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SIT1 is a betaine/proline transporter that is activated in mouse eggs after fertilization and functions until the 2-cell stage

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Betaine (*N,N,N*-trimethylglycine) added to culture media is known to substantially improve the development of preimplantation mouse embryos in vitro, and to be imported into 1-cell embryos by a transporter that also accepts proline. Here, we found that the betaine/proline transporter is active in preimplantation mouse embryos only for a short period of development, between the 1- and 2-cell stages. Betaine/proline transport was activated after fertilization, beginning ~4 hours post-egg activation and reaching a maximum by ~10 hours. One- and 2-cell embryos contained endogenous betaine, indicating that a likely function for the transporter in vivo is the accumulation or retention of intracellular betaine. The appearance of transport activity after egg activation was independent of protein synthesis, but was reversibly blocked by disruption of the Golgi with brefeldin A. We assessed two candidates for the betaine/proline transporter: SIT1 (IMINO; encoded by *Slc6a20a*) and PROT (*Slc6a7*). mRNA from both genes was present in eggs and 1-cell embryos. However, when exogenously expressed in *Xenopus* oocytes, mouse PROT did not transport betaine and had an inhibition profile different from that of the embryonic transporter. By contrast, exogenously expressed mouse SIT1 transported both betaine and proline and closely resembled the embryonic transporter. A morpholino oligonucleotide designed to block translation of SIT1, when present from the germinal vesicle stage, blocked the appearance of betaine transport activity in parthenogenotes. Thus, SIT1 is likely to be a developmentally restricted betaine transporter in mouse preimplantation embryos that is activated by fertilization.

KEY WORDS: Preimplantation, Betaine, Fertilization, Transport, IMINO

INTRODUCTION

Betaine (*N,N,N*-trimethylglycine) is actively taken up by 1-cell embryos via an unidentified saturable transport mechanism (Anas et al., 2007; Hammer and Baltz, 2002). Previous work has shown that betaine can remarkably improve the viability of preimplantation (PI) mouse embryos, at least under in vitro conditions. In particular, the addition of betaine to the PI embryo culture medium protects embryo development against stress imposed by alterations in culture medium composition or increased osmolarity (Biggers et al., 1993; Dawson and Baltz, 1997). Embryos cultured with betaine also maintain more normal intracellular Na^+ and K^+ concentrations (Biggers et al., 1993) and their pattern of protein synthesis more closely resembles that of in vivo embryos (Anbari and Schultz, 1993). Betaine might also function in vivo, as it is present in mouse oviducts (Anas et al., 2007).

One-cell embryos possess a single route for betaine transport that is Na^+- and Cl^--dependent, also transports proline and is inhibited by several methylamino acids and proline derivatives (Anas et al., 2007). This betaine/proline transporter has not, however, been identified at a molecular level and is unlike any transporter previously described in 1-cell mouse embryos (Anas et al., 2007; Hammer and Baltz, 2002). Its transport characteristics do not resemble those of the betaine transporter BGT1 (encoded by *Slc6a12*) (Hammer and Baltz, 2002; Yamauchi et al., 1992), nor of the proline transporters PAT1 and PAT2 (*Slc36a1* and *Slc36a2*, respectively) (Broer, 2008).

One function of betaine in mammalian cells is as an organic osmolyte, one of an array of neutral organic compounds accumulated by specialized transporters to counter increased external osmotic pressure without the deleterious effects of increased ionic strength (Kwon and Handler, 1995). Based on its osmoprotection of PI mouse embryos in culture, Biggers et al. proposed that betaine functions as an organic osmolyte in PI embryos (Biggers et al., 1993). Cleavage-stage embryos are very sensitive to moderately increased osmolarity, and they accumulate large amounts of glycine as an organic osmolyte to balance the osmolarity of their normal in vivo environment (Steeves et al., 2003). Under in vitro conditions, betaine is as effective as glycine at osmoprotection in 1-cell embryos (Anas et al., 2007; Biggers et al., 1993; Dawson and Baltz, 1997; Hammer and Baltz, 2002) and thus might also have a function in cell volume homeostasis. Another established function of betaine is to serve as a donor of methyl groups that are ultimately made available to a wide array of methyltransferases, a function known to be important mainly in the liver (Finkelstein and Martin, 1984; Selhub, 1999).

Based on the dependence of betaine/proline transport in 1-cell mouse embryos on Na^+ and Cl^-, we hypothesized that the embryonic betaine/proline transporter is a member of the neurotransmitter transporter family (NTT; the Slc6 gene family), which contains all known mammalian Na^+- and Cl^--dependent transporters of organic substrates (Chen et al., 2004; Hoglund et al., 2005). Two possible candidates have been proposed (Anas et al., 2007): the brain proline transporter (PROT; encoded by *Slc6a7*) and the intestinal imino acid transporter (SIT1, also known as IMINO and XTRP3s1; encoded by

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Slc6a20a). PROT is a reportedly brain-specific proline transporter with an inhibitor profile (Fremeau et al., 1992; Shafqat et al., 1995) similar to that of the embryonic betaine/proline transporter. However, to our knowledge, whether PROT actually transports betaine is not known. SIT1 was recently identified as the long-sought intestinal proline transporter (Kowalczuk et al., 2005; Takanaga et al., 2005). SIT1 has kinetic properties and an inhibitor profile very similar to those of the embryonic transporter and it accepts betaine as a substrate (Kowalczuk et al., 2005; Takanaga et al., 2005). It was unknown, however, whether either of these transporters is expressed or active in 1-cell mouse embryos.

MATERIALS AND METHODS

Chemicals and media

All chemicals were obtained from Sigma (St Louis, MO) unless otherwise noted. Brefeldin A (BFA) was prepared as a 1000× stock in ethanol, and used at 5 μg/ml (18 μM). Culture media were based on KSOM medium (Lawitts and Biggers, 1993), modified by omitting glutamine and replacing BSA with polyvinyl alcohol (PVA) (1 mg/ml; cold-water soluble, 30-70 kDa). KSOM was equilibrated with 5% CO_2 in air at 37°C. Similarly modified HEPES-KSOM (pH 7.4) was used for oocyte and embryo collection and where specified (Lawitts and Biggers, 1993).

