult to distinguish kinetically from diffusion.

4.2. Extracellular hydrolysis of dipeptides by the mammary gland

The results from the second part of the study clearly show that the mammary gland is capable of hydrolysing dipeptides extracellularly. Thus, anionic dipeptides are capable of *trans*-stimulating D-aspartate efflux from lactating rat mammary tissue. According to the model depicted in Fig. 6 (using L-

Glu-L-Ala as an example), we believe that the mammary gland rapidly hydrolyses the dipeptides followed by uptake of free anionic amino acids via the high affinity anionic amino acid transporter [5,6]. It appears that the mammary gland has a large capacity to hydrolyse dipeptides. This is based on the finding that L-Glu-L-Ala was 75% as effective as free L-glutamate in stimulating D-aspartate efflux from tissue explants. Moreover, the results strongly suggest that the site of hydrolysis is extracellular; anionic amino acids must be at the *trans* aspect of the membrane in order to stimulate D-aspartate efflux via the anionic amino acid transporter. If the peptides were being hydrolysed intracellularly, D-aspartate efflux would have been inhibited as a consequence of competition between the liberated anionic amino acids and D-aspartate for a binding site at the internal face of the anionic amino acid transporter. Furthermore, the finding that certain dipeptides were capable of increasing D-aspartate efflux from the perfused mammary gland suggests that the locus of hydrolysis is at the blood-facing (i.e. basolateral) aspect of the mammary epithelium. This is based on the reasonable assumption that the perfused gland provides a measure of transport across the blood-facing aspect of the mammary epithelium. It is possible that the peptides were also hydrolysed by endothelial cells. However, the finding that anionic dipeptides stimulated D-aspartate efflux from mammary tissue explants, an experimental preparation in which the vast majority of the surface area exposed to the incubation medium is that of the secretory cells (and not the endothelium), suggests that the peptides are hydrolysed by the basolateral membranes of the acinar cells.

Tissue utilization of peptides is not a new concept. There are many studies relating to the use of peptides in human parenteral nutrition which have shown that infused or injected peptides disappear rapidly from the circulation and, since they do not appear in urine, it has been inferred that the peptides are hydrolysed in vivo, with the constituent amino acids entering the free amino acid pool [26–28]. Indeed, direct incorporation into milk casein of leucine and phenylalanine derived from intravascularly infused Gly-L-[^{13}C]Phe and Gly-L-[^{13}C]Leu has been demonstrated in goats [12]. In that study, however, it was not clear whether peptides were hydrolysed systemically or by the mammary gland itself. The present study is the first to show direct mammary tissue hydrolysis of dipeptides and transport of the constituent amino acids. Furthermore, given the experimental conditions, blood or plasma hydrolysis of the peptides can be ruled out.

Interestingly, not all of the anionic peptides stimulated D-aspartate efflux to the same extent. In particular, D-Phe-L-Glu had no effect on D-aspartate efflux supporting the claim that dipeptides containing a D-isomer amino acid at the N terminus resist hydrolysis. In addition, those peptides which contained glycine at the N terminus were weak at *trans*-stimulating D-aspartate release. This is consistent with previous findings that peptides with glycine at the N terminus are more resistant to hydrolysis [26].

4.3. Metabolism of glutathione by the lactating rat mammary gland

The results show that glutathione is capable of stimulating D-aspartate efflux from the mammary gland. This suggests that the mammary gland is able to hydrolyse glutathione and the glutamate residue so released is able to *trans*-stimulate D-aspartate via the high affinity anionic amino acid transport system. It has been previously shown that rat and bovine mammary tissue expresses relatively high levels of γ-glutamyltranspeptidase, an enzyme which can catalyse the release of glutamate from glutathione [29]. The observation that the effect of glutathione on D-aspartate efflux from mammary tissue was inhibited by acivicin is consistent with the notion that γ-glutamyltranspeptidase was responsible for hydrolysing glutathione. Glutathione may be an important physiological source of glutamate (and of cysteine) for the mammary gland. In this connection Pocius et al. [20] presented evidence for an arterio-venous glutathione concentration difference across the mammary gland.

The study reported here does not shed light on the handling of peptides at the apical face of the mammary epithelium. It does, however, indicate that, in addition to nutrition, there are questions of relevance to the mechanisms of drug delivery to the suckling neonate (e.g. protease inhibitors) which may be explored using the experimental protocols described here.

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