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Amino acid carryover in the subzonal space of mouse fertilized ova affects subsequent transport kinetics

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Summary

We have investigated whether culture in glycine-containing medium affects subsequent glycine transport by the specific transport system, GLYT1, which is the sole glycine transporter in fertilized mouse ova. When fertilized ova were maintained for 6 h in culture with a physiological level of glycine (1 mM), subsequent transport of radiolabelled glycine was decreased by 40% compared with fertilized ova that had been maintained in glycine-free medium. Kinetic measurements showed that the apparent glycine affinity was decreased after culture with glycine (K_m increased from 0.20 to 0.41 mM), but maximal transport rate was unchanged (similar V_{max} of 20 and 23 fmol/fertilized ovum/min). These findings could have reflected activation of GLYT1 by prolonged substrate starvation, similar to some other amino acid transport systems. However, our findings were instead consistent with the alteration in glycine transport being due to trapping of glycine within the zona pellucida resulting in competitive transport inhibition even after ova were removed from glycine-containing media. First, even very brief exposures to glycine resulted in decreased subsequent glycine transport rates, with a maximal effect apparent within ~6 min. Second, extensive washing (at least six) reversed the effect. Third, the effect was absent when zona-free fertilized ova were used. Thus, it appears that components of the external environment of preimplantation embryos may continue to affect transport kinetics for a period even after embryos are removed from environments that contain them.

Keywords: Amino acid, Fertilized ovum, Glycine, Transport, Zona pellucida

Introduction

As with other cells, the cells of preimplantation embryos transport a number of substances, including metabolic substrates, inorganic ions and the building blocks for synthesis of macromolecules, across their plasma membranes and into their cytoplasm by means of an array of specific transporters. Not only do embryos express specific transporters for substrates vital to their health, but the array of transporters changes over the course of preimplantation development, to match the needs of the embryo at each stage. For example, cleavage stage embryos have an active pyruvate transport mechanism (Leese & Barton, 1984; Harding *et al.*, 1999), while blastocysts predominantly express facilitated glucose transporters (Carayannopoulos *et al.*, 2000), reflecting stage-specific metabolic needs (Biggers *et al.*, 1967). Developmental stage-specific transporters include a number that are specialized to mediate the uptake of amino acids from the environment (Van Winkle, 2001). One of the most active amino acid transporters in early cleavage stage embryos is the glycine transporter GLYT1, which mediates the Na^+- and Cl^--coupled uptake of glycine and is present in preimplantation embryos before the morula stage (Van Winkle *et al.*, 1988). We have recently shown that GLYT1 regulates cell volume in fertilized mouse eggs via the osmoregulated accumulation of glycine in the embryo and that it is critical to embryo viability (Steeves *et al.*, 2003).

During those investigations, preliminary findings indicated that GLYT1 transport was stimulated after fertilized ova were cultured in medium that lacked

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glycine compared with those cultured in the presence of glycine, as the rate of transport of glycine into fertilized ova was substantially higher in fertilized ova that had been incubated for 6 h in glycine-free media compared to fertilized ova that had been cultured in media containing glycine. This resembled the behaviour of the classical amino acid transporter, System A (ATA2) in fibroblasts, astrocytes and other types of cells (Franchi Gazzola *et al.*, 2001; Tanaka *et al.*, 2005), in which prolonged substrate starvation induces upregulation of transport activity. We therefore performed a series of experiments designed to test whether the increased glycine transport rate observed after incubation of fertilized mouse ova in the absence of glycine was similarly a result of physiological upregulation of GLYT1 by substrate starvation, or alternatively was due to a direct effect of the presence of glycine immediately before transport measurements were performed.

Materials and methods

Chemicals and media

Except when otherwise specified, all chemicals were obtained from Sigma. Components of media were embryo-tested grade when available, or else cell-culture grade. Media were based on KSOM embryo culture medium (Lawitts & Biggers, 1993), with glutamine omitted and polyvinyl alcohol (1 mg/ml) substituted for BSA. HEPES–KSOM (pH 7.4) was used for embryo handling (Lawitts & Biggers, 1993). Glycine was added directly to media when specified.

