Characterization of anionic amino acid transport systems in mouse mammary gland

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Received 14 April 2000; revised 3 August 2000

L-glutamate was transported into mammary tissue via Na+-dependent system X_{AG}^+ that strongly interacted with both D- and L-isomers of aspartate but only with L-isomer of glutamate. Replacement of Cl⁻ by gluconate from the extracellular medium did not affect the uptake of L-glutamate. Although neutral amino acids weakly inhibited the uptake of L-glutamate, there was no evidence for the heterogeneity of anionic amino acid transport system. The X_{AG}^- system was inhibited by sulphydryl group blocking reagent N-ethylmaleimide. Low pH (5) partially inhibited the uptake by L-glutamate by mammary tissue. Prior loading of mammary tissue with L-glutamate slightly down regulated its uptake. Culturing pregnant mouse mammary tissue explants in vitro in the presence of lactogenic hormones (insulin plus cortisol plus prolactin) did not affect appreciably the uptake of L-glutamate.

Several amino acid transport systems in mammary gland have been characterized in the recent past. These systems may be divided into two broad categories based on whether they are Na⁺-dependent or Na⁺-independent, and each of these categories is subdivided into 3 groups depending on whether the systems prefer cationic, zwitterionic or anionic substrates. The zwitterion preferring transport processes in mammary gland are Na⁺-dependent system A₁⁻⁵ and Na⁺-independent systems L₂⁻⁵ and T⁶. System γ⁺ is a Na⁺-independent transporter of cationic amino acidsγ⁻⁸ and X_{AG}^- is a Na⁺-dependent system for anionic amino acids. A (Na⁺+Cl⁻)-dependent system, selective for Β­-amino acids, has been reported in rat mammary tissue¹⁰. In addition to these transport systems, there is yet another class of transporters that have still broader specificity. The Na⁺-dependent systems, BCI⁻-dependent and BCI⁻-independent in mouse mammary tissue¹¹ and a Na⁺-independent system in mouse⁵ and rat¹²,¹³ mammary tissue have been reported to mediate the transport of both zwitterionic and cationic amino acids. A system that may have broad specificity and is activated by cell swelling has also been documented¹⁴.

L-glutamate is the most abundant amino acid in milk proteins. The demand for this amino acid is reflected in the large arteriovenous differences of L-glutamate across the lactating mammary gland in sheep and bovine¹⁵,¹⁶. Indeed, in some species, the extraction of L-glutamate from plasma by lactating mammary gland exceeds 70% of the arterial load, and L-glutamate uptake in some cases is the highest of all non-essential amino acids¹⁶. Millar et al.⁵ have shown that the system X_{AG}^- mediates the transport of L-glutamate into rat mammary tissue. We have now designed experiments to further characterize the properties of L-glutamate transporter in mouse mammary gland.

Lactogenic hormones are required by mammary alveolar cells to acquire the ability to secrete milk¹⁸. The initiation of milk protein synthesis in mammary gland explants from mid-pregnant mice can be accomplished by incubation with insulin, cortisol and prolactin¹⁹. Amino acid transport systems A, L, γ⁺ and BCI⁻-independent are upregulated by lactogenic hormones in pregnant mammary tissue explants²⁰,²¹. The present communication reports that L-glutamate transporter is unaffected by lactogenic hormone in pregnant mouse mammary tissue cultured in vitro.

Materials and Methods

Chemicals and buffers—L-[U-¹³C]glutamic acid (Sp. activity 250 mCi/mmol) was obtained from the Board of Radiation and Isotope Technology, Mumbai 400 094, India. Amin(oxy)acetic acid, D-aspartic acid, D-glutamic acid, ovine prolactin, bovine insulin and Medium 199 with Earle’s salt and glutamine were from Sigma (St. Louis, MO 63178, USA). The other chemicals used were from Himedia (Mumbai 400 086, India) or Loba (Mumbai 400 002, India) or Sarabhai Merk (Mumbai 400 071, India).

