

Cell volume regulation is initiated in mouse oocytes after ovulation

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Fertilized mouse eggs regulate their size principally by accumulating glycine as an intracellular osmolyte using the GLYT1 (SLC6A9) transporter, a mechanism of cell volume homeostasis apparently unique to early embryos before the morula stage. However, nothing was known of cell volume regulation in oocytes before fertilization. We show here that GLYT1 is quiescent in mouse germinal-vesicle-stage oocytes but becomes fully activated within hours after ovulation is triggered. This initiates accumulation of substantial amounts of intracellular glycine in oocytes during meiotic progression, reaching a maximal level in mature eggs. Measurements of endogenous free glycine showed that there were nearly undetectable levels in ovarian germinal-vesicle-stage oocytes, but high levels were present in mature ovulated eggs and in preimplantation embryos through the two-cell stage, but not in morulae. Furthermore, intracellular glycine was regulated in response to changes in external tonicity in eggs and embryos through the two-cell stage, but not in oocytes or embryos after the two-cell stage. Before activation of GLYT1, oocytes were unable to independently regulate their volume. As GLYT1 became active, however, oocyte volume decreased substantially and oocytes gained the ability to regulate their size, which required GLYT1 activity. Before ovulation, oocyte size was instead determined by a strong adhesion to the rigid extracellular matrix of the oocyte, the zona pellucida, which was released coincident with GLYT1 activation. The ability to acutely regulate cell size is thus acquired by the oocyte only after ovulation, when it first develops glycine-dependent cell volume regulation.

KEY WORDS: Glycine, Meiosis, Osmolyte, Zona pellucida

INTRODUCTION

In most mammals, fully grown oocytes in ovarian follicles are arrested in first meiotic prophase as germinal-vesicle-stage oocytes, and released from germinal vesicle (GV) arrest only after ovulation is triggered. The oocyte then progresses through first meiotic metaphase (MI) before re-arresting in second metaphase (MII), where it persists until MII arrest is released at fertilization. At the MI to MII transition, the oocyte extrudes the first polar body, containing maternal genetic material that is not retained in the MII oocyte. The first polar body remains within a perivitelline space (PVS) that develops between the oocyte surface and the inner surface of the zona pellucida (ZP), the extracellular matrix shell surrounding the oocyte (Talbot and Dandekar, 2003). Fully grown oocytes are much larger than typical mammalian somatic cells, and the asymmetric cleavage that gives rise to the first polar body results in a minimal amount of oocyte cytoplasm being lost, thus retaining most of the cytoplasmic volume in the unfertilized MII egg. Only after the egg is fertilized does a series of mitotic cleavages progressively reduce embryonic cell size (Lehtonen, 1980), until the cells reach approximately the size of adult somatic cells at around the morula stage.

Animal cell size is generally maintained by specialized mechanisms that adjust the cytoplasmic concentration of osmotically active solutes (Hoffmann et al., 2009). In mammalian somatic cells, acute decreases in cell volume are typically corrected by importing inorganic ions or a number of organic osmolytes via

several specific transporters, thus increasing intracellular osmotic pressure and restoring volume (Hoffmann et al., 2009). Like somatic cells, one-cell embryos are able to control their volume against unwanted reduction. However, they employ a volume-regulatory mechanism that is apparently unique to early embryos, regulating their size by accumulating glycine to provide intracellular osmotic support and thus control cell volume (Steeves et al., 2003; Steeves and Baltz, 2005). This volume-regulatory glycine accumulation is mediated by the glycine transporter, GLYT1 (SLC9A6 – Mouse Genome Informatics), distinct from the organic osmolyte transporters used by somatic cells (Ho, 2006; Kwon and Handler, 1995; Steeves et al., 2003). The evidence that glycine accumulation via GLYT1 is the major volume-regulatory mechanism in one-cell embryos included the findings that their intracellular free glycine concentration was regulated by osmolarity, that early preimplantation mouse embryos would not develop under conditions isotonic to the oviduct if GLYT1-mediated glycine transport was eliminated, and that one-cell embryos did not maintain normal volume when GLYT1 activity was blocked by a specific inhibitor (Steeves et al., 2003; Van Winkle et al., 1990b). The presence of a cell-volume-regulatory mechanism unique to early preimplantation embryos raised the question of when it first appears during development, and whether it controls the size of the oocyte during its growth and progression through meiosis. We therefore examined volume regulation by the glycine-dependent mechanism in oocytes before fertilization.

MATERIALS AND METHODS

Chemicals and media

Chemicals and components of culture media were obtained from Sigma (St Louis, MO, USA) unless otherwise noted, and those used in media were embryo tested or cell culture grade. The specific GLYT1 inhibitor, ORG23798, was a kind gift of Organon, UK (Steeves et al., 2003). Media were based on KSOM embryo culture medium (Lawitts and Biggers, 1993),

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with glutamine omitted and polyvinyl alcohol (1 mg/ml; cold water soluble, MW 30-70 KDa) substituted for BSA. Hepes-KSOM contained 21 mM Hepes (pH 7.4) with NaHCO_3 reduced to 4 mM. The osmolarities of KSOM and Hepes-KSOM were 250 and 240 mOsm, respectively. Osmolarity was increased with D(+)-raffinose as previously described (Dawson and Baltz, 1997) or decreased to 150 mOsm by lowering NaCl to 45 mM. Osmolarities were confirmed by osmometer (Vapro 5520, Wescor, Logan, UT, USA).