Animals and embryos

Female CF1 strain mice (Charles River) were superovulated by i.p. injection of 5 IU eCG followed 47.5 h later by 5 IU hCG and mated overnight with BDF1 males. Fertilized ova were removed from excised oviducts by flushing with HEPES–KSOM, freed from residual cumulus matrix by brief exposure to 300 μg/ml hyaluronidase and then washed extensively before use. Fertilized ova were identified by the presence of two pronuclei. Fertilized ova were maintained in microdrop culture (15 per drop, comprising one replicate) under mineral oil at 37°C in 5% CO_2/air (Lawitts & Biggers, 1993; Dawson & Baltz, 1997; Steeves *et al.*, 2003). For the experiment in which the zonae pellucidae were removed, fertilized ova were placed in acid Tyrode's (pH 2.5; Sigma) in groups of five, observed until the zonae dissolved and then immediately washed through 10 drops of HEPES–KSOM. Transfer of fertilized ova between medium drops was done using flame-pulled Pasteur pipettes. All animal experiments were approved by the Animal Care Committee of the Ottawa Health Research Institute and conform to the standards of the Canadian Council on Animal Care.

[^{3}H]Glycine transport measurements

[^{3}H]Glycine ([^{2}H]glycine; 10–60 Ci/mmol; Amersham) was added directly to media. Standard curves for conversion of c.p.m. to concentration were generated weekly. [^{3}H]glycine was added to media at the concentrations specified, either from the stock supplied by the manufacturer for low glycine concentrations or as a proportional mixture of ^{3}H-labelled and unlabelled glycine to achieve higher total glycine concentrations. [^{3}H] was measured with a liquid scintillation counter (2200CA TriCarb, Packard Instrument Co.) with each sample counted for 5 min, as previously described (Dawson *et al.*, 1998; Steeves *et al.*, 2003).

The general procedure for the measurement of glycine transport in fertilized mouse ova is described. Fertilized ova were removed from oviducts and washed as described above. They were then placed into microdrop cultures of KSOM under oil (5% CO_2, 37°C) for periods of up to 6 h, as specified. During this culture period, the media were identical except that they either contained 1 mM glycine or no added glycine, as specified. At the end of the culture period, fertilized ova were removed from the culture drops and, if indicated, washed by transferring them through the specified number of drops of HEPES–KSOM with no glycine. A group of 10 fertilized ova was then immediately transferred to a drop of KSOM containing [^{3}H]glycine at the specified concentration for exactly 10 min and then removed for measurement of their [^{3}H]glycine content. The concentration of [^{3}H]glycine was always 10 μM during transport rate measurements except for the experiment in which glycine concentration was varied from 5 μM to 2 mM for determination of transport kinetics.

To measure the [^{3}H] glycine content of fertilized ova, 10 fertilized ova were removed from the [^{3}H] glycine-containing medium after 10 min, immediately washed through 5 drops of ice-cold HEPES–KSOM and then transferred to scintillation vials. Four millilitres of scintillation fluid (Scintiverse BD; Fisher Scientific) was added to each vial on top of the cohort of fertilized ova. Background was always measured by taking medium from the last wash drop for determining the residual counts and this background was subtracted from the counts obtained for the samples. The total glycine content of fertilized ova was calculated using the standard curves and expressed on a per fertilized ova basis. We previously showed that glycine is retained in fertilized ova and preimplantation embryos almost entirely as free glycine for at least 24 h and not significantly incorporated into macromolecules nor

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metabolized (Steeves *et al.*, 2003). Thus, we measured predominantly free glycine in these experiments, even when it had been transported into the fertilized ova.

The rate of glycine transport was calculated from the total amount of glycine taken up by the group of 10 fertilized ova (in fmol, after background was subtracted), divided by the number of fertilized ova (10) and by time of incubation with [^{3}H]glycine (10 min), to yield the rate in fmol/fertilized ovum/min. We have previously confirmed that glycine accumulation by fertilized ova is linear with time up to at least 20 min for glycine concentrations up to at least 1 mM (Steeves *et al.*, 2003).