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Hepes-buffered sodium Ringer phosphate (Na+ plus Cl−-buffer) solution contained (mM) NaCl, 106.5; KCl, 5; NaHCO₃, 5; NaH₂PO₄, 0.6; Na₂HPO₄, 1; CaCl₂, 2; MgCl₂, 1; glucose, 20; and Hepes, 20, and pH adjusted to 7.4 with 1M NaOH. Hepes-buffered sodium-free Ringer (Na+ -free buffer) solution contained (mM) choline chloride, 113; MgSO₄, 1; KHCO₃, 5; CaCl₂, 2; glucose, 20, and Hepes, 20, and pH adjusted to 7.4 with 1M Tris. Hepes-buffered chloride-free sodium Ringer phosphate (Cl−-free Na+ -buffer) solution contained (mM) sodium gluconate, 111.5; KHCO₃, 5; MgSO₄, 1; calcium gluconate, 2; NaH₂PO₄, 0.6; Na₂HPO₄, 1; glucose, 20, and Hepes, 20, and pH adjusted to 7.4 with 1M NaOH. All buffer solutions were equilibrated with air.

Animals and tissue preparation—Swiss mice were maintained on laboratory stock diet in Small Animal House of the Institute. Mammary tissue was taken from 12-14 day pregnant or 10 day post-partum lactating mice. The stage of pregnancy was determined by counting days after the appearance of a vaginal plug. For lactating mice, litters were adjusted to 8 within 1 day of parturition.

The animals were killed by cervical dislocation and mammary glands were dissected into the buffer solution used for amino acid uptake studies. Fat and connective tissue were removed and the mammary gland was diced into 0.5 - 1 mm segments. The tissue was incubated in the same buffer for 30 min at 37°C in a shaking water bath to stabilize its amino acid content.

Measurement of amino acid transport—The amino acid uptake by mammary slices was determined by incubating tissue (40-70 mg) in the selected buffer (details given in table and figure legends) containing appropriate concentration of unlabelled amino acid together with 0.25 μCi/ml of labelled amino acid. Amino(ox)acetic acid (0.5 mM) was included in the buffer to inhibit cell amino acid metabolism. Where the concentration of substrate and competing amino acid exceeded 20 mM, isomolarity of the medium was maintained by decreasing the concentration of NaCl or sodium gluconate or choline chloride. Incubations were carried out in a shaking water bath (90 cycles/min) for the period during which the uptake of amino acid was linear. After incubation, the tissue was washed for 15 min with two changes of chilled buffer to remove extracellular radioactivity. Excess moisture was removed from the tissue by blotting it between the leaves of moist Whatman filter paper. The tissue was dissolved and radioactivity counted in a liquid scintillation counter (Model 1600 CA TriCarb from Packard, Meriden, CT 06450, USA). Portions of incubation medium were also counted. Results were calculated as nmol amino acid accumulated/g mammary cells from measurement of radioactivity and amino acid concentration in the uptake medium and the extracellular space determined using [14C]sucrose in mammary tissue as the conversion factor.

Effect of pH and preloading mammary tissue with amino acids—The effect of decreasing pH on L-glutamate uptake was studied using medium in which Hepes was replaced by phosphate buffer (Na₂HPO₄ and NaH₂PO₄ at a total concentration of 10 mM) and the concentration of CaCl₂ was kept 0.5 mM. The effect of intracellular accumulation of amino acids on L-glutamate uptake was studied by loading the mammary slices with selected amino acid prior to uptake measurement. The diced mammary tissue was incubated with amino acid (10 mM) in Na⁺+Cl⁻ buffer at 37°C for 30 min in a shaking water bath. The control tissue was incubated in the same buffer without amino acid. At the end of loading period, the tissue slices were washed with buffer (ice-cold) used for uptake studies.

Mammary explant culture—The mammary gland explants from 12-14 day pregnant mice were cultured in Medium 199 as described previously. When the effect of hormones were investigated these were added in the culture medium at 5 μg/ml. The culture dishes were incubated at 37°C for 48 hr in humidified CO₂-air (5:95) atmosphere. Explants collected subsequently were washed with buffer (ice-cold) used for uptake studies.

Statistics—Statistical analysis was performed using Student’s unpaired t test and results are expressed as means ± SE.

Results

Time course of L-glutamate uptake—Time course of L-glutamate uptake by lactating mouse mammary tissue was studied using an external L-glutamate concentration of 0.05 mM in Na⁺+Cl⁻ buffer for 30 min. The uptake was linear for 10 min (Fig. 1) and then slowly began to level off. In subsequent experiments, an incubation time of 10 min was used for determining the initial rate of L-glutamate uptake.

