Characterization of glutamine uptake in mouse two-cell embryos and blastocysts

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Summary. Mouse two-cell embryos and blastocysts take up [^3H]glutamine *in vitro* at a constant rate for at least 15 min, depending on the concentration of glutamine and developmental stage of the embryo. Uptake by two-cell embryos can be resolved into two saturable components. The major contributing system is Na^+ independent, inhibited by alanine, methionine, 2-amino-2-norbornanecarboxylic acid (BCH) or leucine and has a K_m of $3856 \pm 672 \mu\text{mol l}^{-1}$ and $V_{\max}$ of $436 \pm 58 \text{ fmol per embryo per 10 min}$. These features are characteristics of the ubiquitous system L transporter. The second component is Na^+ dependent with K_m of $1064 \pm 914 \mu\text{mol l}^{-1}$ and $V_{\max}$ $107 \pm 47 \text{ fmol per embryo per 10 min}$. Similar $V_{\max}$ and inhibition of this component by glycine suggest a low reactivity with the *gly*-system. Blastocyst uptake of glutamine is mainly by a Na^+ -dependent saturable mechanism with K_m of $524 \pm 75 \mu\text{mol l}^{-1}$ and $V_{\max}$ of $1264 \pm 101 \text{ fmol per embryo per 10 min}$ which is inhibited by alanine, isoleucine, leucine and BCH, features characteristic of the system $\text{B}^{0,+}$. The increase in uptake capacity as a consequence of the appearance of the system $\text{B}^{0,+}$ may be related to increased metabolic requirements for glutamine, in the rapidly expanding blastocyst.

Keywords: glutamine transport; blastocyst; embryo; system L; system $\text{B}^{0,+}$; mouse

Introduction

Glutamine is an important component of many mammalian cell culture media. As well as aiding the maturation and development of embryos from many species (hamster: Gwatkin & Haidri, 1973; Carney & Bavister, 1987; rabbit: Bae & Foote, 1975; pig: Petters *et al.*, 1990; mouse: Chatot *et al.*, 1990), it has been identified as a major alternative energy source to glucose for bovine blastocysts (Rieger & Guay, 1988) and human diploid fibroblasts (Zielke *et al.*, 1984).

The concentration of glutamine in mouse oviductal fluid is high (Gardner & Leese, 1990). The uptake of glutamine by preimplantation embryos *in vitro* has been measured with ultramicrofluorometry (Gardner *et al.*, 1989) and radiolabelled glutamine (Chatot *et al.*, 1990), but the specific transporting system into the embryo has not been identified.

Study of amino acid uptake has led to the identification of several specific transport mechanisms. In preimplantation mouse embryos, these include the Na^+ -independent system L (Christensen, 1979), present in all preimplantation stages, and the Na^+ -dependent system *gly* (Hobbs & Kaye, 1985, 1986), most apparent in two-cell embryos, and $\text{B}^{0,+}$ (Van Winkle & Campione, 1987; Van Winkle, 1988), which appears at about compaction and becomes increasingly dominant in activity at the blastocyst stage.

This study aimed to determine the specific mode of glutamine uptake by mouse embryos at the two-cell and blastocyst stages.

*Reprint requests.

Materials and Methods

Superovulation and embryo collection

Randomly bred Quackenbush mice (10 weeks) were superovulated with 5 iu pregnant mares' serum gonadotrophin (PMSG) (Folligon: Intervet (Aust Pty Ltd, Artarmon, NSW, Australia), followed 44–50 h later by 5 iu human chorionic gonadotrophin (hCG) (Chorulon: Intervet (Aust)). Injections were given between 09:00 and 12:00 h. Immediately after hCG injection, females were placed with males and left until 09:00 h on the following day, when they were checked for vaginal plugs. Two-cell and blastocyst embryos were collected from the oviduct (48 h after hCG) or uterus (96 h after hCG), respectively, into M2 medium (Fulton & Whittingham, 1978).

Radioactive media were prepared by the addition of M2 containing glutamine at the appropriate concentrations, to freeze-dried [^3H]glutamine (1 Ci l^{-1} , 46 Ci mmol^{-1} , [G- ^3H]glutamine (Amersham, North Ryde, NSW, Australia)) or [^3H]glycine (1 Ci l^{-1} , 18.5 Ci mmol^{-1} , [2- ^3H]glycine (Amersham)).