Mouse oocytes and embryos

Oocytes and embryos were obtained from female CF1 mice (4-7 weeks old; Charles River Canada, St-Constant, PQ, Canada) that had been superovulated by intraperitoneal injection of equine chorionic gonadotropin (eCG; 5 IU). For ovulated metaphase (M) II oocytes and PI embryos, human chorionic gonadotropin (hCG; 5 IU) was administered by intraperitoneal injection ~47 hours post-eCG. For embryos, the female mice were caged overnight with BDF_1 males (Charles River) immediately after hCG for mating. All procedures were approved by the Animal Care Committee of the Ottawa Health Research Institute.

Germinal vesicle (GV) stage oocytes were obtained by mincing excised ovaries ~44-46 hours post-eCG, collecting fully grown oocytes, and removing adherent cumulus cells by repeated pipetting. MI oocytes were similarly obtained ~4 hours post-hCG, and MII oocytes at ~15 hours post-hCG. One-cell stage embryos were collected (±2 hours) at ~22 hours post-hCG (or, as specified, between 19 and 29 hours), 2-cell at ~44 hours, 4-cell at ~56 hours, 8-cell at ~67 hours, morulae at ~76 hours and blastocysts at ~94 hours. MII oocytes and 1- through 8-cell embryos were removed from excised oviducts by flushing with HEPES-KSOM (containing 300 μg/ml hyaluronidase for MII oocytes and 1-cell embryos to facilitate removal of cumulus), while morulae and blastocysts were flushed from the uterotubular junction and uterus, respectively. Oocytes or embryos were washed through four drops of HEPES-KSOM and then briefly maintained in KSOM according to standard techniques in microdrop cultures (Lawitts and Biggers, 1993) under mineral oil (Sigma-Aldrich, Milwaukee, WI) at 37°C in 5% CO_2 in air until they were used.

For parthenogenetic activation with Sr^{2+}, ovulated MII oocytes were incubated for 2 hours with 10 mM $SrCl_2$ in KSOM ($CaCl_2$ omitted) as described (Phillips et al., 2002). In one experiment, eggs were instead activated using cycloheximide alone (50 μg/ml in KSOM from 1000× stock in water), which inhibits general protein synthesis and thus activates eggs by preventing cyclin B synthesis (Moos et al., 1996; Phillips et al., 2002; Siracusa et al., 1978). The time at which Sr^{2+} or cycloheximide was introduced was designated t=0.

Measurement of ^{3}H-labeled compounds in oocytes and embryos

[^{3}H]betaine ([methyl-^{3}H]betaine, 85 Ci/mmol, 1 mCi/ml) and [^{3}H]proline (L-[2,3-^{3}H]proline, 45 Ci/mmol, 1 mCi/ml) were obtained from Amersham Biosciences (Arlington Heights, IL). [^{3}H]betaine was custom-synthesized by Amersham as previously described (Anas et al., 2007). Stocks were in 2% ethanol in water, stored at –80°C.

For determining betaine transport activity, groups of 5-12 oocytes or embryos were incubated with 1 μM [^{3}H]betaine for 30 minutes, except after parthenogenetic activation (Sr^{2+} or cycloheximide) or for in vivo-maturing oocytes at defined times post-hCG, where incubation with 2 μM [^{3}H]betaine for 10 minutes was used instead to permit more precise timing. Proline transport was measured using 1 μM [^{3}H]proline for 10 minutes in the presence of 10 mM alanine to eliminate the betaine-resistant, alanine-sensitive component of proline transport in PI embryos (Anas et al., 2007). Oocytes or embryos were then immediately processed for measurement of intracellular ^{3}H.

To determine the intracellular content of ^{3}H, oocytes or embryos were removed from [^{3}H]betaine- or [^{3}H]proline-containing medium, washed in ice-cold HEPES-KSOM, and dissolved in 4 ml scintillation fluid (Scintiverse BD; Fisher Scientific, Pittsburgh, PA). ^{3}H was detected using a 2200CA TriCarb liquid scintillation counter (Packard Instruments, Downer's Grove, IL). Background was measured in equivalent volumes of each final wash drop, and the counts obtained were subtracted from the paired embryo sample. The total amount of each compound in oocytes or embryos was calculated using standard curves constructed for each set of experiments. Transport rates were expressed as fmol/oocyte or embryo/minute (normalized to 1 μM substrate when 2 μM [^{3}H]betaine was used).

Measurement of endogenous betaine

Groups of 50 1- or 2-cell embryos, isolated from about five females, were washed extensively with HEPES-KSOM, placed into microcentrifuge tubes with minimal medium, air dried and stored at –20°C. Total betaine was measured using a modification of the high performance liquid chromatography (HPLC)-based method described previously (Storer et al., 2006; Storer and Lever, 2006). Briefly, 25 μl methanol was added to each tube, vortexed, ultrasonicated for 10 minutes, and then 225 μl acetonitrile added prior to derivatization with phenanthrenacyl triflate. HPLC separation was performed using a silica column (3 μm) with a mobile phase of 5 mM dimethylbutylamine, 10 mM succinate and 5% water in acetonitrile at a flow rate of 0.8 ml/minute for a 30-minute run time at 40°C. The sample injection volume was 50 μl. Detection was by fluorescence. Background was determined using a similar amount of medium from the final wash drop. Betaine was quantified by comparison to external standards and the data are reported as pmol/embryo.