Na⁺ and Cl⁻ dependence of L-glutamate uptake—The effects of Na⁺ and/or Cl⁻ replacement on L-glutamate uptake were studied in lactating mouse mammary tissue using an external L-glutamate concentration of 0.05 mM in Na⁺+Cl⁻ buffer for 30 min.
concentration of 0.05 mM. The results show that over 90% of L-glutamate uptake was Na\(^+\)-dependent. The replacement of Cl\(^-\) with gluconate caused no change in the uptake of L-glutamate (data not shown).

**Concentration dependence of L-glutamate uptake**

Fig. 2 shows the rate of L-glutamate uptake as a function of external L-glutamate concentration in lactating mouse mammary tissue. L-glutamate uptake from Na\(^+\)+Cl\(^-\) buffer was a saturable process. In absence of external Na\(^+\), the uptake of L-glutamate was linear with respect to external L-glutamate concentration hence the Na\(^+\)-independent uptake was via diffusion.

**Effect of amino acids on L-glutamate uptake**—In order to get a better appraisal as to the kind of transport system involved in the uptake of L-glutamate, the effect of various amino acids was examined on L-glutamate uptake. With a view to look for possible heterogeneity of L-glutamate transporter, the effect of competing amino acids on L-glutamate uptake was studied at both the low (0.05 mM) and high (1 mM) L-glutamate concentration in the external medium. Table 1 shows that the Na\(^+\)-dependent uptake of L-glutamate was almost completely inhibited by D- and L-aspartate and

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**Table 1**

<table>
<thead>
<tr>
<th>Competing amino acid</th>
<th>Media Na(^+)+Cl(^-) buffer</th>
<th>Na(^+)-free buffer</th>
<th>Na(^+)-dependent uptake(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>82.2±6.0</td>
<td>6.1±0.3</td>
<td>76.1±6.1</td>
</tr>
<tr>
<td>L-aspartate</td>
<td>9.8±1.7</td>
<td>4.8±0.2</td>
<td>5.0±1.7**</td>
</tr>
<tr>
<td>D-aspartate</td>
<td>11.5±0.7</td>
<td>7.2±0.9</td>
<td>4.3±1.5**</td>
</tr>
<tr>
<td>L-cysteate</td>
<td>7.7±1.4</td>
<td>5.5±0.9</td>
<td>2.1±0.8**</td>
</tr>
<tr>
<td>D-glutamate</td>
<td>55.7±2.2</td>
<td>6.3±1.0</td>
<td>49.3±1.7*</td>
</tr>
<tr>
<td>L-alanine</td>
<td>62.1±6.6</td>
<td>9.7±1.2</td>
<td>52.3±7.7*</td>
</tr>
<tr>
<td>L-serine</td>
<td>57.0±6.8</td>
<td>9.9±0.5</td>
<td>47.1±6.8*</td>
</tr>
<tr>
<td>L-histidine</td>
<td>57.4±3.5</td>
<td>9.7±1.1</td>
<td>47.7±3.2*</td>
</tr>
<tr>
<td>L-cystine</td>
<td>ND</td>
<td>11.4±2.9</td>
<td>-</td>
</tr>
<tr>
<td>Experiment 1&lt;sup&gt;\text{b}&lt;/sup&gt;</td>
<td>274.7±2.9</td>
<td>113.5±4.4</td>
<td>156.7±7.6</td>
</tr>
<tr>
<td>None (Control)</td>
<td>141.7±2.9</td>
<td>110.1±4.4</td>
<td>31.6±2.9**</td>
</tr>
<tr>
<td>D-aspartate</td>
<td>127.5±5.3</td>
<td>83.5±6.7</td>
<td>41.8±5.5**</td>
</tr>
<tr>
<td>L-cysteate</td>
<td>124.8±3.0</td>
<td>105.3±11.3</td>
<td>18.4±6.6**</td>
</tr>
<tr>
<td>L-alanine</td>
<td>212.9±4.9</td>
<td>89.2±6.6</td>
<td>122.0±8.1*</td>
</tr>
<tr>
<td>L-serine</td>
<td>209.1±9.4</td>
<td>100.2±7.2</td>
<td>108.9±13.5*</td>
</tr>
</tbody>
</table>

<sup>a</sup>Difference between L-glutamate uptake in Na\(^+\)+Cl\(^-\) buffer and Na\(^+\)-free buffer.