Na^+ -free medium M2

Medium M2 was prepared without Na^+ by replacing NaCl and NaHCO_3 on an equimolar basis with choline chloride and KHCO_3 , respectively. The Hepes was brought to pH 7.4 with KOH. Embryos were incubated for 30 min in this medium.

Glutamine uptake

Glutamine uptake was measured by incubating embryos on a heating block at 37°C , in a 20–50 μl droplet of radioactive medium under 3 ml of light paraffin oil (Caltex, Australia Pty Ltd, Brisbane) in a plastic Petri dish. After a specific time, the embryos were removed from the droplet and washed four times in ice-cold M2 without glutamine. Afterwards they were placed into plastic minicounting vials and 0.1 ml H_2O and 1.0 ml Optiphase Hi-safe 3 counting cocktail (LKB, Wallac, Bromma, Sweden) was added before analysis in a Packard 1900 CA liquid scintillation system. Na^+ -dependent uptake was calculated by subtracting the mean rate of uptake in the absence of Na^+ from the mean rate of uptake in the presence of Na^+ .

Descending paper chromatography

Blastocysts were incubated for 10 min in medium containing $19\text{ }\mu\text{mol } [^3\text{H}]\text{glutamine l}^{-1}$, washed rapidly in ice-cold M2 then placed into $10\text{ }\mu\text{l}$ M2 to which was added $10\text{ }\mu\text{l}$ 20% CCl_3COOH . After centrifuging for 5 min at 5000g, the supernatant was spotted on to 3M filter paper and separated using 12:3:5, $\text{CH}_3(\text{CH}_2)_2\text{CH}_2\text{OH}$: $\text{CH}_3\text{COOH}:\text{H}_2\text{O}$ as the solvent. Nonradioactive marker glutamine was located by ninhydrin. The filter paper was cut into 1.5-cm squares and radioactivity was determined.

Glutamine efflux

Embryos were incubated at 37°C for 10 min in [^3H]glutamine, rapidly washed and replaced into a droplet of medium without glutamine. Samples of embryos were taken at various times and prepared for liquid scintillation analysis as described previously.

Competition

Embryos were incubated in media containing $19\text{ }\mu\text{mol } [^3\text{H}]\text{glutamine l}^{-1}$ and one of the following at 1 mmol l^{-1} : leucine, 2-amino-2-norbornanecarboxylic acid (BCH), alanine, lysine, isoleucine, methylaminoisobutyric acid (MeAIB), tryptophan, D-glutamine or glycine. After 10 min, the embryos were removed, washed and analysed.

Inhibition of [^3H]glycine uptake by glutamine

Embryos were incubated in medium containing $18.5\text{ }\mu\text{mol } [^3\text{H}]\text{glycine l}^{-1}$ and 0.2, 1, 5 or 25 mmol unlabelled glutamine l^{-1} . After 10 min, the embryos were removed, washed and analysed.

Standardization

All measurements of uptake were internally standardized as follows: at the end of an incubation, radioactivity was measured in samples of the medium and the mean was used to calculate the specific radioactivity of the droplet, using

the known glutamine concentration and allowing for the contribution of [^3H]glutamine. Background samples consisted of equivalent volumes to that transferred with the embryos being collected from the final wash medium. The measurements obtained rarely differed from standard background measurements. The counting efficiency was 40% with a standard background of 10–12 c.p.m.

Values presented are means $\pm$ SEM of three experiments containing 6–14 observations unless otherwise indicated. Kinetic parameters for saturable glutamine uptake were determined by nonlinear regression using the equation $v = K_0[\text{Gln}] + (V_{\max}[\text{Gln}]/K_m + [\text{Gln}])$ where v is the initial rate of uptake, K_0 is the rate constant for the nonsaturable transport and K_m and $V_{\max}$ are the kinetic parameters describing the saturable transport component (Duggleby, 1984; Hobbs & Kaye, 1990). Lines of best-fit were calculated from the linear regression for uptake experiments.

Results

Paper chromatography revealed that at least 90% of the radioactivity in blastocysts after incubation for 10 min partitioned with glutamine.

There was no significant loss of pre-accumulated [^3H]glutamine into amino-acid-free medium on ice by two-cell embryos after preloading in medium containing 100 μmol or 1 mmol glutamine l^{-1} . Loss by blastocysts over 15 min was not significant when preloading occurred in 100 μmol [^3H]glutamine l^{-1} , but, when preloading occurred in 1 mmol glutamine l^{-1} , about 30% of accumulated glutamine was lost after 15 min (Fig. 1). Thus, losses during the washing procedure of about 5 min were insignificant.