RT-PCR

RNA was extracted from two independent sets of MII oocytes and PI embryos, with kidney and brain as control tissues, using the RNeasy Micro Kit (Qiagen, Mississauga, ON) and reverse transcribed using the Retroscript Kit (Ambion, Austin, TX). Primer pairs were designed (OligoPerfect, Invitrogen, Carlsbad, CA) using mouse mRNA reference sequences spanning introns. The SIT1 (*Slc6a20a*) forward (sense) primer was 5′-AGCCACCAATGGCCTGATGT-3′ (nt 683-702 of NM_139142) and the reverse was 5′-AGCGATCAGGCTGCCAAAAC-3′ (nt 790-809), yielding a 127 bp amplicon. The PROT (*Slc6a7*) forward primer was 5′-GTGGCAACTGGTGGAACACG-3′ (nt 651-679 of NM_201353) and the reverse was 5′-CACTCCTCGAACCAGCAGCA-3′ (nt 957-976), yielding a 317 bp amplicon. We used, as a positive control, histone 2A family member Z (*H2afz*), previously shown to have a particularly stable expression pattern in PI embryos across different stages and culture conditions (Mamo et al., 2007). The same primer pair as in the original report (202 bp amplicon) was used. Each PCR reaction contained 0.1 embryo equivalent of cDNA. PCR was carried out with Hotstar Taq polymerase (Qiagen) in 20 μl reaction volumes in a Mastercycler thermocycler (Eppendorf, Hamburg, Germany): 95°C for 15 minutes, followed by 40 cycles of 94°C for 60 seconds, 60°C for 30 seconds, and 72°C for 60 seconds. Amplicons were visualized by agarose gel (2%) electrophoresis with ethidium bromide staining using a Typhoon 8600 Phosphorimager (Amersham, Piscataway, NJ). Amplicons derived from MII eggs and kidney for *Slc6a20a* and *Slc6a7* were sequenced by the Ontario Genomics Innovation Centre, Ottawa, Canada.

Expression of murine transporters in *Xenopus laevis* oocytes and transport activity measurements

Mouse SIT1 (*Slc6a20a*) cDNA was cloned into a pGEM-He-Juel vector as described previously (Kowalczuk et al., 2005). Mouse PROT (*Slc6a7*) in a pYX-Asc vector was purchased from Open Biosystems (Huntsville, AL). *Slc6a7* and *Slc6a20a* cRNA were prepared from *Not*I-linearized cDNA templates (Qiagen T7 mMessage Machine). Stage VI *Xenopus laevis*

oocytes were obtained as previously described (Liu and Liu, 2006). Briefly, sexually mature female *Xenopus* (NASCO, Fort Atkinson, WI) were stimulated with eCG (50 IU) 3-7 days before oocyte retrieval. Oocytes were released from excised ovaries by incubation in OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES, pH 7.8; $CaCl_2$ omitted) with 1.5 mg/ml collagenase and 1 mg/ml soybean trypsin inhibitor, and then maintained in OR2 containing 0.1 mg/ml gentamicin. Protocols were approved by the Animal Care Committee of the Ottawa Health Research Institute. Oocytes (in $CaCl_2$-free OR2) were injected with cRNA (625 μg/ml in water) into the cytoplasm, using injection volumes of 20 nl for SIT1 and 10 nl for PROT, or control injections of water. Injected oocytes were incubated in OR2 for 48 hours (PROT) or 72 hours (SIT1) at 16°C before use.

To assess transport, oocytes were washed in ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 1.8 mM $CaCl_2$, pH 7.8), rapidly transferred through two 0.5 ml aliquots of ND96 containing 1 μM [^{3}H]proline or [^{3}H]betaine, and then incubated for 20 minutes at room temperature in 1 ml ND96 with the radiolabeled substrate. Oocytes were then washed in cold ND96, placed in 4 ml of scintillation fluid and vortexed. The amount of substrate in each oocyte was determined as described above for mouse oocytes. Where indicated, potential competitive inhibitors were present during incubation at 5 mM.

Morpholino design and injection

A morpholino antisense oligonucleotide (MO) (Gene Tools LLC, Philomath, OR) of the sequence 5′-CACTGAGGCCGTGCCTTCTCCATGT-3′ (start codon underlined) directed against mouse *Slc6a20a* (NM_139142.2; confirmed unique in mouse with BLASTN) and a standard control MO (human β-globin β-thalassemia mutation) were used. GV mouse oocytes were injected with 5 pl of *Slc6a20a* or control MO (0.5 mM in water). Where indicated, *Xenopus* oocytes were injected with 10 nl of MO (1 mM; MO was co-injected with *Slc6a20a* cRNA, above, where indicated; owing to inclusion of plasmid sequence, the final MO base and cRNA were mismatched). Final MO concentrations in both mouse GV and *Xenopus* oocytes were ~10 μM. Injected and uninjected mouse GV oocytes from the same cohort were cultured overnight (~20 hours) in Minimal Essential Medium α with L-Gln (MEMα; Invitrogen) with 1 mg/ml PVA added, during which time they spontaneously matured to MII eggs, and then were parthenogenetically activated using Sr^{2+} and maintained in culture in KSOM for a further 8.5 hours. The rate of [^{3}H]betaine transport was measured as described above. Injected *Xenopus* oocytes were incubated for 72 hours and [^{3}H]proline measured as described above.

Data analysis

Data are expressed as mean±s.e.m. Graphs were produced using SigmaPlot 8.02 (SPSS, Chicago, IL). Statistical analyses were performed using InStat (GraphPad, San Diego, CA).

RESULTS

Betaine transport activity in oocytes and PI embryos

The rates of saturable betaine transport in germinal vesicle (GV) and metaphase (M) I oocytes, mature MII eggs, and PI embryos at the 1-, 2-, 4- and 8-cell, morula and blastocyst stages were obtained by comparing the total rate of transport of 1 μM [^{3}H]betaine with the non-specific rate measured in the presence of 5 mM unlabeled betaine (Fig. 1A). Substantial saturable transport of betaine was found only at the 1- and 2-cell stages, with a small but significant component persisting into the 4-cell stage. Blastocysts exhibited a high rate of non-saturable betaine transport that was likely to be the result of accumulation of betaine in the blastocoel cavity, as it was not seen in blastocysts that had been mechanically collapsed (not shown). Subtracting the rate of non-specific transport from the total rate yielded specific, saturable transport at each stage and showed that activity is maximal at the 1-cell stage and decreases at the 2-cell stage (Fig. 1B).