Data analysis

Data are presented as means ± standard errors of the mean (S.E.M.). *n* values indicate number of replicates, each containing a pool of the specified number of fertilized ova (usually 10). Calculation of S.E.M. was done using the number of replicates, *n*. Means were tested for significant differences using *t*-tests to compare two means, or ANOVA followed by the Tukey–Kramer multiple comparisons post hoc test for three or more. Statistical analyses were performed using InStat (GraphPad). Curve-fitting was performed by non-linear least squares fits using SigmaPlot 8.02 (SPSS). Graphs were produced using SigmaPlot.

Results

Kinetics of glycine transport by fertilized ova after transfer from glycine-free or glycine-containing media

Fertilized ova were removed from oviducts, washed and immediately placed into culture for 6 h in either glycine-free medium or medium containing 1 mM glycine (unlabelled). A glycine concentration of 1 mM was chosen to be used throughout for groups cultured with glycine, because this approximately equals the amount of glycine in the fertilized ovum's *in vivo* environment in the oviduct, which two reports have found to be 0.6 mM and 3.2 mM in mouse (Guerin *et al.*, 1995; Harris *et al.*, 2005). The rate of glycine transport by embryos immediately after the 6 h incubation was then measured by transferring them from the culture medium into drops containing only 10 μM [^{3}H]glycine for 10 min, washing them and determining total [^{3}H]glycine content within the fertilized ova after the 10 min incubation period. The mean rate of glycine transport by fertilized ova that had been cultured for 6 h in the absence of 1 glycine was 1.10 ± 0.13 fmol/fertilized ovum/min, while after culture in the presence of 1 mM glycine, however, the mean rate of transport was 0.69 ± 0.09 fmol/fertilized ovum/min (Fig. 1*a*). Although we did not measure the rate of transport immediately after fertilized ova were obtained in this particular experiment, the rate of glycine transport after 6 h in the absence of glycine is comparable to the 1.0–1.6 fmol/fertilized ovum/min with 10 μM [^{3}H]glycine, which varies somewhat between groups of oocytes obtained on different days (below and our unpublished data). The approximately 40% reduction in the rate of transport after 6 h exposure to glycine vs. 6 h without glycine was significant ($p = 0.025$; *t*-test).

We had previously shown that glycine transport in fertilized ova essentially followed the Michaelis–Menten relation in its dependence of transport rate on external glycine concentration (Steeves *et al.*, 2003; Steeves & Baltz, 2005). The reduced rate of transport of 10 μM [^{3}H]glycine could have resulted

