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Substrate specificity of the mammary tissue anionic amino acid carrier operating in the cotransport and exchange modes

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Abstract

The substrate specificity of the rat mammary tissue high affinity, Na^+-dependent anionic amino acid transport system has been investigated using explants and the perfused mammary gland. D-Aspartate appears to be transported via the high affinity, Na^+-dependent L-glutamate carrier. Thus, D-aspartate transport by rat mammary tissue was Na^+-dependent and saturable with respect to extracellular D-aspartate with a K_m and V_{max} of 32.4 μM and 49.0 nmol/2 min per g of cells respectively. The uptake of D-aspartate by mammary explants was *cis*-inhibited by L-glutamate and L-aspartate, but not by D-glutamate. L-glutamate uptake by mammary tissue explants was *cis*-inhibited by β-glutamate, L-cysteate, L-cysteine sulfinate and dihydrokainate but not by DL-α-aminoadipate. In addition, dihydrokainate, but not DL-α-aminoadipate inhibited D-aspartate and L-glutamate uptake by the perfused gland. D-Aspartate efflux from mammary tissue explants was *trans*-accelerated by external L-glutamate in a dose-dependent fashion (50-500 μM). The effect of L-glutamate on D-aspartate efflux was dependent on the presence of extracellular Na^+. D-Aspartate, L-aspartate and L-cysteine sulfinate (at 500 μM) also markedly *trans*-stimulated D-aspartate efflux from mammary tissue explants. In contrast, L-cysteine, D-glutamate, L-leucine, dihydrokainate and DL-α-aminoadipate were either weak stimulators of D-aspartate efflux or were without effect. D-Aspartate efflux from the perfused mammary gland was *trans*-stimulated by L-glutamate but not by D-glutamate and only weakly by L-cysteine (all at 500 μM). It appears that the mammary tissue high affinity anionic amino acid carrier can operate in the exchange mode with a similar substrate specificity to that of the co-transport mode.

Keywords: Mammary gland; Anionic amino acid; Membrane transport

1. Introduction

The lactating mammary epithelium has a large demand for amino acids to meet the requirements of milk protein synthesis. Amino acids are taken up by mammary cells via a variety of individual transport systems which have different substrate preferences and ionic requirements. For example, systems A, ASC and L have been identified for neutral amino acids and a system for cationic amino acid transport has also been described [1–5]. In addition, the lactating mammary gland also possesses an amino acid transport system which is activated by cell-swelling that accepts taurine and glycine as substrates [6].

We have recently studied the transport of L-glutamate by the lactating mammary gland [7]. Our interest in L-glutamate transport stems from the find-

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ings that L-glutamate is (a) the most abundant amino acid in milk protein (b) is one of the most concentrated amino acids within mammary cells and (c) is, depending on the species, concentrated in milk with respect to plasma [8]. Accordingly, the lactating mammary gland has a high demand for L-glutamate which is reflected, in a number of species, in large arteriovenous L-glutamate concentration gradients across the mammary epithelium [9,10]. This is consistent with the presence of a transport system for L-glutamate in the lactating mammary gland and suggests that mammary cells do not have to rely solely on L-glutamine as a source of L-glutamate [11]. However, we must stress that we do not yet know the exact contribution of the L-glutamate carrier to the intracellular L-glutamate pool.

Rat mammary tissue expresses a high affinity, Na^+-dependent L-glutamate carrier [7]. The Na^+-dependent L-glutamate system, which appears to be situated in the blood-facing pole of the mammary epithelium, is the predominant, if not only, pathway for L-glutamate uptake by rat mammary tissue. This transport system appears, on the basis of *cis*-inhibition studies, to be selective for anionic amino acids, thus, amino acids such as L- and D-aspartate markedly reduced L-glutamate uptake by mammary tissue in a high affinity fashion. In contrast, neutral and cationic amino acids did not readily interact with the carrier [7]. The ability of the rat mammary L-glutamate transport mechanism to discriminate between the L- and D- isomers of glutamate but not those of aspartate suggests that the carrier bears some resemblance to the high affinity, Na^+-dependent anionic amino acid carriers which have been described in other tissues such as the intestine, placenta, kidney and brain. Indeed, several of the high affinity, Na^+-dependent L-glutamate transport systems, designated EAAC1, GLAST, and GLT, have been cloned and their primary amino acid sequences determined [12–14]. GLAST and GLT appear to be brain specific whereas EAAC1 is expressed in both brain and peripheral tissues.

The purpose of the present study was two-fold. The first objective was to directly confirm that the mammary tissue L-glutamate carrier accepts D-aspartate which, on account of being non-metabolised, would be an ideal substrate to further characterise the mammary Na^+-dependent anionic amino acid transport system. Secondly, we wanted to further characterise the substrate specificity and mechanistics of the carrier which would allow us to compare the properties of the mammary system with those of other high affinity, Na^+-dependent acidic amino acid transporters. Therefore, we have examined the transport of L-glutamate and D-aspartate by isolated rat mammary tissue explants and the perfused in situ rat mammary gland. These two preparations are, in our opinion, the two best experimental systems presently available to study mammary amino acid transport [8]. Explants allow transport systems to be rapidly identified and characterised whereas the perfused mammary preparation allows conclusions to be drawn regarding the location of the transport systems in the mammary epithelium (i.e. apical υ basolateral).