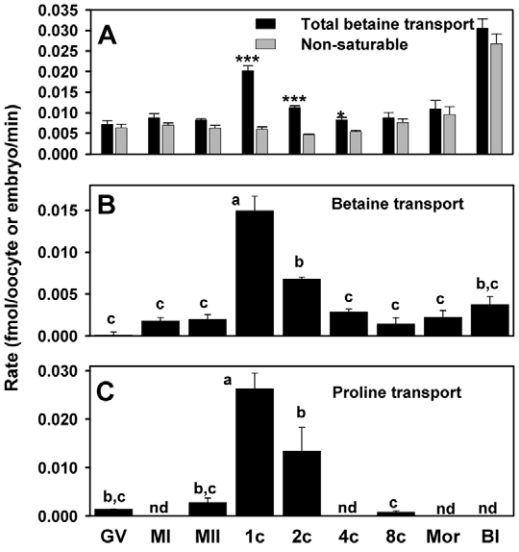

Fig. 1. Betaine and proline transport activity in mouse oocytes and preimplantation embryos. (**A**) Total and non-saturable betaine transport. The total rate of 1 μM [^{3}H]betaine transport at each stage (labeled at bottom of figure) is indicated by the black bars, and the nonspecific, non-saturable rate (with 5 mM unlabeled betaine) by the gray bars. Total betaine transport was significantly different from non-saturable transport at stages indicated by asterisks (*P<0.05, ***P<0.001 within stages, by Student's t-test). Each bar represents the mean±s.e.m. of at least three independent measurements. (**B**) Specific betaine transport. Data in A were used to obtain the rate of specific betaine transporter activity by subtracting the mean non-saturable transport from the total transport at each stage. Bars labeled with different letters are significantly different from each other (P<0.05 by ANOVA and Tukey-Kramer post-hoc test). (**C**) Specific proline transport. Specific transport of 1 μM [^{3}H]proline was calculated as for betaine. Each bar represents the mean±s.e.m. of at least three independent measurements. nd, not determined.

Two routes for proline transport were previously identified at the 1-cell stage: one that is inhibited by alanine and the other being the betaine/proline transporter (Anas et al., 2007). We determined the rate of saturable proline transport in the presence of 5 mM alanine at key developmental stages to reveal proline transport by the betaine/proline transporter. The pattern of proline transport activity (Fig. 1C) was similar to that of betaine (Fig. 1B), with a peak at the 1-cell stage, decline during the 2-cell stage, and no activity in oocytes or 8-cell stage embryos. Thus, the activity of the betaine/proline transporter was essentially restricted to the 1- and 2-cell stages.

Appearance of betaine transporter activity at egg activation

We used parthenogenetic activation with Sr^{2+} to obtain precisely timed activation of mouse MII oocytes with which to investigate the appearance of betaine/proline transport after egg activation. Betaine transport initially appeared at ~4 hours after Sr^{2+}-induced activation and reached a maximum by 10 hours (Fig. 2). Parthenogenotes in the early 2-cell stage (24 hours post-activation) exhibited maximal betaine transport, but activity declined somewhat by 30 hours and was lost by the 4-cell stage. MII oocytes maintained under identical culture conditions exhibited only low betaine transport activity, indicating that egg activation was required.

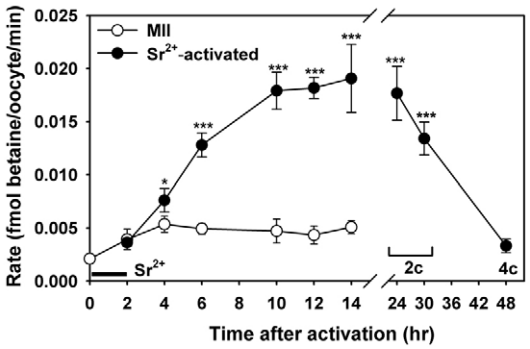

Fig. 2. Initiation of betaine transport after parthenogenetic activation with Sr^{2+}. The rate of betaine transport was measured as a function of time after activation of mouse MII oocytes by exposure to Sr^{2+} (exposure time indicated by bar). Activated oocytes are indicated by black circles. Control oocytes were treated identically but not exposed to Sr^{2+} (white circles). Parthenogenotes developed to the 2- and 4-cell stages (2c and 4c) at the times indicated. Asterisks indicate significant difference from t=0 (*P<0.05, ***P<0.001; ANOVA and Tukey-Kramer post-hoc test). Each point represents the mean±s.e.m. of three independent measurements, except for t=0 where there were ten (s.e.m. smaller than symbol and not shown).

To confirm that betaine transport was initiated with a similar time course in fertilized eggs, and thus that Sr^{2+}-activated parthenogenotes provided an adequate model, we measured the rate of betaine transport by fertilized oocytes immediately after removal from the oviduct at the specified times post-hCG. At 19 hours post-hCG (2-4 hours after fertilization, when fertilization can be first confirmed by second polar body emission), the rate of betaine transport was still low (Fig. 3). By 21 hours, an increased rate was observed, reaching a plateau by 23 hours. Thus, betaine transport appears with a similar time course in vivo.

The appearance of betaine transport activity after fertilization or egg activation could reflect de novo synthesis or the activation of pre-existing transporters. To distinguish between these, we parthenogenetically activated MII oocytes using the protein synthesis inhibitor cycloheximide, and then maintained the parthenogenotes in the continuous presence of cycloheximide for 12 hours. Cycloheximide efficacy was confirmed by (1) greater than 80% parthenogenetic activation and (2) ~90% inhibition of ^{35}S-methionine incorporation into the TCA-insoluble fraction (not shown). Cycloheximide-activated parthenogenotes maintained for 12 hours in the continuous presence of cycloheximide developed betaine transport activity, the rate of which was not significantly different from that of Sr^{2+}-activated parthenogenotes (Fig. 4). This indicated that the appearance of betaine transport activity after oocyte activation did not require protein synthesis, and showed that an independent method of parthenogenetic activation also resulted in the appearance of betaine transport.

We used the fungal toxin brefeldin A (BFA), which has been widely employed to inhibit Golgi-based membrane vesicle fusion (Dinter and Berger, 1998), to determine whether the appearance of betaine transport requires an intact Golgi. We previously confirmed that BFA causes the physical disruption of Golgi aggregates in mouse oocytes (Wang et al., 2008), consistent with previous findings (Moreno et al., 2002). Betaine transport was measured in fresh MII oocytes (t=0) and in parthenogenotes (Sr^{2+}-activated) cultured in the continuous presence or absence of BFA for up to 30 hours. A sample was removed from BFA at 7 hours and then cultured to 12 hours in its absence (Fig. 5, arrow a), and another was removed from BFA at 10 hours and then cultured to 30 hours (Fig. 5, arrow b). The effect of BFA was reversible, as transport activity developed in parthenogenotes after removal from BFA (Fig. 5, arrows). BFA also did not directly inhibit betaine transport because the rate of transport in 1-cell embryos was not significantly decreased upon 4 hours exposure to BFA (not shown). Since, in many systems, Golgi-derived vesicles are transported by cytoskeleton-dependent mechanisms, we assessed the effect of several cytoskeleton-perturbing agents. However, there was no effect on betaine transport activity at 10 hours post-Sr^{2+} activation of the continuous presence of demecolcine or nocodozole (microtubule depolymerization), cytochalasin D (F-actin depolymerization), or jasplakinolide (F-actin stabilization) (not shown).