Mouse oocytes and embryos

Animal protocols were approved by the Animal Care Committee of the Ottawa Health Research Institute. Oocytes and preimplantation embryos were obtained from CF1 female mice (4-7 weeks old, Charles River, St-Constant, PQ, Canada) that were superovulated with equine chorionic gonadotropin (eCG, 5 IU i.p.). GV-stage oocytes were released from antral follicles by mincing ovaries in Hepes-KSOM 43-45 hours post-eCG, collecting fully grown oocytes in cumulus-oocyte complexes (COCs), and removing adherent cumulus cells by repeated pipetting as previously described (Phillips et al., 2002).

Mouse GV oocytes undergo spontaneous meiotic maturation when removed from the follicle. Thus, oocytes progressing through MI in vitro were obtained by culturing GV oocytes in KSOM for up to 8 hours (Phillips et al., 2002). Where maintaining GV arrest in vitro was required, dbcAMP (100 μM) was present during collection and culture.

To obtain oocytes undergoing meiotic maturation in vivo, ovulation and release from GV arrest were induced by injecting female mice with human chorionic gonadotropin (hCG, 5 IU i.p.) at ~47 hours post-eCG. Oocytes were removed at various times, as indicated, to obtain oocytes in MI (~2-10 hours post-hCG) or in MII (10-20 hours post-hCG).

For embryos, females were caged overnight individually with B6D2F1 males after hCG administration. One-cell embryos were collected at ~22 hours post-hCG, two-cell embryos at ~44 hours, four-cell embryos at ~56 hours, eight-cell embryos at ~67 hours, morulae at ~76 hours and blastocysts at ~94 hours (± 2 hours of times indicated). MII oocytes and one- through to eight-cell embryos were removed from excised oviducts by flushing with HEPES-KSOM (with 300 $\mu\text{g}/\text{ml}$ hyaluronidase for MII oocytes and one-cell embryos to facilitate removal of expanded cumulus), whereas morulae and blastocysts were flushed from the uterotubular junction and uterus, respectively. After collection, oocytes and embryos were washed and then maintained in KSOM drops under mineral oil in 5% CO_2/air at 37°C (Lawitts and Biggers, 1993).

Glycine measurements

[^3H]Glycine ([2- ^3H]glycine; ~20 Ci/mmol; Amersham Pharmacia, Arlington Heights, IL, USA) was added directly to media. ^3H in oocytes was measured by liquid scintillation counter (2200CA TriCarb, Packard Instrument) and quantitated as previously described (Steeves et al., 2003), with 5-10 oocytes or embryos pooled for each replicate. The rate of glycine transport was determined by incubating oocytes for 10 minutes in the presence of 10 μM [^3H]glycine and calculating the rate of transport as the molar amount of glycine per oocyte or embryo per minute per μM glycine in the medium, reported as fmol/(oocyte or embryo)/minute. Specific glycine transport by GLYT1 was calculated by subtracting the rate of glycine transport in the presence of the GLYT1 inhibitor ORG23798 (Steeves et al., 2003) (5 μM) from the rate of glycine transport in vehicle alone (0.1% DMSO). Longer-term glycine accumulation by oocytes or embryos was determined by incubating them for the specified times (up to 8 hours) in 1 mM total glycine (1:1000 ^3H -labeled:unlabeled) as previously described (Steeves et al., 2003).

Endogenous unlabeled glycine in freshly isolated oocytes and embryos was measured by amino acid microanalysis (BioLC with AminoPac P10 column, Dionex, Sunnyvale, CA, USA) with each replicate being the TCA-soluble fraction from a pool of 90 oocytes or embryos, as previously described (Steeves et al., 2003). Total glycine was expressed as pmol/(oocyte or embryo).

Oocyte volume measurements

Digital images of oocytes were obtained using a Nikon Coolpix 4500 digital camera on a Zeiss IM35 microscope fitted with Hoffman optics. Oocyte volumes were calculated from images by measuring two orthogonal

diameters and assuming that the oocyte was approximately a sphere with a diameter equal to the mean of the two measured diameters. Calibration was achieved using a stage micrometer.

Transmission electron microscopy

Oocytes were processed for transmission electron microscopy (TEM) essentially as previously described (Dandekar et al., 1995), except that Ruthenium Red was omitted. Briefly, oocytes were fixed in 3% glutaraldehyde for 2 hours at room temperature and then placed into melted (37°C) agar. The agar was trimmed to small blocks containing the oocytes after solidification at room temperature; the blocks were then post-fixed in 1% osmium tetroxide for 45 minutes at room temperature. All solutions were in 0.1 M cacodylate buffer (pH 7.4). The blocks with oocytes were dehydrated through a graded ethanol series (30, 50, 75, 95, 100% in water, 15 minutes each), infiltrated with Spurr's plastic (SPI Supplies, West Chester, PA, USA) 1:1 in ethanol for 24 hours followed by 100% Spurr's for 24 hours, and then polymerized at 70°C for 16 hours. Thin sections were cut on a Sorvall microtome with a diamond knife. TEM images were obtained with a Phillips 500 Transmission Electron Microscope.

Data analysis

Plots were prepared using SigmaPlot 8.2 (SPSS, Chicago, IL, USA). Data were expressed as the mean \pm s.e.m. Comparisons between means were made by ANOVA followed by the Tukey-Kramer multiple comparison test (more than two groups compared), or by Student's two-tailed *t*-test (two groups compared), using InStat (GraphPad, San Diego, CA, USA).