<sup>b</sup>The internal L-glutamate and competing amino acid concentrations were 0.05 mM and 5 mM, respectively and n=4.

<sup>\text{c}</sup>The external L-glutamate and competing amino acid concentrations were 1 mM and 15 mM, respectively and n=6.

Values for Na\(^+\)-dependent uptake were compared with their control value for significance: P values; *<0.05, **<0.01.
The effect of D-glutamate and neutral amino acids on L-glutamate uptake was much weaker. The pattern of inhibition by amino acids of L-glutamate was similar at both the low and high L-glutamate concentration, suggesting the absence of low affinity L-glutamate transporter in mouse mammary gland. Since the Na⁺-independent uptake of L-glutamate was not inhibited by any anionic or neutral amino acid or cysteic acid, the possibility of Na⁺-independent transport system for anionic amino acids in lactating mouse mammary tissue is also ruled out.

Effect of D-aspartate and L-serine on the uptake of L-glutamate at low pH—Effect of D-aspartate and L-serine at pH 6 on the uptake of L-glutamate was studied to examine the possibility of the transport of anionic amino acid by protonated ASC system. The inhibition of L-glutamate uptake reached maximum (77%) at an external D-aspartate concentration of 2 mM (Table 2), thus saturating the anionic amino acid specific transport system. Inclusion of L-serine in the external medium containing 5 mM D-aspartate caused no further significant inhibition of L-glutamate uptake which rules out the mediation of protonated ASC system in the uptake of L-glutamate by lactating mouse mammary tissue.

Uptake of L-glutamate in mammary tissue loaded with amino acids—The characteristics of L-glutamate transporter were further studied by examining the effect of preloading mammary cells with L-serine and L-glutamate individually. Preloading mammary tissue with L-serine had no effect, while prior loading with L-cysteate. The effect of D-glutamate and neutral amino acids on L-glutamate uptake was much weaker.

Effect of pH on L-glutamate uptake—Effect of decreasing pH on L-glutamate uptake was studied in lactating mouse mammary tissue (Fig. 4). The uptake of L-glutamate slightly decreased at pH 6.5 and 6.

Effect of N-ethylmaleimide on L-glutamate uptake—Prior to uptake measurement, the mammary tissue slices were incubated with N-ethylmaleimide at 37°C for 15 min. This treatment diminished the uptake of L-glutamate, with maximal inhibition at 2 mM N-ethylmaleimide (Fig. 5).

Effect of lactogenic hormones on L-glutamate uptake in mammary tissue cultured in vitro—Mammary gland explants prepared from pregnant mice were cultured in vitro for 48 hr in presence of absence of lactogenic hormones, insulin, cortisol and prolactin. The uptake of L-glutamate from Na⁺+Cl⁻ buffer was 15.2±1.6 and 19.8±1.9 nmol/g cells/10 min (n=6) in mammary explants cultured in the absence and presence of lactogenic hormones, respectively. The increase in L-glutamate uptake was insignificant compared to several fold increase observed for the hormone responsive amino acid transport systems A, L and y⁰⁻⁵⁺.

Discussion

Three transport systems capable of transporting anionic amino acids have been characterized in mammalian cells: i) a high affinity (Kₐ 1-290 μM) Na⁺-
Fig. 3—Effect of preloading mammary tissue with amino acids on uptakes of L-glutamate [Prior to uptake measurement, mammary tissue slices were incubated in a shaking water bath (90 cycles/min) at 37°C for 30 min in Na⁺-Cl⁻ buffer containing individual amino acids at 10 mM. Control tissue slices were incubated in the same buffer with no added amino acid. At the end of the loading period, tissue slices were washed with ice-cold buffer. Uptakes of L-glutamate were measured using an external L-glutamate concentration of 0.05 mM in Na⁺-Cl⁻ buffer. Values are means for 10 determinations with SE indicated by vertical bars].