Endogenous betaine content of 1-cell and 2-cell mouse embryos

To determine whether mouse embryos contain endogenous betaine at the developmental stages at which we have found betaine transport activity, in vivo-derived 1- and 2-cell embryos were isolated from oviducts, washed extensively, and then groups of 50 placed in tubes and dried for betaine measurements. The total endogenous betaine contents of three independent samples each of 1- and 2-cell embryos were determined. Very little betaine (0.05±0.03 pmole per embryo equivalent of medium, mean±s.e.m., n=6) was found in wash drop samples, indicating that there was no significant contamination from extracellular betaine (not significantly different from 0 by one-sample Student's t-test, P=0.20). The embryo samples (after background subtraction), by contrast, contained betaine at 0.98±0.23 pmole/1-cell embryo and 1.30±0.23 pmole/2-cell embryo (both significantly different from 0, P<0.05).

Expression of candidate betaine/proline transporters in mouse eggs and PI embryos

Two candidates had been identified that might mediate betaine/proline transport activity in 1-cell embryos: the brain proline transporter (PROT, encoded by *Slc6a7*) and the intestinal imino acid transporter (SIT1, encoded by *Slc6a20a*). To determine whether

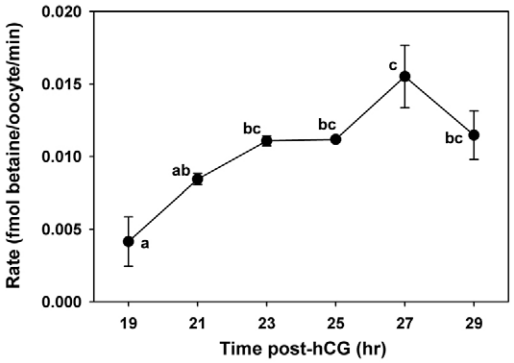

Fig. 3. Betaine transport development in vivo. The rate of betaine transport in mouse oocytes is shown 19-29 hours post-hCG. Fertilization was assumed to take place in the oviduct ~14 hours after treatment with human chorionic gonadotropin (post-hCG). Points not sharing the same letters are significantly different (P<0.05; ANOVA and Tukey-Kramer post-hoc test). Each point represents the mean±s.e.m. of three independent measurements.

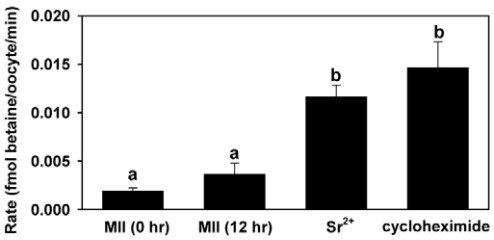

Fig. 4. Effect of inhibition of protein synthesis on development of betaine transport activity after egg activation. The rate of betaine transport was measured in freshly obtained mouse MII oocytes (MII 0 hr), MII oocytes maintained in culture for the entire period (MII 12 hr), Sr^{2+}-activated oocytes 12 hours post-activation (Sr^{2+}), and oocytes that were activated with 50 μg/ml cycloheximide. Bars with different letters are significantly different ($P<0.05$; ANOVA and Tukey-Kramer post-hoc test). Each bar represents the mean±s.e.m. of three independent measurements.

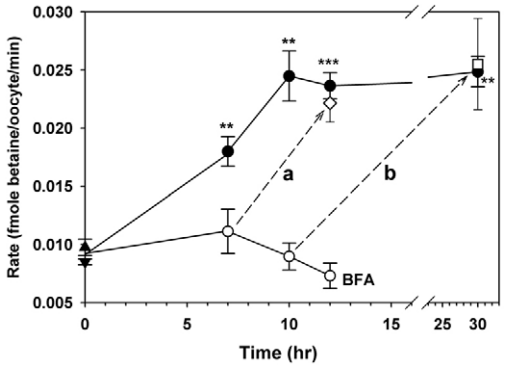

Fig. 5. Brefeldin A (BFA) reversibly inhibits development of betaine transport activity after MII oocyte activation. Betaine transport activity in mouse MII oocytes parthenogenetically activated with Sr^{2+} was determined in the presence or absence of BFA. Two separate experiments are shown: (a) 7 and 12 hours and MII oocytes at $t=0$ (triangle); (b) 10 and 30 hours and MII oocytes (inverted triangle). The results are combined in this figure, but statistical analysis was performed separately. Parthenogenotes were cultured in control medium (black circles) or with BFA (white circles). The dashed arrows indicate the transfer of parthenogenotes from BFA to BFA-free culture at the time indicated by the arrow tail (7 or 10 hours), with betaine transport measured at the time indicated by the arrowhead (12 or 30 hours). Asterisks indicate significant difference from MII oocytes at $t=0$ (**$P<0.01$, ***$P<0.001$; ANOVA and Tukey-Kramer post-hoc test). The rates after transfer from BFA (arrows) were not significantly different at 12 hours (white diamond) or 30 hours (white square) from rates after culture in the continuous absence of BFA at the same times (black circles at 12 and 30 hours). Each point is the mean±s.e.m. of three independent measurements of the total rate of betaine transport (non-saturable rate was not subtracted in this set of experiments).

mRNA for either transporter was present in PI embryos, we carried out RT-PCR on RNA isolated from MII eggs, PI embryos from the 1-cell to blastocyst stages, kidney and brain, with primers designed to amplify *Scl6a7*, *Slc6a20a* and *H2afz* (control). Surprisingly, we found that both *Scl6a7* and *Slc6a20a* mRNAs were present in MII eggs and 1-cell embryos (Fig. 6). Amplicons derived from MII eggs and kidney for *Slc6a20a* and *Slc6a7* were excised from gels and sequenced to confirm their identity. We had previously confirmed the *H2afz* amplicon by sequencing. *Slc6a7* and *H2afz*, but not *Slc6a20a*, were detected in brain as expected (not shown). We also used independent sets of primers for each gene (*Slc6a20a* and *Slc6a7*) in 1-cell embryos and confirmed their expression (not shown). Thus, both PROT and SIT1 remained as possible candidates for the betaine/proline transporter, as their mRNAs were present before transport activity appears.