RESULTS

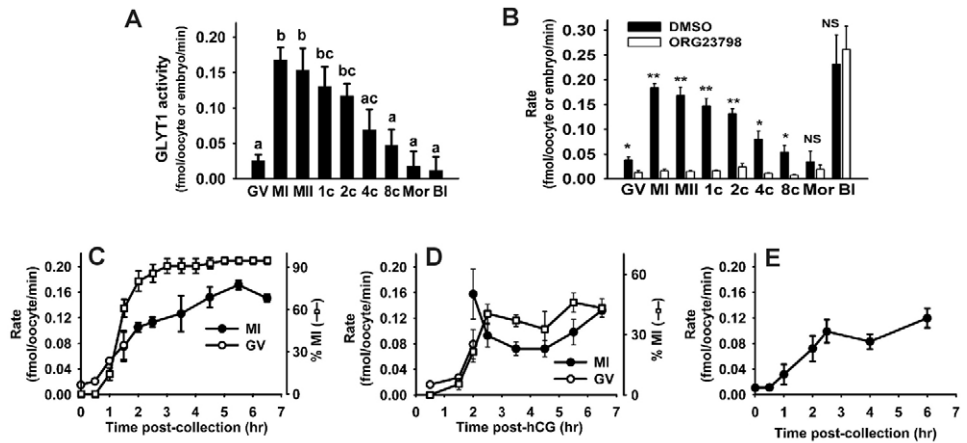
Initiation of GLYT1 activity during meiotic maturation

To determine when GLYT1 is active during meiotic maturation of oocytes and preimplantation embryogenesis, we assessed GLYT1 transport activity in freshly isolated oocytes and preimplantation mouse embryos at stages from GV oocyte through to blastocyst by measuring the total rate of uptake of [^3H]glycine and subtracting the rate of uptake in the presence of the GLYT1-specific inhibitor, ORG23798 (Fig. 1A,B). Specific GLYT1 activity was high in MII eggs and one- and two-cell embryos, and gradually decreased after the two-cell stage as previously reported for 'System Gly' activity in preimplantation embryos (Van Winkle et al., 1988). However, in freshly isolated GV oocytes before initiation of meiotic maturation, glycine transport was nearly quiescent (Fig. 1A,B), indicating that GLYT1 must be activated during meiotic maturation of oocytes.

To determine the timing of GLYT1 activation, we measured glycine transport during meiotic maturation in vitro. Because saturable glycine transport was found to be entirely via GLYT1 in oocytes, eggs and embryos before the morula stage (Fig. 1A,B) (Steeves et al., 2003), the rate of glycine transport in oocytes was subsequently measured without subtracting the small non-specific component. As mouse oocytes spontaneously resume meiosis when removed from the follicle, the rate of glycine transport was measured in GV oocytes placed into culture and allowed to progress into MI (Fig. 1C). There was little uptake of glycine evident up to ~0.5 hours after removal from the ovary, but robust transport activity developed within ~2 hours, during which time essentially all oocytes had entered MI. To confirm that a similar initiation of GLYT1 activity took place in vivo, oocytes were retrieved from ovaries at different times after ovulation was induced by intraperitoneal injection of hCG, and the rate of glycine transport immediately measured (Fig. 1D). This revealed that GV oocytes retrieved within ~1.5 hours post-hCG exhibited almost negligible glycine transport, whereas maximal glycine transport had developed by ~2-3 hours, around the time that oocytes entered MI in vivo.

Fig. 1. Onset of glycine transport in mouse oocytes.

(A) Rate of specific glycine transport by GLYT1 in GV oocytes during meiotic progression (MI and MII) and the earliest stages of preimplantation embryogenesis (one- to eight-cell as indicated). The specific GLYT1 transport rate was calculated from the data shown in B. Bars that do not share letters are significantly different ($P < 0.01$; ANOVA and Tukey-Kramer multiple comparison test). (B) Rates of total glycine transport in the presence of vehicle alone (DMSO) and transport in the presence of the GLYT1 inhibitor, ORG23798, as indicated in the key. Rates in the presence of ORG23798 were subtracted from total transport at each stage to obtain GLYT1-specific rates of glycine transport shown in A. Within each stage, the significance of differences in the presence and absence of ORG23798 was determined by *t*-test. The large component of glycine transport in blastocysts not inhibited by ORG23798 probably represents transport by the B^0+ transporter that is highly active in blastocysts but not earlier (Van Winkle et al., 1990a). (C) Appearance of glycine transport during oocyte meiotic progression in vitro coincident with entry into MI, as indicated by similar timing of increased glycine transport (circles; left axis) and percentage MI oocytes (squares; right axis). At 1.5 hours, there was a mixture of GV (open circles) and MI (filled circles), but rates were similar and thus symbols nearly coincide. (D) The appearance of glycine transport during in vivo oocyte meiotic maturation after hCG-induced triggering of ovulation is coincident with entry into MI. There was a mixture of GV and MI at 2 hours post-hCG. GV oocytes collected more than 2 hours post-hCG did not exhibit GLYT1 activity (not shown). Symbols and axes are as in C. (E) Appearance of glycine transport in oocytes arrested at the GV stage with dbcAMP showing essentially the same time course as during meiotic maturation. In each panel, each bar or point represents the mean \pm s.e.m. of three to five independent measurements. *, $P < 0.05$; **, $P < 0.001$. Bl, blastocysts; Mor, morulae; NS, not significant.



These results showed that glycine transport via GLYT1 developed coincident with the transition into MI. Therefore, we assessed whether GLYT1 activation required meiotic progression. Unexpectedly, when oocytes in vitro were prevented from spontaneously entering MI by the presence of the cell-permeant cAMP analog dibutyryl cAMP (dbcAMP; 100 μ M), GV-arrested oocytes still developed glycine transport with a similar timecourse (Fig. 1E).