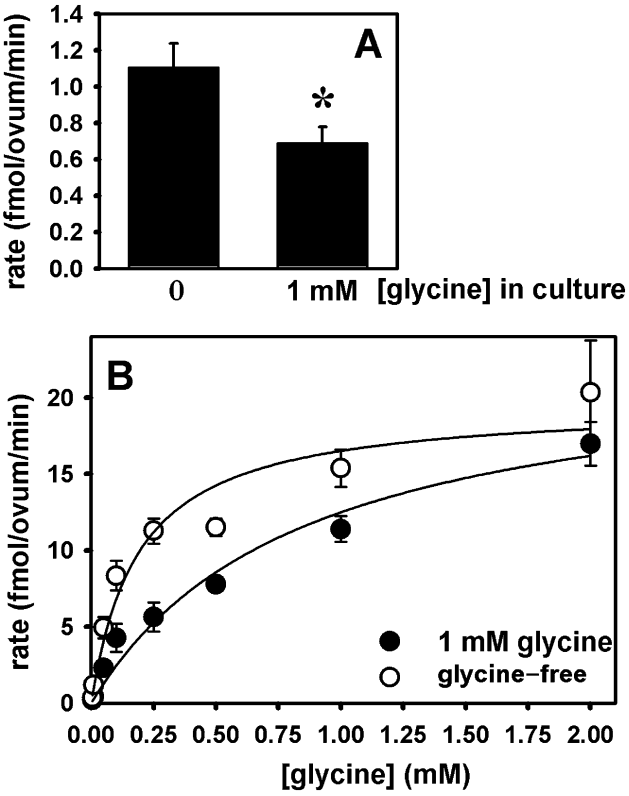

Figure 1 Effect of culture for 6 h with or without glycine on subsequent glycine transport. (*a*) The rate of transport of 10 μM [^{3}H]glycine after 6 h of culture in medium with 0 or 1 mM glycine, as indicated, was measured. The glycine transport rate was significantly lower after culture with glycine ($p = 0.025$, *t*-test). $n = 7$ replicates in each group. (*b*) Rate of glycine transport as a function of glycine concentration, after culture for 6 h with 1 mM glycine (closed circles) or without glycine (open circles). Curves were fit to the Michaelis–Menten relation as described in the text. Each point represents 5–6 replicates, except $n = 3$ for 2 mM.

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from either a reduced V_{max} or an increased K_m (i.e., decreased affinity), or a combination of both. To distinguish these possibilities, we measured the rate of glycine transport by fertilized ova following a 6 h incubation in either glycine-free or glycine-containing (1 mM) media, as above, using concentrations of [^{3}H]glycine from 5 μM to 2 mM during the 10 min incubation. The rate of transport increased more quickly with increasing [^{3}H]glycine for fertilized ova that had been incubated for 6 h in glycine-free medium compared with those incubated in the presence of 1 mM glycine (Fig. 1*b*). Non-linear least squares curve fits of the Michaelis–Menten equation: rate = V_{max}[glycine]/([glycine] + K_m) to each set of data yielded comparable V_{max} values (20 vs. 23 fmol/fertilized ovum/min for those incubated in glycine-free vs. 1 mM glycine-containing media, respectively). However, the K_m obtained from the curve fit increased from 0.19 mM to 0.84 mM after culture with 1 mM glycine vs. without added glycine. Qualitatively similar findings were obtained when analyzing the data using Eadie–Hofstee plots (rate/[glycine] vs. rate) to linearize the data (not shown), which yielded comparable V_{max} values of 20 and 17 fmol/ fertilized ovum /min, while K_m increased from 0.20 mM to 0.41 mM, after culture without or with glycine, respectively. Thus, there was an apparent decrease in affinity (i.e., increased K_m) for glycine transport without a change in the maximal transport rate after 6 h culture of fertilized ova in the presence of 1 mM glycine.

Effect of length of exposure to glycine on subsequent glycine transport

An apparently decreased substrate affinity can result from physiological changes in the transport system or can be a result of direct inhibition. Physiologically based changes in K_m are more likely to take some time to develop, while direct inhibition should appear as soon as the inhibitor is present in the immediate environment of the transporter. Therefore, we assessed how long a period of incubation in glycine-containing medium was needed for a decrease in glycine transport rate to be induced. We found that 3.0, 1.0 and 0.5 h incubations in medium containing 1 mM glycine resulted in similar decreases in the subsequent measured rate of transport of 10 μM [^{3}H]glycine. After 3 h, the rate of glycine transport by fertilized ova exposed to 1 mM glycine was 0.68 ± 0.09 fmol/fertilized ovum/min, vs. 1.50 ± 0.09 fmol/fertilized ovum/min after culture in glycine-free medium ($p < 0.001$; $n = 5$, *t*-test); after 1 h, the rates were 0.86 ± 0.08 vs. 1.49 ± 0.12 fmol/fertilized ovum/min ($p = 0.003$; $n = 5$); and after 0.5 h, the rates were 0.74 ± 0.09 vs. 1.26 ± 0.14 fmol/fertilized ovum/min ($p = 0.014$; $n = 5$). In the same set of experiments, the rate of glycine transport by two separately handled cohorts of freshly isolated fertilized ova (no culture period) were 1.18 ± 0.05 and 1.19 ± 0.06 fmol/fertilized ovum/min ($p = 0.91$; $n = 6$ each). There were no significant differences between the transport rate by fresh fertilized ova and those after incubation in glycine-free media for 0.5, 1.0, or 3.0 h ($p > 0.05$ by ANOVA with Tukey–Kramer post-hoc test). Thus, glycine transport rates did not change during incubation in glycine-free medium, but were lower after incubation for any time from 0.5–3.0 h in 1 mM glycine. This fact indicated that the decrease in glycine transport rate occurred within 0.5 h of exposure to 1 mM glycine.