Transport characteristics of SIT1 and PROT

In order to determine whether either of the candidate transporters had proline and betaine transport characteristics that resembled those in 1-cell mouse embryos (Anas et al., 2007), we expressed mouse SIT1 and PROT in *Xenopus* oocytes, and determined the characteristics of betaine and proline transport by each. Expression of SIT1 in *Xenopus* oocytes resulted in a ~9-fold increase in the uptake of 1 μM [^{3}H]proline over 20 minutes (Fig. 7A). Induced proline transport was reduced to the level in control (water-injected) oocytes by 5 mM unlabeled betaine, and thus was saturable. Proline transport was also eliminated in the presence of 5 mM 2-methylaminoisobutyric acid (MeAIB) but was unaffected by 5 mM histidine. SIT1-expressing *Xenopus* oocytes similarly showed an ~8-fold increase in [^{3}H]betaine uptake, which was reduced to the level in control oocytes by 5 mM unlabeled proline, was eliminated in the presence of MeAIB, but unaffected by histidine (Fig. 7B).

Expression of PROT in *Xenopus* oocytes resulted in a ~4-fold increase in proline uptake (Fig. 7C). In contrast to that in SIT1-expressing oocytes, proline transport in PROT-expressing oocytes was not inhibited by the presence of either 5 mM unlabeled betaine or 5 mM MeAIB, but was reduced to the level in control oocytes by 5 mM histidine. Uptake of betaine by PROT-expressing *Xenopus* oocytes was not greater than that of control oocytes (Fig. 7D), indicating that PROT does not transport betaine at detectable levels.

Effect on betaine transport in 1-cell mouse embryos of a morpholino directed against *Slc6a20a*

To determine whether knockdown of SIT1 expression affected endogenous betaine transport in mouse parthenogenotes, we used a morpholino (MO) designed to block *Slc6a20a* translation. De novo protein synthesis is apparently not required for the appearance of betaine transport activity after egg activation (see above, Fig. 4). Therefore, we injected GV mouse oocytes and then in vitro matured and parthenogenetically activated them, hypothesizing that the presence of the *Slc6a20a* MO for the ~28 hours required for oocyte maturation and activation might interfere with the maintenance of SIT1 protein. GV oocytes that were in vitro matured and then parthenogenetically activated transported betaine at a rate similar to parthenogenetically activated in vivo matured oocytes, indicating that in vitro maturation itself did not affect the post-activation development of betaine transport. In oocytes injected with the *Slc6a20a* MO, however, transport was significantly reduced (to approximately the level of non-specific transport; see Fig. 1A), whereas the control MO had no effect (Fig. 8A). Therefore, the appearance of betaine transport after egg activation is substantially inhibited when SIT1 protein translation is specifically blocked in mouse oocytes.

To confirm that the MO we used could inhibit functional expression of SIT1, we injected it into *Xenopus* oocytes expressing exogenous mouse SIT1 (as described above). Induced [^{3}H]proline transport was significantly inhibited by the *Slc6a20a* MO, but not the control MO (Fig. 8B), confirming that the *Slc6a20a* MO decreased functional expression of mouse SIT1.

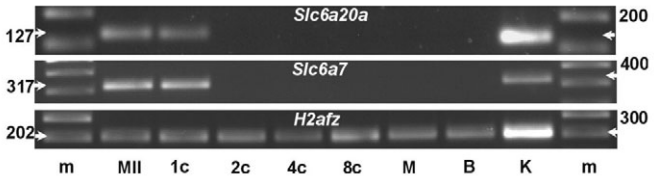

Fig. 6. RT-PCR detection of mRNA for SIT1 (*Slc6a20a*) and PROT (*Slc6a7*). PCR was performed on cDNA from mouse MII eggs (MII), 1-cell (1c), 2-cell (2c), 4-cell (4c), 8-cell (8c), morula (M) and blastocyst (B) stage embryos, with kidney (K) as a positive control to indicate the position of amplicon (the band intensity from kidney relative to those of the embryos is of no physiological relevance). *Slc6a20a* and *Slc6a7* mRNAs were most strongly detected in MII eggs and 1-cell embryos. *H2afz* was used as a positive control for the presence of cDNA. The expected amplicon sizes (bp) are indicated on the left and by arrows in each marker lane (m, a 100-bp ladder, the largest visible band of which is sized). Only portions of each gel are shown, but no other bands were visible below or above. The example shown is one of two independently collected sets of RNA. In the other, intense bands were evident at the MII and 1-cell stages as shown here, but faint bands were also visible at other stages.

DISCUSSION

Betaine/proline transport is activated by fertilization and restricted to a short period of development

Virtually all transporters, including a number of amino acid and metabolite transporters, that have been found to be active in 1-cell embryos are also similarly active in MII eggs and thus do not change upon fertilization (Harding et al., 1999; Van Winkle et al., 1988; Van Winkle et al., 1990). Indeed, to our knowledge, none of the diverse array of transporters of biologically important organic compounds has been found to be activated (or inactivated) shortly after fertilization in mammalian eggs, although several ion channels and intracellular pH-regulatory mechanisms change in activity during the immediate post-fertilization period (Day et al., 1998; Lane et al., 1999; Phillips and Baltz, 1999), and a cysteine transporter is reportedly activated shortly beforehand, during meiotic maturation (Van Winkle et al., 1992). Here we have found that the betaine/proline transporter previously identified in mouse 1-cell embryos is quiescent in oocytes prior to fertilization and then becomes activated within several hours of egg activation, providing the first example of such a transporter that is activated upon fertilization in mammals. Once betaine/proline transport is activated, it persists for only a little more than one cell cycle before becoming inactive again. We found high transporter activity only at the 1-cell and 2-cell stages, both in embryos that had developed in vivo and in parthenogenotes that developed in vitro. We are not aware of any other transporters, the functional expression of which is restricted to so short a developmental period of very early PI embryogenesis.