We also used several other treatments that maintain GV arrest by maintenance of high intracellular cAMP: namely hypoxanthine (4 mM), forskolin (100 μ M) or IBMX (200 μ M), and additionally assessed oocytes maintained in GV arrest with roscovitine (50 μ M), which inhibits cyclin-dependent kinase activity. When rates of glycine transport were measured after 4 hours in vivo, the rates measured for GV-arrested oocytes after culture with hypoxanthine (0.10 ± 0.02 fmol/oocyte/minute; mean \pm s.e.m.), IBMX (0.08 ± 0.003), forskolin (0.08 ± 0.003) or roscovitine (0.13 ± 0.02) were not significantly different from rates measured in MI oocytes cultured for 4 hours in KSOM (0.12 ± 0.03) or DMSO vehicle (0.1% ; 0.15 ± 0.02), nor from the rate in GV oocytes maintained in arrest for 4 hours with dbcAMP (0.08 ± 0.01) as above ($P > 0.05$, ANOVA with Tukey-Kramer post-hoc test, $n = 3$ for all groups except $n = 6$ for DMSO). Thus, although resumption of meiosis and activation of glycine transport normally occurred simultaneously, they could be decoupled, indicating entry into MI was not required for the initiation of GLYT1 activity.

We also tested whether the appearance of GLYT1 activity required de novo protein synthesis, possibly indicating that new GLYT1 transporter proteins or an obligate regulatory protein were being synthesized. GV oocytes were cultured for 4 hours in the presence or absence of cycloheximide (50 μ g/ml), the presence of which caused an 87% inhibition of 35 S-methionine incorporation into the TCA-precipitable fraction over 4 hours (not shown). The rate of glycine transport was 0.14 ± 0.02 fmol/oocyte/minute (mean \pm s.e.m.) following culture with cycloheximide versus

0.12 ± 0.01 in its absence ($P = 0.44$, *t*-test; $n = 3$), indicating no requirement for protein synthesis during development of GLYT1 activity.

Glycine accumulation in oocytes during meiotic maturation

The results above indicated that glycine transport only begins several hours after ovulation is triggered, implying that, in vivo, GV and early MI oocytes should not have accumulated appreciable glycine, whereas MII eggs, which have been transporting glycine actively since the early MI stage, could have amassed considerable intracellular glycine. To determine whether this predicted pattern of glycine accumulation was occurring in vivo, we measured the endogenous soluble glycine content of GV oocytes, MII eggs and preimplantation embryos immediately after removal from the female reproductive tract. We found that one- and two-cell embryos contained high levels of endogenous free glycine (Fig. 2A). Morulae, however, contained little free glycine, showing that embryos do not retain high levels of glycine after GLYT1 is no longer active (cf. Fig. 1A). Consistent with the development of GLYT1 activity only after ovulation or removal of oocytes from the ovary, free cytosolic glycine was nearly undetectable in GV oocytes and very low in early MI oocytes, whereas, by contrast, large amounts were present in ovulated MII eggs at levels similar to those in one- and two-cell embryos (Fig. 2A). Thus, glycine is accumulated to high levels by oocytes during meiotic maturation in vivo.

GV oocytes are connected to surrounding cumulus cells by gap junctions (Anderson and Albertini, 1976; Simon et al., 1997), and thus cumulus cells might serve as a reservoir for glycine. We thus measured endogenous glycine in COCs, but found that it was undetectable (0.0 ± 0.1 pmol/COC; $n = 3$ groups). Similarly, MI oocytes with cumulus attached (MI oocytes shown in Fig. 2 included cumulus) had little endogenous glycine.

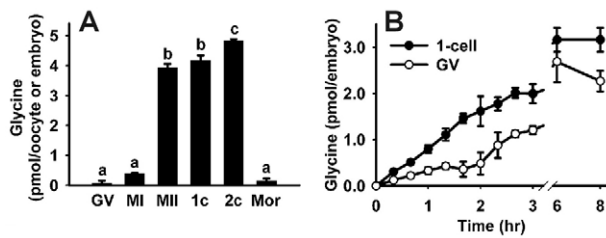


Fig. 2. Glycine accumulation by oocytes and embryos.

(A) Endogenous soluble glycine in freshly isolated GV, MI (4.5-5.5 hours post-hCG) and MII (14-15 hours post-hCG) mouse oocytes and embryos at the one-cell, two-cell and morula stages. Bars that do not share letters are significantly different ($P < 0.01$; ANOVA and Tukey-Kramer multiple comparison test). (B) Total glycine accumulated as a function of time by one-cell embryos and by GV oocytes maintained in arrest by dbcAMP. In each panel, each bar or point represents the mean \pm s.e.m. of three independent measurements. Mor, morulae.

Finally, we determined whether freshly isolated COCs could transport glycine, by measuring the rate of uptake of [3 H]glycine by COCs immediately after removal from the ovary (as above for denuded oocytes). Freshly isolated COCs transported glycine at a rate not different from zero (0.07 ± 0.07 fmol/COC/minute; $n=3$), which was also not significantly different from the rate of transport by freshly isolated denuded GV oocytes measured in parallel (0.03 ± 0.02 ; $n=3$; $P=0.6$, t -test). Thus, cumulus cells would not appear to serve as a significant pool of accumulated glycine for enclosed GV oocytes. When we cultured intact COCs for more than 1 hour, however, we found that glycine transport increased, and reached a higher rate than in denuded GV oocytes alone (data not shown), implying that GLYT1 activity may also become upregulated in granulosa cells with time after removal from the follicle, perhaps accounting for the previously reported presence of GLYT1 activity in COCs (Haghighat and Van Winkle, 1990).

To demonstrate that glycine accumulation begins in oocytes around the time GLYT1 is activated, we incubated GV oocytes and one-cell embryos in medium containing 1 mM glycine (3 H-labelled), approximating the glycine concentration in mouse oviductal fluid (Harris et al., 2005), and measured the total amount of glycine that had accumulated as a function of time. As previously shown (Steeves et al., 2003; Steeves and Baltz, 2005), one-cell embryos immediately began accumulating glycine, and had reached a plateau level by 6-8 hours (Fig. 2B). By contrast, GV oocytes accumulated glycine slowly for the first ~2 hours, and only then began to accumulate glycine at a rate similar to that of one-cell embryos (Fig. 2B). After 6-8 hours, however, glycine in GV oocytes had reached approximately the same level as accumulated by one-cell embryos. Thus, glycine accumulation begins around the time that GLYT1 is activated.