2. Materials and methods

2.1. Animals

Lactating Wistar rats 10–15 days post partum were used. Animals were maintained on a 12 h light–12 h dark cycle and allowed free access to water and chow.

2.2. Preparation of mammary tissue explants

Mammary tissue explants were prepared according to the method described in detail by Shennan [15]. Rats were killed by cervical dislocation and the abdominal mammary glands were immediately removed and placed in an ice-cold buffer similar in composition to that required for the flux studies. The mammary glands were finely dissected to produce explants weighing between 0.5–2.0 mg for the uptake studies and 4.0–8.0 mg for the efflux experiments.

2.3. Measurement of L-glutamate and D-aspartate transport in mammary tissue explants

2.3.1. L-Glutamate and D-aspartate uptake

L-Glutamate and D-aspartate uptake, respectively using L-[^{3}H]glutamate and D-[^{3}H]aspartate as tracers was measured according to the methods described in detail by Millar et al. [7]. Briefly, tissue explants were incubated in 4 or 6 ml of buffer (compositions

given in figure and table legends) at 37°C for 20 min prior to the addition of isotope (0.25–0.5 μCi/ml). Following the addition of the radiolabel tissue explants were removed at pre-determined intervals and immediately washed with 4 ml of an ice-cold buffer. The tissue explants were lightly blotted and weighed. Once the weight had been determined the tissue explants were placed in 4 ml of either 10% trichloroacetic acid (for the L-glutamate experiments) or water (for the D-aspartate experiments) and were allowed to stand for at least 16 h. Afterwards the tubes were centrifuged at $13\,000 \times g$ for 3 min and a portion of the resulting supernatant was counted to determine the amount of isotope associated with the explants. The specific activity of the radioisotopes in each incubation medium was determined by counting the radioactivity in 100 μl samples. The tissue extracellular space was estimated in each experiment by measuring the distribution of [^{3}H]sucrose between the incubation medium and the tissue explants.

L-glutamate and D-aspartate uptake by tissue explants was calculated according to Eq. (1):

$$[\text{amino acid}]_c = \{[\text{amino acid}]_t - ([\text{amino acid}]_m . F)\} / 1 - F \quad (1)$$

where $[\text{amino acid}]_c$ is the cellular concentration of L-glutamate or D-aspartate in nmol/g of cells, $[\text{amino acid}]_t$ is the total tissue concentration of L-glutamate or D-aspartate in nmol/g of tissue wet weight, $[\text{amino acid}]_m$ is the concentration of L-glutamate or D-aspartate in the incubation medium in nmol/ml and F is the sucrose space (%).

2.3.2. D-Aspartate efflux

D-Aspartate efflux, using D-[^{3}H]aspartate as tracer, was measured according to the method of Shennan [15]. Mammary tissue explants, prepared as described above, were loaded with radiolabelled D-aspartate by incubation at 20°C for 30–40 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 (+2–4 μCi/ml of D-[^{3}H]aspartate). Following the loading period the tissue was transferred through a series of tubes containing 2 ml of radioactive free solutions after which the tissue was allowed to stand in 4.0 ml of distilled water for at least 16 h in order to leach out the remaining isotope. The fractional efflux (min^{-1}) of D-aspartate from the tissue explants was calculated according to Eq. (2):

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

where ΔX represents D-aspartate released (dpm) from the tissue in the time interval Δt and X_t is the tissue D-aspartate content (dpm) at time t. The radioactivity left in the tissue at a given time was determined by adding the radioactivity left in the explant at the end of the incubation to the radioactivity lost by the explant in each of the collection periods in reverse order.

We also analysed the D-aspartate efflux time courses by plotting ln [radiolabelled D-aspartate] remaining in the tissue as a function of time. The negative slopes of these plots provided a measure of the unidirectional efflux rate constants (see Table 2).

2.4. Perfusion of the lactating rat mammary gland

The lactating rat mammary gland was perfused according to the method of 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), and the tissue was perfused via the superficial epigastric blood vessels (see figure legends for precise composition of the perfusates). The perfusate was delivered to the tissue with a flow and pressure profile similar to that in vivo [16].

2.4.1. Measurement of L-glutamate and D-aspartate uptake by the perfused gland

The transport of L-glutamate and D-aspartate, respectively using L-[^{3}H]glutamate and D-[^{3}H]aspartate as tracers, was measured using a rapid, paired-tracer dilution technique as described by Millar et al. [7]. A mixture containing the radiolabelled amino acid and [^{14}C]sucrose was injected close arterially (60 μl in < 2 s) and 20 venous outflow samples were collected at 6-s intervals. The uptake (U) of L-glutamate or D-aspartate for each collection period was calculated according to Eq. (3):

$$U(\%) = 1 - (R_t / R_i) \quad (3)$$

where R_t is the ratio of radiolabelled L-glutamate (or D-aspartate)/radiolabelled sucrose in the venous out-

flow samples at time t and R_i is the ratio of radiolabelled L-glutamate (or D-aspartate)/radiolabelled sucrose in the injectate. The unidirectional uptake (V_t) of L-glutamate and D-aspartate was calculated from the maximum uptake (U_{max}) according to Eq. (4):

$$V_t = -\ln(1 - U_{max}) \times F \times C \quad (4)$$

where F is the perfusate flow and C is the perfusate concentration of unlabelled L-glutamate or D-aspartate. The clearances of L-glutamate and D-aspartate (i.e., the volume of perfusate notionally cleared of isotope) was calculated by multiplying the perfusate flow rate (μl/min per g of tissue) by the uptake (%) for each collection period.