The appearance of betaine/proline transport after egg activation apparently involves pre-existing transporters, rather than de novo protein synthesis, as parthenogenotes developed the same level of betaine transport in the continuous presence of cycloheximide as in Sr^{2+}-activated eggs. We further found that the Golgi disruptor BFA reversibly prevented the development of betaine transport activity in activated eggs, while not affecting activity that had already developed in 1-cell embryos. Thus, we infer that insertion of presynthesized proteins into the plasma membrane is required for activation. One route for membrane protein insertion into the egg plasma membrane after egg activation is cortical granule exocytosis. However, this occurs very rapidly after egg activation and is blocked by perturbation of F-actin (Sun and Schatten, 2006), unlike the betaine/proline transporter. A better candidate is a distinct process of protein trafficking that has been reported to be more slowly induced by egg activation in mammals and is blocked by BFA but insensitive to disruption of F-actin or microtubules (Clayton et al., 1995), similar to our findings here for betaine/proline transport. This mechanism mediates the appearance of the cell adhesion molecule uvomorulin (E-cadherin) in the plasma membrane over a period of ~6 hours following egg activation (Clayton et al., 1995), and occurs during the same period that the fragmented, inactive Golgi in MII oocytes becomes reorganized following fertilization (Payne and Schatten, 2003).

Identification of the betaine/proline transporter in mouse embryos

It was previously proposed (Anas et al., 2007) that the betaine/proline transporter in 1-cell embryos was most likely a member of the neurotransmitter transporter (Slc6) gene family, and two candidates were identified based on their acceptance of proline as a substrate:

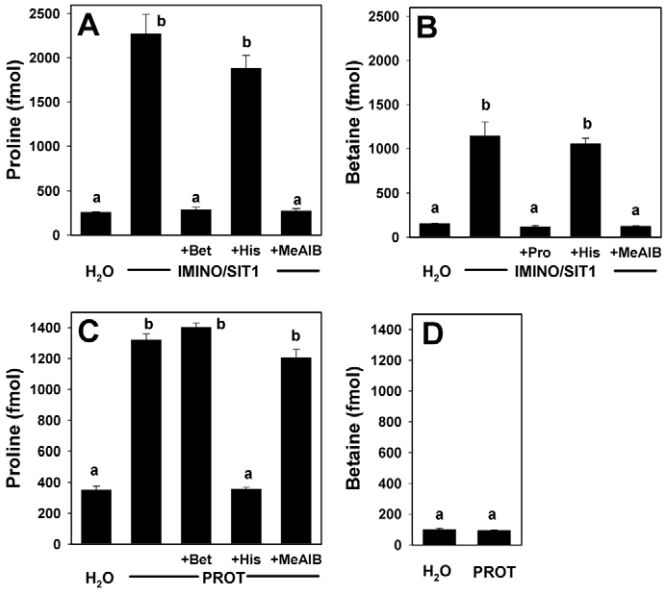

Fig. 7. Proline and betaine transport by *Xenopus* oocytes expressing mouse SIT1 or PROT. (**A**,**B**) Transport characteristics of *Xenopus* oocytes expressing mouse SIT1. Proline (A) and betaine (B) transport were measured in oocytes expressing SIT1 or in control water-injected oocytes (H_2O) as indicated. The effect of 5 mM betaine (+Bet), histidine (+His) or 2-methylaminoisobutyric acid (+MeAIB) is shown. Bars with different letters (a, b) are significantly different within each panel (P<0.05 by ANOVA and Tukey-Kramer post-hoc test). Each bar represents the mean±s.e.m. of four to seven individual oocytes, except for the water-injected control (n=10). (**C**,**D**) Transport characteristics of *Xenopus* oocytes expressing mouse PROT. Proline (C) and betaine (D) transport were measured in oocytes expressing PROT (labels as in A,B). Since there was no saturable betaine transport (D), competitive inhibitors were not tested for betaine. Each bar represents the mean±s.e.m. of five to eight oocytes, except the water-injected control (n=10). One additional set of injections of PROT was performed (not shown), which confirmed that proline but not betaine uptake was increased in PROT-expressing oocytes as compared with water-injected oocytes (n=5-7 each).

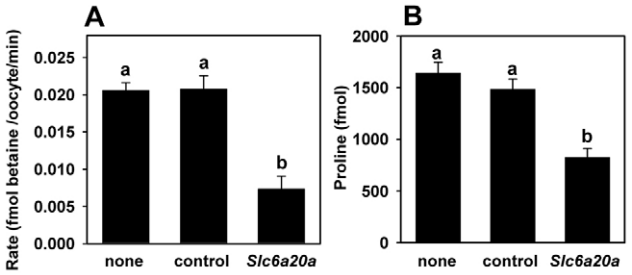

Fig. 8. Morpholino against *Slc6a20a* suppresses betaine transport in mouse parthenogenotes. (**A**) Effect of morpholinos on betaine transport in mouse parthenogenotes. Rates of betaine transport were determined after injection of MOs into GV oocytes followed by in vitro maturation and parthenogenetic activation with Sr^{2+}. The MO designed to block translation of SIT1 (*Slc6a20a*) significantly decreased the rate of betaine transport (a versus b, $P<0.01$ by ANOVA and Tukey-Kramer post-hoc test) relative to uninjected parthenogenotes (none) or those injected with a control MO. Each bar represents the mean±s.e.m. of three independent experiments, each containing 8-12 oocytes. (**B**) Effect of MOs on functional expression of exogenous mouse SIT1 in *Xenopus* oocytes. *Xenopus* oocytes were co-injected with *Slc6a20a* cRNA and MOs (labeled as in A). Induced proline uptake was significantly decreased by the *Slc6a20a* MO (a versus b, $P<0.001$ by ANOVA and Tukey-Kramer post-hoc test). Each bar represents the mean±s.e.m. proline uptake in seven to eight individual oocytes after subtraction of the mean uptake in water-injected controls.