Initiation of glycine-dependent cell volume regulation

It has previously been shown that a hallmark of cell volume control at the one-cell stage is that the total intracellular concentration of glycine increases with external osmolarity (Steeves et al., 2003; Steeves and Baltz, 2005). We therefore examined when during meiotic progression such osmotic regulation of glycine accumulation appears, by exposing oocytes, eggs and preimplantation embryos to 1 mM glycine for 8 hours and determining total accumulated glycine at 250 versus 350 mOsM. An

accumulation period of 8 hours was chosen because it fits within the MI stage of oocyte maturation and within embryonic cell cycles. Although 8 hours is not sufficient to reach plateau levels of glycine, particularly at 350 mOsM, it is sufficient to easily detect differences in accumulation (Steeves et al., 2003). We found that glycine accumulation was lower at 350 than at 250 mOsM in GV and MI oocytes, and in embryos at the four-cell, eight-cell and morula stages, and thus was not stimulated by hypertonicity (Fig. 3). By contrast, glycine accumulation was significantly stimulated by hypertonicity during the restricted period of development starting with the mature, ovulated MII egg and ending at the two-cell stage (Fig. 3), identifying the developmental period during which cell volume regulation is likely to be mediated by glycine and GLYT1.

It has previously been established that one-cell embryos require glycine uptake and GLYT1 activity to maintain their volume (Steeves et al., 2003). However, when this mechanism is first used during development was not known. To determine whether oocytes could regulate their volume using glycine as soon as GLYT1 activity develops, we compared the abilities of freshly obtained GV oocytes (before GLYT1 activation) and GV oocytes first maintained in vitro for 3 hours (so that GLYT1 was fully activated) to maintain their volume in the presence or absence of glycine. GV oocytes were exposed to high osmolarity (350 mOsM) or lower osmolarity (250 mOsM) in the presence or absence of glycine, and their volume was determined at the end of 2 hours. The volume of freshly obtained GV oocytes was significantly smaller after 2 hours at higher than at lower osmolarity, and glycine had no effect on their volume (Fig. 4A). By contrast, GV oocytes assessed after maximal GLYT1 activity had already developed (Fig. 4B) were significantly better able to maintain their volume in the presence of glycine. Furthermore, inhibition of GLYT1 activity with the specific inhibitor ORG23798 eliminated this ability (Fig. 4C). Thus, GV oocytes began to gain the ability to maintain their volume using a glycine-dependent mechanism by 3 hours after removal from the follicle.

Release of oocyte-ZP adhesion and decrease in oocyte volume

It is well known that a PVS between the oocyte plasma membrane and the ZP, the glycoprotein extracellular matrix shell enclosing the oocyte (Wassarman, 2008), is present in MII eggs but not GV oocytes (Talbot and Dandekar, 2003). The rigidity of the ZP implies

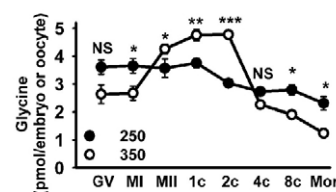


Fig. 3. Osmotic regulation of intracellular glycine. The amount of glycine accumulated by oocytes at different stages of meiotic maturation (GV, MI, MII) and preimplantation mouse embryos [one-cell to eight-cell stages as indicated and morulae at low (250 mOsM) and high (350 mOsM) osmolarity (as indicated in the key)], after 8 hours in the presence of 1 mM glycine. GV oocytes were maintained in arrest with dbcAMP. MI oocytes were cultured in the absence of dbcAMP, and transitioned from GV oocytes within the first 2-4 hours. MII eggs and preimplantation embryos were collected at the stage indicated and remained at that stage for the entire incubation. Comparisons were made between osmolarities within each stage (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's t -test). Each point represents the mean \pm s.e.m. of three to five independent measurements. Mor, morulae.

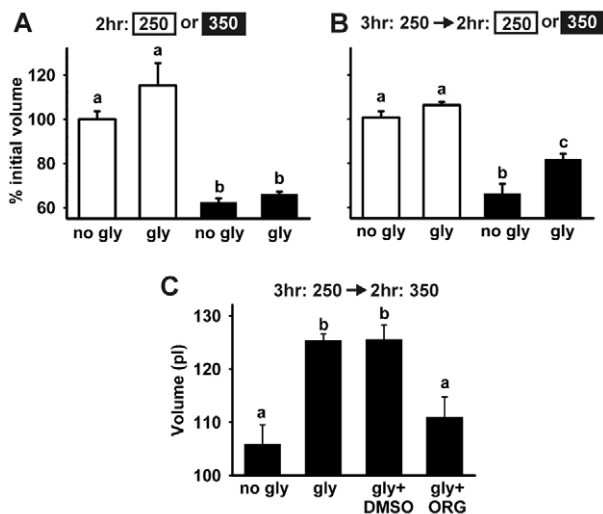


Fig. 4. Regulation of oocyte cell volume. (A) Volumes of GV oocytes maintained in GV arrest with dbcAMP. Oocytes collected from mouse ovaries were placed immediately (before GLYT1 activity developed) in media of 250 or 350 mOsM in the absence or presence of 1 mM glycine. Mean oocyte volume was measured after 2 hours (experimental protocol indicated above each panel). Although any volume recovery at 350 mOsM was likely to still be incomplete at 2 hours, this period was chosen to allow measurements before GLYT1 activity could develop in these freshly obtained GV oocytes. (B) Oocyte volume measured similarly to those in A, but following 3 hours of culture to allow maximal GLYT1 activity to develop before the 2-hour incubation. For A and B, volumes were normalized to the mean volume at 250 mOsM in the absence of glycine. (C) Effect of inhibiting GLYT1 transport with ORG23798 (5 μ M, gly+ORG; gly+DMSO indicates control with 0.1% DMSO vehicle alone) on oocyte volume at 350 mOsM in the presence or absence of glycine. In each panel, bars represent the mean \pm s.e.m. of three to five independent determinations. Different letters indicate significant differences among means within each panel ($P < 0.05$ by ANOVA and Tukey-Kramer multiple comparison test). gly, presence of glycine; no gly, absence of glycine.