Dependent system, designated $X_{AG}^-$ has been described\(^{20}\). This system plays an important role in neurotransmission\(^{21}\) and in transport of L-glutamate in mammalian tissue\(^{22}\); ii) a low affinity ($K_m$ 2-4 mM), Na⁺-dependent L-glutamate transporter has been identified in the fibroblasts\(^{20}\), hepatocytes\(^{22}\) and intestine\(^{23}\); iii) a Na⁺-independent system $Xc^-$ capable of transporting L-glutamate and cystine has been described\(^{24}\).

Heterogeneity of anionic amino acid transport systems has been reported in several tissues such as central nervous system\(^{25}\), renal brush border membrane vesicles from rat and rabbits\(^{26}\) and rabbit jejunal brush border membrane vesicles\(^{27}\). The present results show that the transport of L-glutamate in mouse mammary tissue is via a Na⁺-dependent system having characteristics similar to system $X_{AG}^-$. Na⁺-dependent uptake of L-glutamate in mouse mammary tissue was strongly inhibited by both D- and L-isomers of aspartate and only L-isomer of glutamate. This stereo-selectivity for glutamate but not for aspartate is similar to the pattern described\(^{22}\) for system $X_{AG}^-$. L-glutamate transport in mouse mammary tissue was partially inhibited at low pH similar to system $X_{AG}^-$ in human placental trophoblast microvillus membrane vesicles\(^{28}\). Therefore, it is concluded that the anionic amino acid

![Graph of pH vs. L-glutamate uptake](image)

Fig. 4—Effect of pH on uptakes of L-glutamate by lactating mouse mammary tissue [Uptakes were measured using an external L-glutamate concentration of 0.05 mM in modified Na⁺-Cl⁻ solution buffered with NaH₂PO₄ and Na₂HPO₄ at final concentration of 10 mM. Values are means for 10 determinations with SE indicated by vertical bars].

![Graph of N-ethylmaleimide concentration vs. L-glutamate uptake](image)

Fig. 5—Effect of N-ethylmaleimide on L-glutamate uptake by lactating mouse mammary tissue [Prior to uptake measurement, mammary slices were incubated in a shaking water bath (90 cycles/min) at 37°C for 15 min with N-ethylmaleimide in Na⁺-Cl⁻ buffer. Control tissue slices were incubated in the same buffer with no added N-ethylmaleimide. At the end of incubation period, tissue slices were washed with ice-cold buffer. Uptakes of L-glutamate were measured using an external L-glutamate concentration of 0.05 mM in Na⁺-Cl⁻ buffer. Values are means for 5 determinations with SE indicated by vertical bars. *Values are significantly different from control: $P<0.001$].

$\text{Control} \hspace{1cm} \text{Serine} \hspace{1cm} \text{Glutamate}$
transporter in mouse mammary gland is system Xag-

The present study further established that the Xag-

transporter is highly sensitive to treatment with N-

ethylmaleimide, suggesting the participation of its

sulfhydryl groups in the transport of L-glutamate.

Down regulation of system Xag was also observed in

mouse mammary tissue preloaded with L-glutamate.

L-glutamate uptake in mouse mammary tissue was

weakly inhibited by neutral amino acids suggesting the

possibility for a low affinity system similar to that

characterized in other tissue. To look for such

possibility, the inhibition of Na+-dependent uptake of

L-glutamate by L-serine was studied in the presence of

system Xag saturating concentration of D-aspartate

under slightly acidic (pH 6) conditions. Low pH was

selected keeping in view of the observation that ASC-

like activity may serve upon protonation as a low

affinity pathway for anionic amino acids. The choice

of inhibitory amino acid was dictated by the fact that

the Xag system displays no stereo-specificity for

aspartate, whereas, ASC-like system excludes D-

aspartate. L-serine was chosen for being a preferred

substrate of the ASC system. The present results show

that there was no significant inhibition of L-glutamate

uptake by L-serine in the presence of 5 mM D-aspartate

at pH 6, hence ruled out the possibility of low-affinity

Na+-dependent ASC-like system. This conclusion is

further supported by recent observation that the ASC

system is absent in mouse mammary gland.

At physiological concentration, over 90% of L-

glutamate uptake by mouse mammary tissue was via

Na+-dependent Xag system. The Na+-independent

uptake of L-glutamate was linear with respect to its

external concentration (Fig. 3), and was not inhibited

by any amino acid including cystine and anionic amino

acids, thus, rules out the possibility of any Na+-

independent transporter of L-glutamate in mouse

mammary tissue.

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