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

Mammary tissue, perfused as described above, was loaded with radiolabelled D-aspartate by perfusing with a buffer containing (mM) 118 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, 25 $NaHCO_3$ and 10 glucose plus 0.1–0.2 μCi/ml of D-[^{3}H]aspartate for approximately 20 min. All perfusates were supplemented with BSA (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 radiolabelled aspartate (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 homogenised. Samples of the homogenate (150–250 mg) were incubated in distilled water for at least 16 h in order to leach out the remaining 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 experiment. Venous outflow samples (0.75 ml) were mixed with an equal volume of 10% TCA and were spun at $13\,000 \times g$ for 3 min. 1 ml samples of the resulting supernatants were removed and prepared for counting. The fractional efflux (min^{-1}) of D-aspartate from the perfused gland was calculated according to Eq. (2).

2.4.3. Materials

L-[^{3}H]Glutamate and D-[^{3}H]aspartate were purchased from Amersham International (UK). All other chemicals were obtained from Sigma (UK).

2.4.4. Statistics

Differences were assessed by Students's paired or unpaired t tests as appropriate and were considered significant when $P < 0.05$.

3. Results

3.1. D-Aspartate transport by mammary tissue explants and the perfused mammary gland

Cis inhibition studies of L-glutamate uptake by rat mammary tissue suggest that D-aspartate is a substrate of the L-glutamate carrier [7]. If D-aspartate does share a pathway for transport with L-glutamate then it follows that D-aspartate transport should display similar characteristics to that of L-glutamate transport. Thus, D-aspartate transport should be a Na^+-dependent process and should be inhibited by amino acids that block L-glutamate uptake. Furthermore, Na^+-dependent D-aspartate transport should be situated in the basolateral pole of the mammary epithelium [7]. In view of this we tested the Na^+-dependency of D-aspartate transport by mammary tissue explants and the perfused mammary gland. In addition, we examined the effect of L-aspartate, L-glutamate and D-glutamate on the clearance of D-aspartate by the perfused mammary gland.

Fig. 1A shows that the uptake of D-aspartate (measured at a concentration of 5 μM) by mammary tissue explants was time dependent being linear for at least 3 min. Fig. 1A also shows that D-aspartate uptake by mammary explants was a Na^+-dependent process: replacing extracellular Na^+ with choline ions abolished D-aspartate uptake. We tested the effect of reversing the Na^+ gradient on D-aspartate efflux from mammary tissue explants preloaded with radiolabelled D-aspartate. It was predicted that D-aspartate efflux should be influenced by the extent of the *trans*-membrane Na^+ gradient given that we have previously shown that the mammary tissue L-glutamate transporter can run in the reverse mode [7]. Preliminary experiments revealed that D-aspartate efflux, like that of L-glutamate, from mammary tissue explants could be resolved into at least two components (results not shown). The fast component, which was minimal by 20 min, represented the efflux of

radiolabelled D-aspartate from the tissue extracellular space. In view of this, we allowed the efflux of D-aspartate to proceed for 40 min prior to testing the effect of reversing the Na^+-gradient. In this set of experiments, the results of which are shown in Fig. 1B, we found that reversing the Na^+-gradient by transferring the tissue from a medium containing Na^+ as the principal cation to one in which choline had replaced Na^+ led to a marked increase in the