that the volume of the oocyte decreases during PVS formation. This decrease in volume was generally assumed to result from decreased oocyte size due to first polar body emission. We wondered whether PVS formation might instead be related to the onset of cell volume regulation.

We first determined the timecourse of oocyte volume change after ovulation was triggered. When we measured the volume of oocytes immediately (within 5 minutes) after removal from ovaries as a function of time from 0-15 hours post-hCG, we found that that oocyte volume progressively decreased (Fig. 5A), resulting in the formation of a visible PVS (Fig. 5B). Virtually all of the cell volume decrease was completed during MI before first polar body emission (~10 hours post-hCG). A similar volume decrease also occurred in GV oocytes maintained in arrest in vitro (Fig. 5C) where no polar body was emitted. We also observed oocyte volume decreases and PVS development in 150, 250 and 350 mOsM media (not shown) similar in timecourse and extent to that in 300 mOsM (isotonic to follicular and oviductal fluid) shown in Fig. 5A-C, indicating that the volume decrease was not the result of any change in tonicity upon transfer to culture.

To more closely examine the early stages of this process, GV oocytes were fixed 0-3 hours after removal from the ovary and processed for TEM. The plasma membrane of freshly isolated

oocytes was invariably observed to be closely apposed to the inner ZP surface, with oocyte microvilli surrounded by zona matrix (Fig. 5D). By 0.5 hours, a small gap appeared, and by 2-3 hours oocyte microvilli were free of the ZP matrix. Thus, it appeared that the oocyte and ZP were closely associated before meiotic progression was initiated, but this association was lost within the first few hours after the oocyte was removed from the ovary, during the period when it normally transitions into MI.

The above observations led to the hypothesis that oocyte size was constrained by a physical oocyte-ZP interaction before PVS development. To test this, we hypertonic (450 mOsM) shocked GV oocytes, and found that freshly isolated oocytes were unable to shrink independently of the zona, but instead became concave and deformed the ZP or developed concavities that we interpreted as indicating a local adhesion failure (Fig. 6A). By contrast, GV oocytes that were hypertonic shocked after 3 hours in vitro shrank uniformly and remained spherical in response to hypertonic shock, and the ZP was unaffected. When fresh GV oocytes were fixed immediately after hypertonic shock and examined by TEM, it was revealed that the oocyte and ZP remained in contact, and that many microvilli were extended with their tips appearing to remain in contact with the ZP matrix (Fig. 6B). By contrast, at 250 mOsM, microvilli often lay parallel to the surface.

As another test of oocyte-zona adhesion, we microinjected mineral oil between the ZP and GV oocyte to determine whether the oocyte could be displaced from the ZP. At 3-4 hours after oocyte collection, the membrane was always easily displaced by the oil droplet within the ZP (Fig. 6C). However, in fresh GV oocytes the oil instead remained trapped in a restricted cavity and did not displace the oocyte from the ZP. Thus, it appears that the GV oocyte strongly adheres to the inner ZP surface.

Finally, removal of the ZP from fresh GV oocytes using acid Tyrode's solution accelerated the initial volume decrease (shown in Fig. 5C) in GV oocytes by ~30 minutes (not shown), consistent with oocyte size being constrained by the ZP before the oocyte-ZP adhesion is released.

DISCUSSION

GLYT1-mediated glycine transport begins in the mouse oocyte after ovulation is triggered

GLYT1 is quiescent in freshly isolated GV oocytes, which also contain very little endogenous glycine. However, within several hours of the initiation of ovulation in vivo, or removal of the GV oocyte from its normal context in the ovarian follicle, GLYT1-mediated glycine transport becomes activated in the oocyte, and glycine accumulation commences. Concomitantly, oocytes become able to use glycine to regulate their volume. Thus, the glycine-dependent cell volume regulation previously described in one-cell embryos is activated as soon as GLYT1 activity appears, within several hours of the induction of ovulation.

The amount of glycine that was accumulated was regulated in response to changes in external osmolarity in MII eggs similarly to one- and two-cell embryos. By contrast, hypertonic stimulation of intracellular glycine accumulation did not occur in GV oocytes (either maintained in arrest or allowed to progress into MI), nor in four-cell or later embryos. Instead, at these developmental stages, glycine levels were generally lower when external osmolarity was increased. The lower total glycine at higher osmolarity at these stages may simply be due to a passive effect, as cytoplasmic volumes would be expected to be decreased under hypertonic conditions, so that similar intracellular glycine concentrations would result in a reduction in total glycine. In any event, the ability to