PROT (*Slc6a7*) and SIT1 (*Slc6a20a*). We unexpectedly found mRNA for both in eggs and 1-cell embryos. In addition, gene array data for PI embryo development including MII oocytes (Zeng et al., 2004) deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/projects/geo) show similar expression patterns for each gene to those we report here (GEO accession/array ID/gene name: GDS813/1422899_at/*Slc6a20a* and GDS814/1455469_at/*Slc6a7*). Thus, we could not rule out either candidate based on mRNA expression.

We expressed each candidate in *Xenopus* oocytes and tested their ability to transport betaine or proline and whether, like the betaine/proline transporter in 1-cell embryos, this was inhibited by MeAIB. In addition, inhibition by histidine was tested because it had previously been reported to block proline transport by PROT (Fremeau et al., 1992). The results appear to rule out PROT because although *Xenopus* oocytes expressing PROT showed increased transport of proline, there was no increase in betaine transport. Also, proline transport was blocked by histidine but not inhibited by MeAIB and, most importantly, it was not inhibited by excess betaine. Although we cannot rule out the possibility that accessory proteins required for normal PROT function are absent from *Xenopus* oocytes, the close correspondence of proline transport characteristics in PROT-injected *Xenopus* oocytes to those previously reported in mammalian cells (Fremeau et al., 1992) makes this unlikely. By contrast, the transport characteristics of mouse SIT1 in *Xenopus* oocytes closely matched those of betaine/proline transport in 1-cell mouse embryos (Anas et al., 2007). Both betaine and proline were transported, inhibited by MeAIB, and resistant to histidine. In addition, the rate of transport of 1 μM proline was approximately twice that of 1 μM betaine, which is the same relationship as that found in 1-cell mouse embryos (Anas et al., 2007). Taken together, these results implicate SIT1 as the transporter that is likely to mediate betaine and proline transport in 1-cell mouse embryos.

This conclusion was supported by the results obtained with a MO targeted against *Slc6a20a*, which showed a substantial loss of betaine transport by 1-cell mouse embryos in which the *Slc6a20a* MO had been present from the GV stage. Since inhibition of global protein synthesis with cycloheximide did not block the appearance of betaine/proline transport activity at 12 hours post-egg activation, the efficacy of the MO when present from the GV stage implies SIT1 synthesis during meiotic maturation. Further investigations into the mechanism underlying the appearance of transport activity after fertilization are clearly needed. Immunolocalization of the transporter protein in mouse oocytes and early embryos would help address this question. However, thus far we have not been able to successfully use the available antiserum raised against SIT1 (a kind gift of Dr F. Verrey) for immunocytochemistry in mouse oocytes.

Function of the betaine/proline transporter SIT1 in PI mouse embryos

The activation of betaine/proline transport after fertilization and its presence only during a very short period of development implies that it serves an important function during PI embryo development. Although, in vitro, SIT1 transports betaine and proline, we propose that its physiological function in the early embryo is to mediate betaine transport. Betaine is apparently accumulated to relatively high levels in vivo. Assuming a volume of cleavage-stage embryos of ~180 pl, the measured amounts of endogenous betaine in 1- and 2-cell embryos correspond to intracellular concentrations of ~6-7 mM. This is comparable to the average concentration of ~4 mM in liver, which is the tissue with the highest known endogenous betaine level, and much higher than the ~0.1 mM in rodent blood plasma (Slow et al., 2008) or ~0.5 mM in mouse oviducts (Anas et al., 2007). The apparent accumulation of betaine in vivo would appear to imply that a major function of SIT1 is to mediate betaine accumulation or retention by early PI embryos.

The betaine/proline transporter in PI embryos that we have identified here as SIT1 is the sole route of betaine transport in PI embryos, whereas there are at least two routes of proline transport (Anas et al., 2007). The second proline transport route in 1-cell embryos does not correspond to PROT despite the presence of its mRNA, but instead resembles the classical transport system ASC (Anas et al., 2007). Although proline is likely to be transported into early embryos, the importance of SIT1-mediated uptake relative to other routes is not known.

The mechanism by which betaine exerts a beneficial effect on PI embryo development is not yet known. As discussed above, two established functions for betaine in mammals are as an organic osmolyte and as a methyl group donor. We (Dawson and Baltz, 1997; Hammer and Baltz, 2002) and others (Biggers et al., 1993) have established the ability of betaine to protect PI embryo development against increased osmolarity in vitro, although it is not yet certain that it performs this role in vivo. Previous work has established glycine as a major organic osmolyte in cleavage-stage PI mouse embryos. Glycine is transported by the specific transporter GLYT1 (SLC6A9) (Steeves et al., 2003), and endogenous glycine is present in 1-cell mouse embryos at high levels [~25 mM free glycine in freshly obtained 1- and 2-cell embryos; our unpublished data, measured as described by Steeves et al. (Steeves et al., 2003)]. Thus, betaine present at ~6-7 mM might play an important role along with glycine in osmoprotection in vivo.

An alternative hypothesis is that intracellular betaine might instead serve as a methyl pool during PI embryogenesis. One crucial set of events occurring during PI embryogenesis is the

global DNA demethylation and remethylation between fertilization and implantation, which mediates the switch from parental to embryonic epigenetic marking, while maintaining DNA methylation of uniparentally imprinted genes (Lucifero et al., 2004). Although the folate pathway is usually thought to provide the methyl groups for the large array of methyltransferases in most cells outside liver, it might be that betaine participates in PI embryos because late PI embryos appear to express betaine-homocysteine methyltransferase (BHMT), the key enzyme in liver that mediates the transfer of methyl groups from betaine (our unpublished data). Further work, however, is needed to explore this possibility, including determining whether betaine accumulated at the 1- to 2-cell stage can be retained long enough to be utilized in the peri-implantation period.

In summary, we have found that the betaine/proline transporter is expressed for only a restricted period of development starting a few hours after fertilization and continuing through the 2-cell stage. This transport activity is likely to correspond to SIT1, arising by activation of pre-existing transporter proteins after fertilization. Although betaine has been clearly shown to have beneficial effects on PI embryos, acting as an organic osmolyte and promoting in vitro development past the 2-cell stage, further work is needed to fully establish the physiological functions of the betaine that is accumulated by early PI mouse embryos.

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