Fig. 1. Efflux of [^3H]glutamine on ice from two-cell mouse embryos preloaded at 37°C with 100 μmol (●) or 1 mmol glutamine l^{-1} (■) and blastocysts at 100 μmol (○) and 1 mmol (□) glutamine l^{-1} . Results from two experiments, mean $\pm$ SEM each with four to seven embryos per time point; *significantly less than value at zero time ($P < 0.05$) for blastocysts preloaded with 1 mmol glutamine l^{-1} .

Uptake

Two-cell embryos took up [^3H]glutamine at a constant rate of 1.8 fmol per embryo min^{-1} ($r = 0.92$, $n = 48$, $P < 0.001$) for at least 30 min at 37°C in 100 μmol glutamine l^{-1} (Fig. 2a) and 5.6 fmol per embryo min^{-1} ($r = 0.87$, $n = 56$, $P < 0.001$) for at least 15 min in 1 mmol glutamine l^{-1} (data not shown). Afterwards the uptake rate appeared to plateau.

Blastocysts maintained a constant uptake rate of 33 fmol per embryo min^{-1} over 60 min ($r = 0.93$, $n = 52$, $P < 0.001$) in 100 μmol glutamine l^{-1} (Fig. 2b).

Na⁺-free conditions

Uptake of glutamine by two-cell embryos (Fig. 2a) and blastocysts (Fig. 2b), incubated in Na⁺-free M2 containing 100 μmol glutamine l^{-1} , was constant over at least 60 min. In the absence

Fig. 2. Glutamine ($100 \mu\text{mol l}^{-1}$) uptake by two-cell mouse embryos (a) or blastocysts (b) incubated at 37°C . Each point represents 4–6 embryos, mean $\pm$ SEM from three experiments: Na⁺ present (○) or absent (●) from medium M2.

of Na⁺, the uptake of glutamine by two-cell embryos was reduced by about 30% and in blastocysts by about 98%.

Saturation kinetics

Total uptake of glutamine by two-cell embryos did not reach a plateau even at 12 mmol l^{-1} , indicating a nonsaturable component. The uptake could be divided into a saturable Na⁺-dependent component and a Na⁺-independent component that appeared not to be saturable (Fig. 3a). The kinetic parameters for the Na⁺-dependent component were $K_m 1064 \pm 914 \mu\text{mol l}^{-1}$ and $V_{\max} 107 \pm 47 \text{ fmol per embryo per 10 min}$, fitted using nonlinear regression analysis and allowing for a nonsaturable component.

The Na⁺-independent component had the following values: $K_m 3856 \pm 672 \mu\text{mol l}^{-1}$ and $V_{\max} 436 \pm 58 \text{ fmol per embryo per 10 min}$ by nonlinear regression, when the nonsaturable rate constant was set at 9 pl per embryo per 10 min as reported by Hobbs & Kaye (1990). Graphical analysis using Eadie–Hofstee plots (Van Winkle *et al.*, 1990) allowing for a nonsaturable component with rate constant 9 pl per embryo per 10 min (Hobbs & Kaye, 1990) showed only one saturable component of Na⁺-independent uptake (Fig. 3) with $K_m 2767 \pm 525 \mu\text{mol l}^{-1}$ and $V_{\max} 369 \pm 50 \text{ fmol per embryo per 10 min}$; $n = 8$, $r = 0.900$, not significantly different from values estimated by nonlinear regression.

Total glutamine uptake by blastocysts appeared to be saturable up to 2 mmol l^{-1} at 37°C (Fig. 4a). Nonlinear curve fitting showed $K_m 524 \pm 75 \mu\text{mol l}^{-1}$ and $V_{\max} 1264 \pm 101 \text{ fmol per embryo per 10 min}$, when the rate constant for the nonsaturable component was set at 40 pl per embryo per 10 min (Hobbs & Kaye, 1990). Graphical analysis using Eadie–Hofstee plots with a set