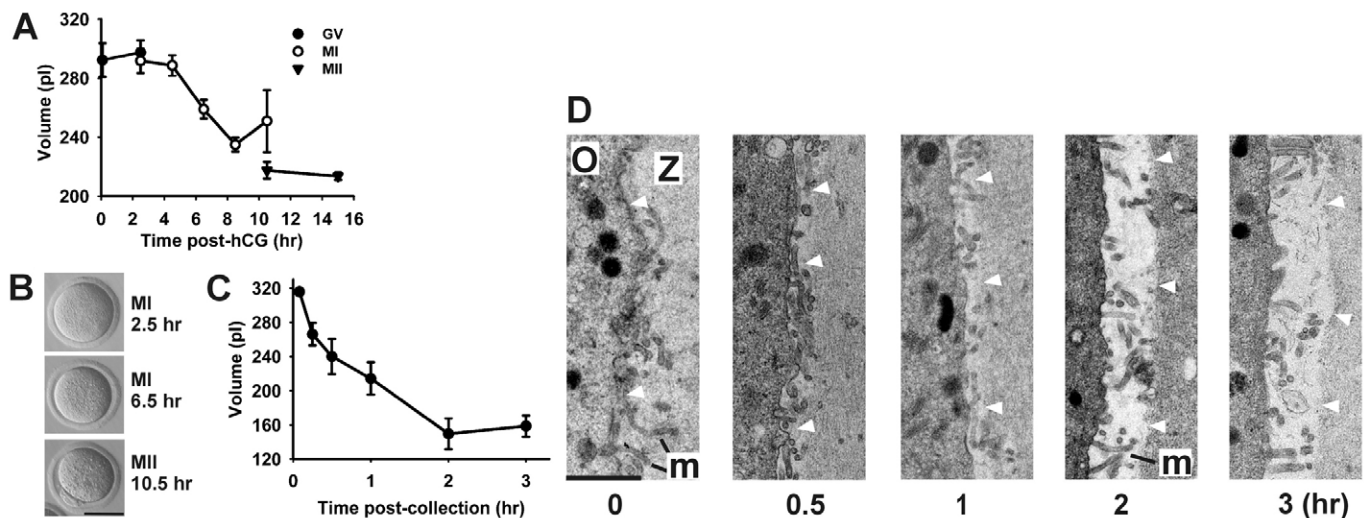


Fig. 5. Change in oocyte volume and oocyte-zona pellucida contact after ovulation. (A) Oocyte volume as a function of time after ovulation had been triggered with hCG (meiotic stage indicated in key). (B) Examples are shown at the meiotic stages and times post-hCG indicated on the right. (C) Volume of GV oocytes (maintained in arrest with dbcAMP) as a function of time in vitro after removal from the ovary at $t=0$. Medium osmolarity was 300 mOsM to approximate follicular fluid. (D) TEM of oocytes treated as in A,B and fixed at the times after removal from the ovary indicated below the panel, showing a gap developing between oocyte and ZP. Arrowheads indicate approximate position of inner ZP surface. Scale bars: 50 μ m in B; 1 μ m in D. m, microvilli; O, oocyte; Z, zona pellucida.

increase intracellular glycine levels in response to decreased cell volume is strikingly restricted to a narrow developmental window from the MII egg through to the two-cell embryo stage.

Endogenous glycine was found at high intracellular levels starting in MII eggs and persisting through the one- and two-cell stages but was very low in morulae or GV or MI oocytes, so that high endogenous glycine coincided with the period when glycine accumulation in vitro can be stimulated by increased external osmolarity. Although endogenous glycine has not previously been assessed in GV or MI oocytes, our results are consistent with

previous reports indicating that MII eggs and one- and two-cell embryos contain high levels of glycine (4-10 pmol per egg or embryo), whereas four- to eight-cell embryos contained less than half, and blastocysts less than one-tenth, the amount in MII eggs or two-cell embryos (Schultz et al., 1981; Van Winkle and Dickinson, 1995).

The concentration of glycine accumulation by oocytes and early embryos is considerable. Here, we measured ~4-5 pmol per egg or embryo of endogenous free glycine at the MII through to two-cell stages. Assuming a volume of ~180 pl for eggs and early embryos