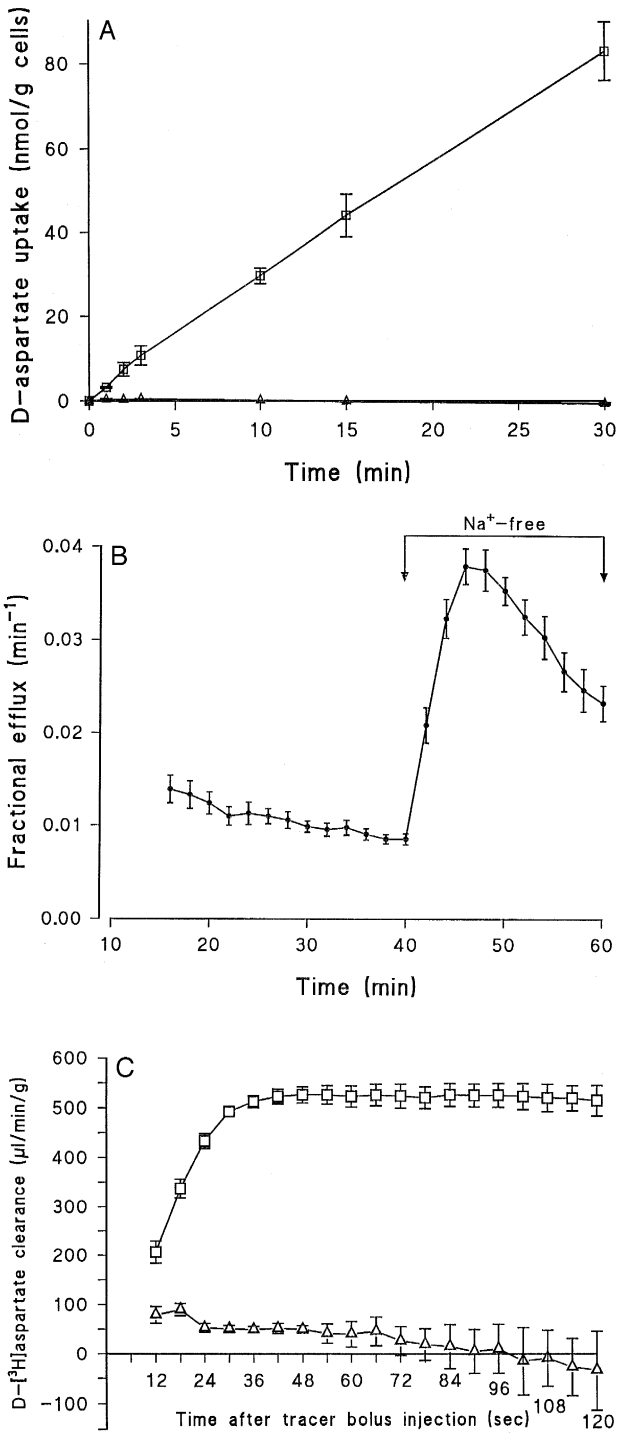

Fig. 1. A. The uptake of D-aspartate by rat mammary tissue explants in the presence (□) and absence (△) of extracellular Na^+. Tissue was incubated in a medium containing (mM) 0.005 D-aspartate, 135 XCl (where X = Na or choline), 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-Mops, pH 7.4. The results shown are the means ± SEM of 3 experiments. B. The effect of reversing the Na^+-gradient on D-aspartate efflux from rat mammary tissue explants. Tissue was first incubated in a buffer 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 except that choline replaced the Na^+ (indicated by the bar). The results shown are the means ± SEM of 5 experiments. C. The clearance of D-aspartate by the perfused rat mammary gland in the presence (□) and absence (△) of extracellular Na^+. The tissue was perfused with a buffer containing (mM) 118 XCl (where X = Na^+ or $NMDG^+$), 4 KCl, 2 $CaCl_2$, 1 KH_2PO_4, 1 $MgSO_4$, 10 glucose and 20 Tris-Hepes, pH 7.4. The perfusate was supplemented with BSA (5%) and gassed with 100% O_2. The results shown are the mean ± SEM of 4 experiments.

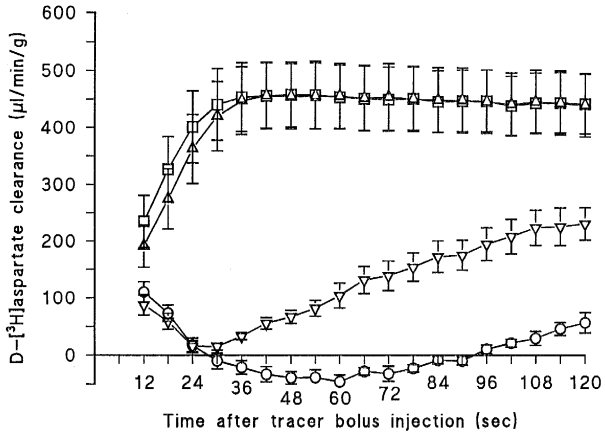

Fig. 2. The effect of unlabelled (○) L-aspartate, (▽) L-glutamate and (△) D-glutamate on the clearance of D-aspartate by the perfused rat mammary gland. The clearance in the absence of any additions (i.e. the control) is shown by the open squares. The perfusate contained (mM) 118 NaCl, 4 KCl, 2 $CaCl_2$, 1 NaH_2PO_4, 1 $MgSO_4$, 10 glucose and 25 $NaHCO_3$. The perfusate also contained BSA (5%) and was gassed to pH 7.4 with 95% O_2 /5% CO_2. Each amino acid was added to the injectate at a concentration of 50 mM. The results shown are the mean ± SEM of 4 experiments.

fractional release of D-aspartate. We found that D-aspartate clearance by the perfused mammary gland was a Na^+-dependent process. Fig. 1C illustrates that D-aspartate clearance by the perfused rat mammary gland was markedly reduced when Na^+ was replaced with $NMDG^+$ suggesting that the (Na^+-D-aspartate) cotransport mechanism is situated, along with Na^+-dependent L-glutamate transport, in the blood-facing aspect of the mammary epithelium. We tested the effect of unlabelled anionic amino acids on the clearance of D-aspartate by the perfused mammary gland. In this set of experiments the amino acids were co-injected with the radiotracers. Although each amino acid was present at a concentration of 50 mM in the injectates it must be borne in mind that the concentration of the amino acids at the site of transport would have been much less owing to the diluting effect of the perfusate. Fig. 2 shows that extracellular L-glutamate and L-aspartate, but not D-glutamate, were effective inhibitors of D-aspartate clearance by the perfused mammary preparation. Importantly, the lack of inhibition of D-aspartate clearance by D-glutamate rules out the possibility that the other amino acids are exerting their effect as a consequence of changing the osmolality of the perfusate.These results taken together appear to confirm the prediction that D-aspartate and L-glutamate are transported by a single carrier in lactating rat mammary tissue and therefore justify the use of D-aspartate to further characterise the high affinity, Na^+-dependent anionic amino acid carrier which is expressed in mammary tissue.