Fig. 3. Saturation curves of glutamine uptake by two-cell mouse embryos at 37°C. Each point represents 4–8 embryos, mean $\pm$ SEM from five experiments, total glutamine uptake (●), Na⁺-independent uptake (■) and Na⁺-dependent curves (◆). (a) The lines were fitted to the equation $v = K_0[\text{Gln}] + V_{\max}[\text{Gln}]/K_m + [\text{Gln}]$ with K_0 9 pl per embryo per 10 min, K_m 3856 $\mu\text{mol l}^{-1}$ and $V_{\max}$ 436 fmol per embryo per 10 min in the absence of Na⁺, K_0 0, K_m 1064 $\mu\text{mol l}^{-1}$ and $V_{\max}$ 107 fmol per embryo per 10 min for Na⁺ dependent. (b) The data for Na⁺-independent uptake were transformed into an Eadie-Hofstee plot with same allowance for diffusion and fitted by linear regression, $n = 8$, $r = 0.90$, $P < 0.05$.

nonsaturable rate constant of 40 pl per embryo per 10 min showed only one saturable component (Fig. 4b) with K_m $252 \pm 110 \mu\text{mol l}^{-1}$ and $V_{\max}$ 1049 ± 212 fmol per embryo per 10 min, which are comparable to the values obtained by nonlinear regression.

Competition with other amino acids

When incubated with 1 mmol isoleucine, tryptophan or leucine l⁻¹, two-cell embryos showed a significant reduction in glutamine uptake (Fig. 5). There was no significant inhibition by alanine, glycine, MeAIB, D-glutamine or BCH in normal M2, but, when embryos were incubated in Na⁺-free M2 (Fig. 5b) with 1 mmol alanine, leucine, methionine or BCH l⁻¹, there was a significant inhibition, the last three being about five times more effective than alanine.

Glutamine inhibited the uptake of [³H]glycine (23 $\mu\text{mol l}^{-1}$) when two-cell embryos were incubated at 37°C for 10 min in the presence of Na⁺ (Fig. 6). There was no inhibition with 0.2 mmol glutamine l⁻¹, but at 1, 5 and 25 mmol l⁻¹ glycine uptake was inhibited by at least 30%.

At the blastocyst stage (Fig. 7), glutamine uptake was significantly inhibited by alanine, isoleucine, tryptophan, leucine, lysine, glycine (50–70%), and by BCH (about 30%). There was no significant inhibition by MeAIB.

Fig. 4. Na^+ -dependent uptake of glutamine by mouse blastocysts at 37°C . Each point represents 5–10 embryos, mean $\pm$ SEM from five experiments. (a) The line was fitted to the equation $v = K_0[\text{Gln}] + V_{\text{max}}[\text{Gln}]/K_m + [\text{Gln}]$ with K_0 40 pl per embryo per 10 min, K_m $524 \mu\text{mol l}^{-1}$ and V_{max} 1264 fmol per embryo per 10 min. (b) After allowance for diffusion (as above), the data were transformed to an Eadie–Hofstee plot and the line was fitted by linear regression, $n = 11$, $r = 0.61$, $P < 0.05$.

Discussion

The chromatography established that at least 90% of the radiolabel taken up by blastocysts over 10 min remained as glutamine. In addition to confirming that embryo-derived ^3H represents accumulated glutamine, this result indicates that the conversion of glutamine to other metabolites is slow, as observed by Chatot *et al.* (1990).

Glutamine was readily taken up by two-cell embryos, confirming previous studies (Gardner *et al.*, 1989; Chatot *et al.*, 1990). However, the present results show that the rate of glutamine uptake at 0.1 and 1 mmol l^{-1} varies with time and was constant only over the initial 30 min in two-cell embryos. The initial rate varied with the concentration of glutamine, thus indicating that the kinetics of uptake are complex (Fig. 3).

In these studies, each embryo accumulated radioactivity. This contrasts with the 'negative uptake' or loss of embryonic glutamine by some embryos reported by Gardner *et al.* (1989). It is not possible to measure efflux of ^3H glutamine without preaccumulation. However, it is possible that the small efflux apparent after 15 min in our studies could become significant over 2 h and approach the rates of loss observed in that earlier study. Our aim was primarily to determine the extent of reversibility of the transport system, and the data suggest this to be a minor component of the net flux of glutamine across the embryonic membranes.

Fig. 5. Uptake of $[^3\text{H}]$ glutamine by two-cell mouse embryos (a) in the presence or (b) in the absence of Na^+ and in the presence of alanine (Ala), isoleucine (Iso), tryptophan (Trp), leucine (Leu), 2-amino-2-norbornanecarboxylic acid (BCH), methylaminoisobutyric acid, (MeAIB), D-glutamine (D-Gln), methionine (Met), or glycine (Gly); *significantly lower than control value ($P < 0.05$), mean $\pm$ SEM from three experiments each with 6–8 embryos.