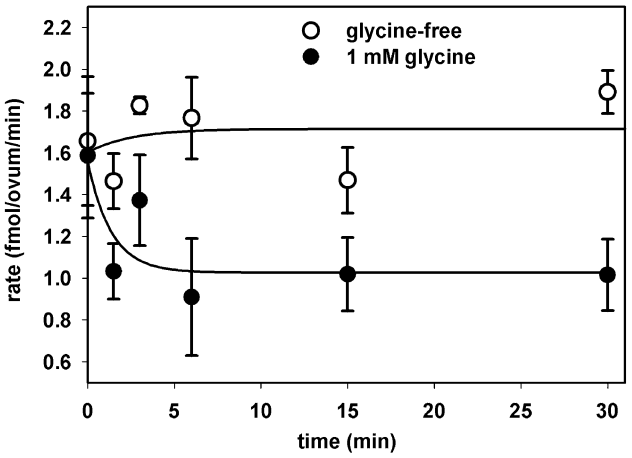

Figure 2 Effect of length of time of exposure to glycine on subsequent rate of glycine transport. Fertilized ova were exposed to 1 mM glycine (closed circles) or glycine-free medium (open circles) for 0–30 min and the subsequent rate of glycine transport immediately measured. Exponential curve fits (as described in text) indicated no change with time of exposure to glycine-free medium, but a decrease that was complete by ~6 min of exposure to glycine-containing medium. Each point represents $n = 3$ replicates.

Therefore, in another set of experiments, we measured glycine transport rates after shorter periods of exposure to 1 mM glycine of between 0 and 30 min. Fertilized ova were exposed for 0, 1.5, 3, 6, 15 and 30 min to either glycine-free medium or medium with 1 mM glycine and the rate of transport of 10 μM [^{3}H]glycine then immediately measured after removal from the incubation medium, as above. The rates were plotted as a function of time and could be adequately fit by single exponentials of the form rate = $r_0 + ae^{-bt}$, which were used to facilitate visualization and for determination of the steady-state rate achieved after sufficient time. The rate decreased only after exposure to glycine-containing, but not glycine-free, medium (Fig. 2). When exposed to glycine, the subsequent rate

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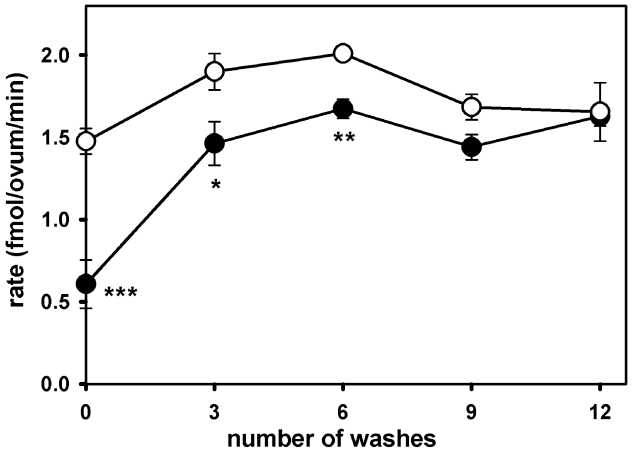

Figure 3 Effect of number of washes on glycine transport rate. The rate of glycine transport was measured after fertilized ova had been cultured for 6 h in the presence of 1 mM glycine (closed circles) or in the absence of glycine (open circles), following the number of washes indicated (0–12). The rate of transport was lower after up to six washes. Treatment groups (glycine vs. no glycine) were paired for each number of washes and so differences were assessed only between glycine vs. no glycine for a given number of washes (by *t*-test). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. $n = 6$ replicates for 0 washes and four replicates each for others.

of glycine transport appeared to reach a minimum after an exposure that was only at most ~6 min.