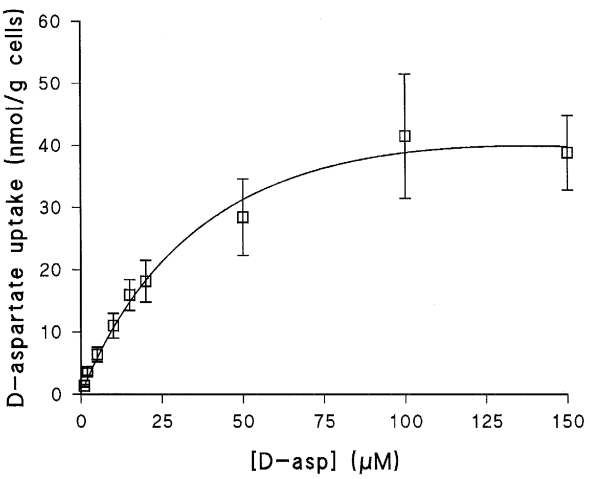

Fig. 3. The D-aspartate concentration dependence of D-aspartate uptake by mammary tissue explants. Tissue was incubated in a buffer containing (mM) 0.005–0.15 D-aspartate, 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-Mops, pH 7.4. D-Aspartate uptake was assayed at 37°C after 2 min of incubation. The points shown are the mean ± SEM of 3 experiments.

Table 1
The effect of a range of anionic amino acids on L-glutamate and D-aspartate uptake by lactating rat mammary tissue explants

	L-Glutamate uptake	D-Aspartate uptake
Inhibitor	IC_{50} (μM)	
L-Aspartate	40.6 ± 0.7	
D-Aspartate	15.9 ± 8.6	
D-Glutamate	> 1000	
β-Glutamate	46.1 ± 11.3	
L-Cysteate	13.4 ± 7.4	
CSA	8.4 ± 4.7	
AAD	> 1000	> 1000
DHK	17.5 ± 6.1	42.1 ± 8.7

Mammary tissue was incubated in a buffer containing (mM) 0.005 L-glutamate or D-aspartate, 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-Mops, pH 7.4 plus 5–1000 μM of the amino acids when required. Uptake was assayed after 2 min of incubation at 37°C. The data for the effects of L-aspartate, D-aspartate and D-glutamate on L-glutamate are taken from [7].

3.2. The kinetic characteristics of D-aspartate uptake by mammary tissue explants

The kinetic parameters (i.e. K_m and V_{max}) of D-aspartate uptake by lactating rat mammary tissue explants were examined. Fig. 3 shows that D-aspartate uptake was saturable with respect to the extracellular D-aspartate concentration. The calculated K_m and V_{max} (from Hanes-Woolf plots) were respectively 32.4 ± 5.0 μM and 49.0 ± 9.1 nmol/2 min per g of cells (± SEM, $n = 3$).

3.3. The effect of other anionic amino acids on L-glutamate and D-aspartate transport

To gain more information on the substrate specificity of the carrier with respect to anionic amino acids, we tested the effect of several other anionic amino acids on L-glutamate and D-aspartate uptake by both mammary explants and the perfused mammary preparation. The amino acids tested were β-glutamate and the sulfur containing amino acids L-cysteate and L-cysteine sulfinate. In addition, we also examined the effect of DL-α-aminoadipate (AAD) and

dihydrokainate (DHK) both of which have been widely used to characterize L-glutamate transport in other cell types (e.g. see [17]). Table 1 shows the effect of these amino acids on L-glutamate uptake by mammary tissue explants. The IC_{50} for each amino acid was calculated from plots of $[J_c/J_c - J_i]\upsilon 1/I$ where J_c is the uptake in the absence of the inhibitor, J_i is the uptake in the presence of the inhibitor and I is the concentration of inhibitor. Shown for comparison is the effect of L- and D-aspartate and D-glutamate on L-glutamate uptake. All of the amino acids tested, with the exception of AAD, inhibited L-glutamate uptake with high affinity in a dose dependent fashion. Table 1 shows that DHK also inhibited D-aspartate uptake with high affinity. In contrast AAD had no effect on D-aspartate uptake. Consistent with these results was the finding that DHK, but not AAD, inhibited D-aspartate and L-glutamate uptake by the perfused mammary gland (Fig. 4).