Fig. 6. Glutamine inhibition of $23 \mu\text{mol l}^{-1}$ $[^3\text{H}]$ glycine uptake by two-cell mouse embryos. Significantly less than value with no glutamine, * $P < 0.05$, ** $P < 0.005$. Each point represents mean $\pm$ SEM from two experiments with 4–8 embryos per trial.

Fig. 7. Uptake of [³H]glutamine by mouse blastocysts in the presence of alanine (Ala), isoleucine (Iso), tryptophan (Trp), leucine (Leu), 2-amino-2-norbornanecarboxylic acid (BCH), methylaminoisobutyric acid (MeAIB), lysine (Lys) or glycine (Gly). Each point comprises 5–8 embryos from two experiments, mean $\pm$ SEM; *significantly less than control value ($P < 0.05$).

The total uptake of glutamine by two-cell embryos was resolved into two saturable components. The major component was Na⁺-independent and accounted for about 85% of the total glutamine uptake at 0.1 mmol l⁻¹, to about 70% at 10 mmol l⁻¹. This component is probably the transporter system L, which is the principal transporter system of leucine in cleaving mouse embryos (Holmberg & Johnson, 1979). This system is also responsible, but to a lesser degree, for the transport of tryptophan, isoleucine, methionine, alanine and BCH (Van Winkle, 1988), all of which effectively inhibited Na⁺-independent glutamine uptake in two-cell mouse embryos.

The significant inhibition of glycine uptake in two-cell embryos by glutamine at concentrations > 1 mmol l⁻¹ (Fig. 6) indicates that there is a Na⁺-dependent component of glutamine uptake, accounting for approximately 35% of the total uptake in two-cell embryos. This is probably due to transport via system *gly*, which is the exclusive glycine transporter in two-cell embryos (Hobbs & Kaye, 1985, 1986; Van Winkle *et al.*, 1988). The $V_{\max}$ value for glutamine (107 ± 47 fmol per embryo per 10 min) is within the range reported for glycine uptake (Hobbs & Kaye, 1990).

In blastocysts, uptake of glutamine occurred at a constant rate of 33 fmol embryo⁻¹ per min for at least 60 min at 100 μ mol l⁻¹. This is comparable with the rate reported previously under similar conditions (Gardner *et al.*, 1989; Chatot *et al.*, 1990).

A small amount of glutamine entered blastocysts in the absence of Na⁺ and may be due to simple diffusion (Hobbs & Kaye, 1990), since there was no evidence for a second saturable system. Most glutamine uptake (99%) was Na⁺-dependent and saturable. The $V_{\max}$ value is close to that reported for alanine and glycine by system B^{0,+} (Van Winkle *et al.*, 1988). As glutamine uptake was inhibited by alanine, isoleucine, tryptophan, leucine, lysine, BCH and glycine, all known to be transported in blastocysts by system B^{0,+} (Hobbs & Kaye, 1985; Van Winkle & Campione, 1987) and not by MeAIB, a specific competitor for system A, we conclude that this Na⁺-dependent system (B^{0,+}) is the major means of glutamine uptake in blastocysts.

Recently, alanine uptake in blastocysts has been resolved into two components, B^{+,0} and B, on the basis of differential inhibition by lysine and BCH (Van Winkle *et al.*, 1990). The residual uptake of glutamine by blastocysts in the presence of these inhibitors may indicate a similarly small contribution of system B to glutamine uptake, but this was not investigated because of the number of embryos required.

In conclusion, our results show that glutamine, in common with most amino acids, enters mouse embryos predominantly by facilitated transport systems. During development there is a change in the major transporter systems responsible for glutamine uptake from systems L and *gly* in two-cell embryos to system B^{0,+} in blastocysts. This shift probably occurs at about the morula

stage of development similar to that reported for the transport of other amino acids such as alanine (Van Winkle *et al.*, 1990).

The threefold increase in $V_{\max}$ for glutamine uptake during development to the blastocyst stage may account for the threefold increase in the embryonic pool of glutamine (Shultz *et al.*, 1981). This increased uptake capacity may enable blastocysts to provide for the large increase in protein synthesis that occurs after compaction (Brinster, 1971; Sellens *et al.*, 1981; Harvey & Kaye, 1988).

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