Effect of extent of washing on subsequent glycine transport

As very brief exposures to glycine-containing media affected the subsequent rate of glycine transport (above), we hypothesized that the decrease in glycine transport rate and increase in the effective K_m was due to persistent presence of glycine in the vicinity of the fertilized ovum plasma membrane. If this were the case, then sufficient washing, providing enough time for any glycine to diffuse away and be removed, should reverse the decreased transport rate. Therefore, we tested the effect of different numbers of wash steps with glycine-free medium on glycine transport by fertilized ova after incubation for 6 h in medium with 1 mM glycine. Each wash consisted of placing the fertilized ova in a drop (~100 μl) of glycine-free medium in a Petri dish and mixing them around the drop briefly (with the pulled Pasteur pipette used for transferring fertilized ova), then transferring them to the next drop. The effect of 0, 3, 6, 9, or 12 washes was assessed. A plot of glycine transport rates vs. number of washes (Fig. 3) shows that, for the group that had been incubated in 1 mM glycine, the rate of glycine transport increased with the number of washes up to six washes, while at nine washes there was only a small difference that did not quite reach significance and with 12 washes there was no difference.

Effect of zona removal on subsequent glycine transport

A possible cause of the apparent persistence of glycine in the vicinity of the fertilized ovum membrane during glycine transport measurements was trapping in the zona pellucida matrix or in the perivitelline space between the membrane and zona pellucida. Therefore, we tested the effect of removing the zona pellucida. The zona pellucidae of fertilized ova were removed using acid Tyrode's medium, washed extensively and then incubated for 30 min in medium either with 1 mM glycine or no glycine. The rate of glycine transport was then immediately measured using 10 μM [^{3}H]glycine for 10 min, as above. When glycine transport rates were measured immediately after incubation with 1 mM glycine or without glycine, the measured rates were 1.13 ± 0.19 and 1.40 ± 0.41 fmol/fertilized ovum/min, respectively ($p = 0.59$, *t*-test; $n = 3$ each). Washing the embryos through three drops of glycine-free medium before the measurements yielded corresponding rates of 1.43 ± 0.07 and 1.23 ± 0.19 fmol/fertilized ovum/min, which were not different from the rates without washing nor from each other ($p = 0.80$, ANOVA; $n = 3$ each).

Discussion

We have found here that exposure to physiological levels of a transport substrate, glycine, can have very significant effects on subsequent transport of the substrate by its specific transport system in fertilized ova, GLYT1. One possible cause for an increased transport rate after extended culture in substrate-free medium could be the stimulation of transport by substrate starvation, which is a well established feature of some transporters, notably the amino acid transporter System A (ATA2) whose activity is strongly upregulated after substrate deprivation (Pastor-Anglada *et al.*, 1996; Franchi Gazzola *et al.*, 2001; Tanaka *et al.*, 2005). Such an effect could plausibly have been induced after the 6 h culture period initially used. However, several of our results make this an unlikely cause of the higher rate of glycine transport by GLYT1 in fertilized ova cultured in the absence of glycine compared to those cultured in the presence of glycine. First, the effect could be elicited to the maximal extent by exposures to glycine that were as short as ~6 min. Second, extensively washing the fertilized ova eliminated the apparent stimulation of transport.

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Finally, the difference in transport rates was not seen in zona pellucida-free fertilized ova.