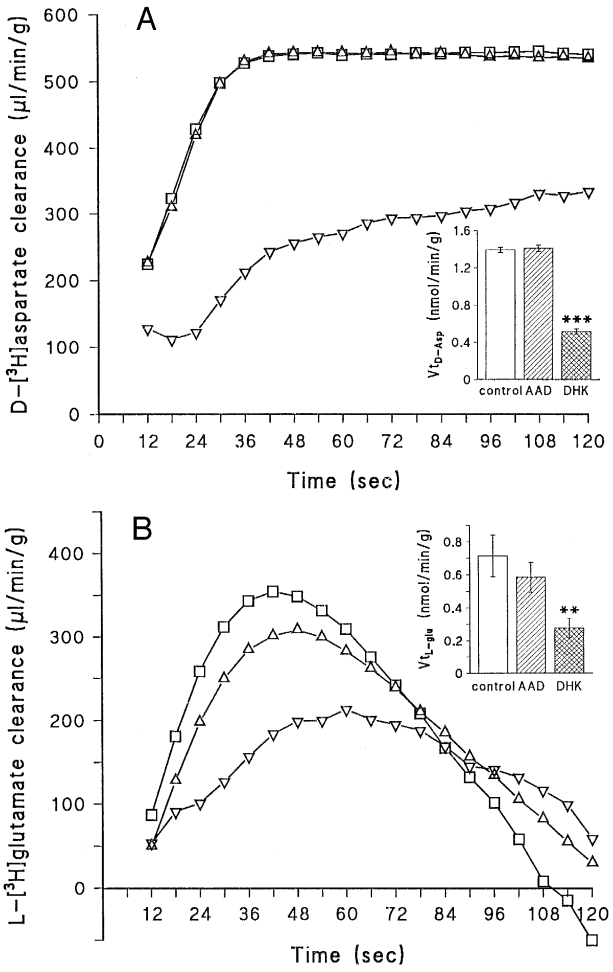

Fig. 4. The effect of dihydrokainate (▽) and DL-α-aminoadipate (△) on the clearance of (A) D-aspartate and (B) L-glutamate by the perfused rat mammary gland. The control clearance (i.e. no additions) of D-aspartate and L-glutamate is shown by the open squares. The perfusate contained (mM) 0.001 D-aspartate or L-glutamate, 118 NaCl, 4 KCl, $2CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, 10 glucose and 25 $NaHCO_3$. The perfusate also contained BSA (5%) and was gassed to pH 7.4 with 95% O_2 /5% CO_2. When required, DHK and AAD were added to the perfusate to give a final concentration of 300 μM for the D-aspartate experiments and 100 μM for the L-glutamate studies. The insets show the effects of DHK and AAD on the unidirectional uptakes of D-aspartate and L-glutamate. The results shown are means ± SEM of 5 and 3 experiments for L-glutamate and D-aspartate, respectively. ** $P < 0.01$; *** $P < 0.001$.

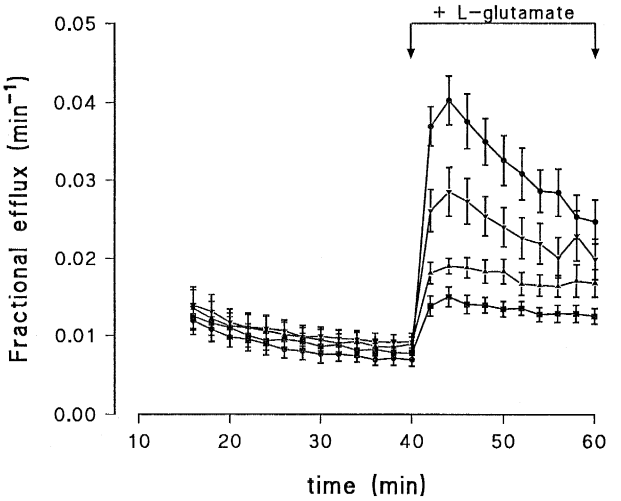

Fig. 5. The effect of extracellular L-glutamate on D-aspartate efflux from rat mammary tissue explant. D-Aspartate efflux was first measured into a medium containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-Mops, pH 7.4 followed by one of similar composition supplemented with (■) 50 μM, (▲) 100 μM, (▼) 200 μM or (●) 500 μM L-glutamate (indicated by bar). The results shown are means ± SEM of 5 experiments.

3.4. The effect of extracellular amino acids on D-aspartate efflux.

Kanner and Bendahan [18] found that the efflux of L-glutamate from synaptic plasma membrane vesicles could be stimulated by external L-glutamate. Recently, Zerangue and Kavanaugh [19] have shown that a neuronal subtype Na^+-dependent L-glutamate transporter (designated EAAT3) can transport anionic

Table 2
The effect of external amino acids (at 500 μM) on D-aspartate efflux from lactating rat mammary tissue explants

Amino acid	*n*	Efflux rate constants ($min^{-1} \cdot 10^{-4}$) control	+test	difference	*P* <
L-Glutamate	7	75±10	362±32	287±38	0.001
L-Aspartate	6	82±12	311±32	229±43	0.01
D-Aspartate	4	86±8	415±32	329±36	0.01
CSA	4	58±18	369±55	311±70	0.05
L-Cysteine	5	103±9	161±14	58±9	0.01
L-Leucine	3	80±12	88±23	8±11	n.s
D-Glutamate	5	87±20	105±19	18±9	n.s
DHK	4	54±11	100±14	46±3	0.001
AAD	3	73±16	87±22	14±9	n.s.

D-Aspartate efflux was measured into a medium containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-Mops, pH 7.4, and then to a similar medium supplemented with a 'test' amino acid at 500 μM (CSA = L-cysteine sulfinate).

amino acids by heteroexchange. These findings led us to test whether the mammary anionic amino acid transport mechanism can also function in the exchange mode. We tested the effect of a range of extracellular amino acids on D-aspartate efflux from mammary tissue explants. We chose D-aspartate in preference to L-glutamate since the former is more resistant to metabolism. For this set of experiments the efflux of D-aspartate was initially measured into an amino acid free medium and following this the tissue was transferred into a medium supplemented with a test amino acid. Fig. 5 shows that extracellular L-glutamate *trans*-stimulated D-aspartate efflux from rat mammary tissue explants in a dose-dependent manner: this is consistent with heteroexchange. We also examined the effect of D-glutamate, D-aspartate, L-aspartate, L-cysteine, L-leucine, L-cysteine sulfinate,