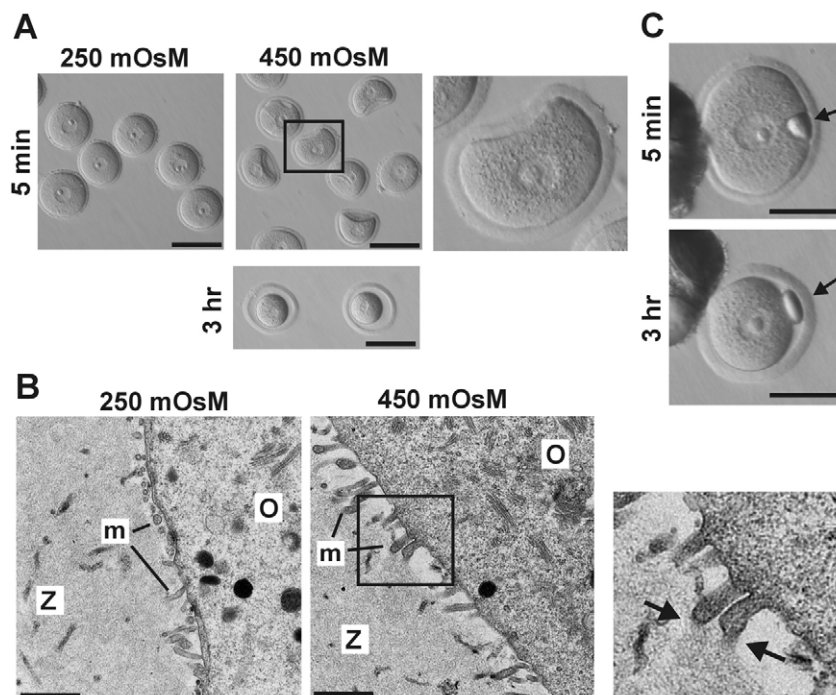


Fig. 6. Oocyte-zona pellucida adhesion. (A) Mouse GV oocytes maintained at normal culture osmolarity (250 mOsM) or hypertonically shocked (450 mOsM) for ~5 minutes (indicated at left) after removal from the ovary (osmolarities indicated at the top). The oocyte within the box is shown at higher magnification on the right. The panel beneath (3 hr, 450 mOsM) shows similar hypertonically shocked oocytes after they had first been in culture for 3 hours. Examples shown are typical of oocytes from three independent repeats. (B) TEM of oocyte-ZP contact at 250 mOsM (left) and 450 mOsM (center). The boxed area is shown at higher magnification on the right. Arrows indicate an example of apparent adhesion between microvilli and ZP in hypertonically shocked oocytes. Scales and labeling are as in Fig. 5D. (C) Mineral oil (arrows) microinjected into the interface between oocyte and ZP in freshly collected (top) and arrested (bottom) GV oocytes showing oocyte surface displaced from the ZP in arrested GV oocytes 3 hours after removal from the ovary (3 hr) but not in freshly collected GV oocytes (5 min). Examples are typical of oocytes in three independent sets of microinjections. Scale bars: 50 μ m in A,C; 1 μ m in B.

(Lehtonen, 1980), the endogenous free glycine measured here corresponds to at least ~20-30 mM intracellular glycine, sufficient to balance ~20-30 mOsm in external osmolarity. Thus, only small relative changes in total glycine would be needed to mitigate against normal volume fluctuations in eggs and embryos.

Oocyte volume decreases following release of oocyte-zona pellucida adhesion during the initial stages of meiotic maturation

During essentially the same period in which GLYT1 activity appears, the volume of the oocyte decreases markedly, both in vitro and in vivo. This re-setting of oocyte size, normally occurring soon after ovulation is triggered, results in a loss of ~20% in total oocyte volume between 4 and 8 hours after hCG and a similar volume decrease in vitro in oocytes after their removal from the follicle. Notably, this occurs well before the first polar body is emitted at ~10 hours post-hCG, and in GV-arrested oocytes where polar body emission does not occur. Indeed, the emission of the polar body only appears to result in a further volume decrease of only a few percent of initial volume.

The decrease in oocyte size during this period causes the formation of a PVS, which is not present in GV oocytes in the follicle. The initial formation of the PVS has not been extensively studied previously, although the composition of the matrix that is present in this space after first polar body emission has been examined (Dandekar and Talbot, 1992; Talbot and Dandekar, 2003). There is, however, a recent paper (Inoue et al., 2007) that indicates a strong correlation between the ability of GV oocytes to form a PVS within the first hour after removal from the ovary and their subsequent ability to complete meiosis, implying that such early PVS formation occurs in healthy fully grown oocytes. Our experiments using hypertonic shock or sub-zonal oil drop injection indicated a strong physical adhesion between GV oocytes and the ZP, which is released as the PVS forms. Ultrastructural studies confirmed a close apposition between oocyte and ZP when the GV oocyte is freshly isolated, possibly mediated by an interaction between microvillar tips and the zona matrix, that is lost concomitantly with the release of adhesion. Thus, it appears that, before ovulation is triggered, GV oocyte volume is constrained by the fixed size of the ZP cavity, preventing any significant independent volume regulation by the oocyte. Oocyte-ZP adhesion is normally released just after ovulation, when the oocyte decreases considerably in volume. As this process occurs concomitantly with the activation of GLYT1, the loss of oocyte-ZP adhesion and the decrease in oocyte volume represents a transition from passive oocyte size control in the follicle, determined by the inner ZP diameter, to independent control of oocyte size employing GLYT1-mediated glycine transport for volume regulation.

A possible reason for only initiating independent cell volume control in the oocyte after ovulation may be found in the architecture of the ovarian follicle. Until ovulation, the oocyte is intimately connected with its surrounding somatic granulosa cells (GC) through GC processes that traverse the ZP to mediate direct oocyte-GC communication through gap junctions (Anderson and Albertini, 1976). Bidirectional oocyte-GC communication is required for oocyte growth and viability (Matzuk et al., 2002), including direct communication through oocyte-GC gap junctions (Simon et al., 1997). If the GV oocyte were to independently regulate its volume, however, such connections could not be easily maintained through the rigid ZP, and thus, a close association between oocyte and ZP may be required to prevent disruption of

oocyte-GC communication. As gap-junction-mediated communication between the oocyte and GC is normally lost ~2 hours after ovulation is triggered (Gilula et al., 1978), it would then no longer be necessary for the oocyte to maintain close contact with the ZP, thus permitting the initiation of cell volume regulation that we have described here.

Cell volume regulation is initiated in oocytes after ovulation

The results we have reported here are consistent with independent cell volume regulation beginning in the oocyte after ovulation. Thus, at least in mice, the potential new organism first gains the ability to regulate its own cell volume only after ovulation is triggered. The glycine-dependent cell-volume-regulatory mechanism that then develops is restricted to the egg and very early preimplantation embryo. This may be a general feature of mammals, as GLYT1 activity is also found in early human embryos (Hammer et al., 2000). Presumably, the cells of the developing embryo in the later preimplantation stages and during early postimplantation development adopt more conventional mechanisms of cell volume regulation similar to those of cells in the fetus and somatic cells, although this has yet to be investigated.

Thus, a significant event at the very earliest stages of development that has not previously been described is the initiation of cell volume control. We propose a model in which the initial step is the release of a strong adhesion between the GV oocyte and ZP, followed by a decrease in oocyte volume that occurs near the time of GLYT1 activation and the development of the glycine-dependent mechanism of cell volume regulation that is apparently unique to eggs and early preimplantation embryos. It is unknown, however, what mediates the release of oocyte-ZP adhesion, nor the mechanism by which GLYT1 is activated, although our data indicate the latter is independent of synthesis and thus must involve the activation of pre-existing GLYT1. Future investigations are needed to address these questions.

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