The results obtained here are instead consistent with competitive inhibition of glycine transport by glycine trapped inside of the zona pellucida that persists after transfer of the fertilized ova out of the glycine-containing medium. Competitive inhibition results in the apparent decrease of substrate affinity, consistent with the increased K_m and unchanged V_{max} measured here. Although we did not carry out the more extensive set of measurements which would be required to unequivocally demonstrate competitive inhibition, i.e., measuring inhibition by a range of concentrations of unlabelled glycine at several different fixed concentrations of [^{3}H]glycine, it seems most probable that unlabelled glycine would serve as a competitive inhibitor of a [^{3}H]-labelled analogue of itself, which is biochemically nearly identical.

An effect that is due to persistence of glycine within the zona pellucida is consistent with our findings that extensive washing quickly eliminated the difference in glycine transport rates and that transport rates were not affected by incubation with glycine for fertilized ova that were zona pellucida-free. The ability of washing to remove glycine trapped within the zona also probably explains why freshly obtained oocytes do not exhibit depressed glycine transport rates, despite being exposed to endogenous glycine in the female tract, since oocytes were flushed from the oviduct and washed extensively in glycine-free HEPES–KSOM and then kept in fresh medium until used.

Trapping of amounts of free glycine sufficient to affect subsequent kinetics is somewhat surprising, as the zona pellucida is reported to be a porous structure through which small molecules diffuse essentially unimpeded. Significant exclusion of macromolecules by the zona pellucida has been reported to occur only for molecular weights exceeding ~110 kDa (Legge, 1995) and even viruses have been reported to be able to penetrate the zona to some extent (Gwatkin, 1967), while glycine has a molecular weight of only 75 Da. Direct measurements of permeability of small molecules using a series of dyes showed that most, including those with physicochemical characteristics similar to glycine, passed through the mouse zona pellucida unhindered (Turner & Horobin, 1997), although the time dependence of permeation was not assessed. However, our data indicate that even very small, hydrophilic molecules with no net charge like glycine can persist within the subzonal space for significant time after they are no longer present in the bulk external fluid. This perhaps reflects the need to diffuse through long, narrow pathways across the zona pellucida, which has a layered, lattice-like ultrastructure (Phillips & Shalgi, 1980). The tortuosity of the diffusion path could thus significantly slow the exit of glycine from the subzonal space. There could, in addition, be a slowing of diffusion due to the viscous material reported to exist within the perivitelline space between the ovum surface and the inner surface of the zona pellucida (Dandekar & Talbot, 1992). Thus, it appears that physiologically significant amounts of soluble compounds can be trapped under the zona at levels sufficient to affect subsequent transport, implying also that they themselves may continue to be transported into the oocyte or embryo for some time after the compound is no longer present, unless extensively washed.

The findings reported here may have implications for preimplantation embryo culture and assisted reproduction, since embryo metabolism and their ability to adjust to new environments can be significantly affected even by very soluble compounds that can persist near the plasma membrane because o subzonal trapping. Interestingly, Gardner & Lane (Gardner & Lane, 1996) reported that a 5 min exposure to amino acids was sufficient to affect subsequent development of fertilized ova to blastocysts, more than doubling the rate of development to blastocysts and increasing cell number. This amount is a comparable exposure to the ~6 min exposure to glycine that we found elicited the maximum effect on glycine transport rate. A possible explanation for the ability of very short exposures to amino acids to have such profound effects on preimplantation development could be the trapping of amino acids within the zona and their subsequent transport into the fertilized ovum even after the amino acid exposure is terminated. The trapping phenomenon may thus have some effect on embryo culture, both by prolonging exposures to compounds that become trapped within the zona even after they have been ostensibly washed away and by providing a small store of substrates that continue to be available to the embryo even after the external supply is removed. However, more significant effects are likely to be on experimental measurements of transport or signaling, in which transport substrates or extracellular ligands may continue to be present even after switching media, as was seen here. Thus, when physiological measurements are made on oocytes or preimplantation embryos either freshly isolated from the female tract or immediately after transfer between different media, it is clearly important to ensure that the extent of washing and period of time elapsed is sufficient to completely eliminate any soluble compounds from the perivitelline space.

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