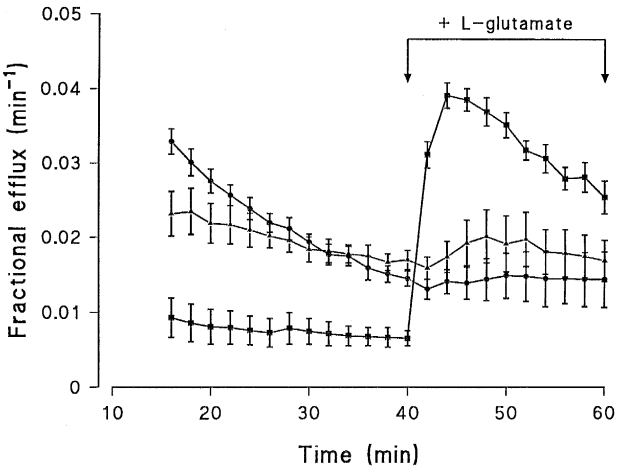

Fig. 6. The effect of extracellular L-glutamate on D-aspartate efflux from mammary tissue explants in the presence and absence of extracellular Na^+. D-Aspartate was first measured into a medium containing (mM) 135 XCl (where X = Na^+ (■), Choline (●) or Li^+ (▲)), 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 20 Tris-Mops, pH 7.4 followed by one of similar composition except that it also contained 500 μM L-glutamate. The results shown are the means ± SEM of 4 experiments.

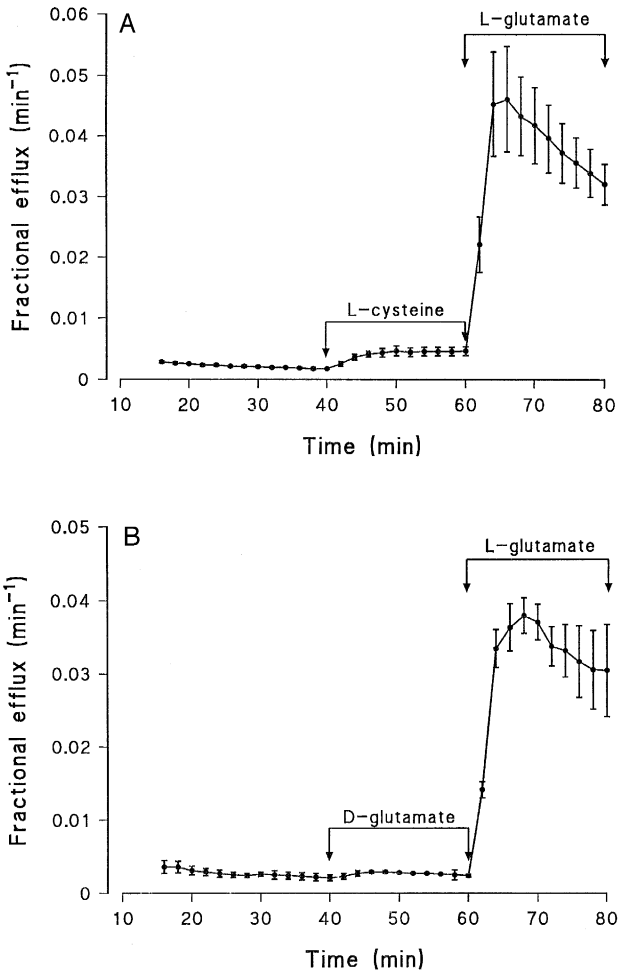

Fig. 7. A,B. The effect of extracellular L-cysteine, D- and L-glutamate on D-aspartate efflux from the perfused lactating rat mammary gland. The gland was initially perfused with a buffer containing (mM) 118 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, 10 glucose and 25 $NaHCO_3$ (supplemented with 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 L-cysteine, D-glutamate or L-glutamate (500 μM) (as indicated by bars). The results shown are the mean ± SEM of 3 experiments.

AAD and DHK on D-aspartate efflux from mammary explants (Table 2). The results show that D-aspartate, L-aspartate and L-cysteine sulfinate were as effective as L-glutamate at *trans*-accelerating D-aspartate efflux. On the other hand, L-cysteine and DHK only weakly interacted with the carrier whilst D-glutamate, L-leucine and AAD had no effect. The effect of extracellular L-glutamate on D-aspartate efflux was dependent upon extracellular Na^+. Thus, L-glutamate had no effect on D-aspartate efflux when extracellular Na^+ was replaced with either choline or Li^+ (Fig. 6). Note from Fig. 6 that the fractional efflux of D-aspartate, in the absence of extracellular L-glutamate, was higher when the medium was Na^+-free. This is a consequence of reversing the Na^+-gradient and is consistent with the results shown in Fig. 1B.

We also examined the effect of extracellular amino acids on the efflux of radiolabelled D-aspartate from the perfused lactating rat mammary gland to confirm that the exchange of amino acids was occuring at the basolateral aspect of the mammary epithelium. As with explants, the release of D-[^{3}H]aspartate from the gland was initially high as a consequence of efflux from the extracellular compartment. Therefore, the efflux of D-aspartate was allowed to proceed for 40 min prior to testing the effect of extracellular amino acids. Fig. 7(A and B) shows that extracellular L-glutamate, but not D-glutamate or L-cysteine, markedly *trans*-accelerated D-aspartate efflux from the perfused lactating rat mammary gland when tested at a concentration of 500 μM.

4. Discussion

The first objective of this study was to confirm that D-aspartate was transported by the mammary tissue high affinity, Na^+-dependent L-glutamate carrier. We did this because D-aspartate would be an excellent substrate to further characterise mammary tissue anionic amino acid transport, particularly efflux, on account of being non-metabolized. The results of the present study confirm the prediction, based on *cis*-inhibition experiments [7], that D-aspartate is transported by the mammary tissue L-glutamate carrier which is situated in the blood-facing aspect of the mammary epithelium. Thus, D-aspartate transport by mammary tissue is a Na^+-dependent process and is inhibited by L-glutamate and other amino acids which inhibit L-glutamate transport. Similarly, D-aspartate transport, like L-glutamate transport, was insensitive to D-glutamate. The uptake of D-aspartate by mammary tissue explants was saturable with a K_m of 32.4 μM. Therefore, it appears that the mammary anionic amino acid carrier has a higher affinity for D-aspartate than L-glutamate given that we have previously shown that the K_m for L-glutamate by mammary explants is 112.5 μM [7]. Importantly, the Na^+-dependent system appears to be the main, if not only, route for D-aspartate transport in mammary tissue. There was, however, a difference between the profiles of D-aspartate and L-glutamate transport by the perfused mammary gland. Thus, the clearance of D-aspartate, unlike that of L-glutamate, did not decrease in the latter stages of the time course. This may reflect that D-aspartate is more avidly retained than L-glutamate by mammary tissue. Thus, L-glutamate may exit mammary tissue via other transport pathways. On the other hand, it may reflect that D-aspartate is not metabolised. The mammary tissue anionic amino acid transport system, which bears many of the characteristics of high affinity, Na^+-dependent anionic amino acids transport systems (system X^-_{AG}) described in other epithelia, also appeared to interact with the sulfur containing amino acids L-cysteate and L-cysteine sulfinate. We also examined the effect of two other anionic amino acids, namely AAD and DHK. We found that L-glutamate and D-aspartate accumulation was inhibited by DHK but not AAD. This profile of inhibition has not been seen with other high affinity, Na^+-dependent anionic amino acid transport systems. For example, EAAC1 is inhibited by AAD but not DHK [17], GLT-1 is sensitive to both AAD and DHK whereas GLAST is not inhibited by either drug [17,20].

High affinity, Na^+-dependent anionic amino acid carriers can operate in the exchange mode. For example, it has recently been shown that external L-glutamate can stimulate D-aspartate efflux via a high affinity neuronal L-glutamate transporter expressed in *Xenopus* oocytes. [19]. It is apparent that L-glutamate is capable of stimulating D-aspartate efflux from mammary tissue explants. The question is: is the *trans*-stimulation of D-aspartate efflux via the high affinity, Na^+-dependent carrier or via another system? The finding that *trans*-stimulation of D-aspartate

efflux by L-glutamate was dependent upon the presence of extracellular Na^+ is consistent with the exchange mode being a function of the high affinity, Na^+-dependent system. Moreover, the Na^+-dependence rules out system x_c^-, a mechanism which is capable of exchanging L-glutamate and L-cystine [21]. In addition, it is evident from the experiments using the perfused mammary preparation that the site of amino acid exchange, like that of the cotransporter, is in the blood-facing aspect of the epithelium. Our results show that D-aspartate efflux from mammary tissue can also be markedly *trans*-accelerated by amino acids such as D-aspartate, L-aspartate and L-cysteine sulfinate. The finding that an amino acid can *trans*-accelerate the efflux of D-aspartate suggests that the particular amino acid is actually transported by the carrier. *Trans*-stimulation of D-aspartate efflux by L-glutamate, L-aspartate and L-cysteine sulfinate is therefore consistent with their effects on L-glutamate uptake by mammary tissue. In this connection, Bouvier et al. [22], have shown that L-cysteine sulfinate is transported by the L-glutamate carrier which is expressed in Muller cells from the salamander retina. Interestingly, amino acids such as D-glutamate, AAD, L-cysteine and L-leucine, which do not readily interact with the mammary tissue Na^+-dependent, anionic amino acid cotransporter do not, or only weakly, elicit *trans*-stimulation of D-aspartate efflux suggesting that the exchange mode of the carrier has a substrate specificity similar to that of the cotransport mode. However, we found that DHK, which inhibits the uptake of L-glutamate and D-aspartate, did not markedly stimulate the efflux of D-aspartate. This suggests that DHK, whilst binding to the carrier, is not readily transported. The intracellular concentrations of L-glutamate and L-aspartate (and possibly other anionic amino acids) in mammary tissue may be regulated by the anionic amino acid transport system working in the exchange mode as well as the cotransport mode. For example, L-glutamate uptake may influence the intracellular concentration of L-aspartate (and vice versa). In support of this notion is the observation that D-aspartate efflux from mammary tissue explants was stimulated by physiological levels of extracellular L-glutamate. It is apparent from this study that examining the effect of extracellular amino acids on the efflux of D-aspartate is a rapid and convenient way of testing whether an amino acid is actually transported by the high affinity Na^+-dependent anionic amino acid carrier. This would be useful particularly when the putative substrate is not readily available in the radiolabelled form.

Finally, the observation that L-cysteine did not readily stimulate D-aspartate efflux from mammary tissue to the same extent as L-glutamate is worthy of further comment. Zerangue and Kavanaugh [19] found that L-cysteine was as capable as L-glutamate at accelerating D-aspartate efflux via EAAT3, a human homologue of EAAC1, expressed in *Xenopus* oocytes. In contrast, L-cysteine did not readily interact with the human homologues of GLAST and GLT.

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