Inositol transport in mouse oocytes and preimplantation embryos: effects of mouse strain, embryo stage, sodium and the hexose transport inhibitor, phloridzin

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The uptake of *myo*-inositol by mouse oocytes and preimplantation embryos of a crossbred (DBA × C57BL/6) and a purebred outbred strain (MF1) was measured using [2-³H]*myo*-inositol. Uptake in crossbred embryos increased about 15-fold between the one- and two-cell stages and increased again by about sixfold at the blastocyst stage compared with the morula stage. Uptake in purebred embryos increased about 42-fold between the one- and two-cell stages and increased more than threefold at the blastocyst stage compared with the morula stage. In all stages examined, except two-cell crossbred embryos, inositol uptake was, depending on the stage, either largely or partly sodium dependent and could be inhibited by the sodium-dependent hexose transport inhibitor, phloridzin. This is consistent with the hypothesis that transport occurs via a sodium *myo*-inositol transporter (SMIT) protein.

In addition, there was strong evidence that a sodium-independent mechanism of uptake, possibly a channel, was switched on at the two-cell stage coincident with zygotic gene activation which resulted in 141-fold and 71-fold increases in sodium-independent uptake from the one-cell to two-cell stages in crossbred and purebred embryos, respectively. This mechanism was either abolished or drastically downregulated at the blastocyst stage, whereas sodium-dependent uptake was markedly upregulated. In two-cell crossbred embryos, there was a complete abolition of sodium-dependent uptake, again possibly regulated by zygotic gene activation. The hypothesis that the changes in mechanism of inositol uptake at about the two-cell stage are due to zygotic gene activation was supported by the finding that these changes did not occur in parthenogenetic two-cell embryos.

Introduction

myo-Inositol has several important functions in mammalian cells. First, it is a precursor of phosphoinositides which, in addition to their role as structural components of cell membranes, have key roles as signalling molecules. Receptor-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP₂) by phospholipase C, leading to the production of the second messengers Ins(1,4,5)P₃ and diacylglycerol, is a major mechanism for the coupling of cell surface receptors to cellular responses (for review, see Berridge, 1992). Second, inositol is a precursor of the glycosylphosphatidylinositols, which act as anchoring moieties for many membrane-bound proteins (for review, see Low and Saltiel, 1988). Some glycosylphosphatidylinositols generate water-soluble oligosaccharides termed inositol phosphoglycans, which also act as signalling molecules (Saltiel *et al.*, 1988; Jones and Varela-Nieto, 1998). Third, inositol itself acts as an osmolyte, controlling cell volume and protecting a wide

variety of cells exposed to hyperosmotic stress (Burg, 1994; Strange *et al.*, 1994; Handler and Kwon, 1996).

Transport of inositol is usually controlled by the sodium inositol co-transporter (SMIT), the dominant inositol uptake system for mammalian cells and tissues (Kwon *et al.*, 1992). Similarities in kinetics, Hill coefficients and inhibition by phloridzin between SMIT and the sodium-glucose transporter (SGLT1) in *Xenopus* oocytes indicate that the two transporters share a common mechanism and many structural features (Hager *et al.*, 1995).

Embryo culture studies have demonstrated that inositol has an essential role in the growth of rabbit blastocysts (Kane, 1989) and in the hatching of hamster blastocysts (Kane and Bavister, 1988). Inositol improves development of IVF-produced bovine embryos to blastocysts in defined medium in the absence of serum (Holm *et al.*, 1999). Inositol is transported into mouse, rabbit and bovine embryos and then incorporated into various phospholipid and inositol phosphates (Kane *et al.*, 1992; Fahy and Kane, 1993; Hynes *et al.*, 2000). In addition, there is strong evidence from inhibitor studies (Stachecki and Armant, 1996a,b) that the phosphatidylinositol system plays an important role in

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mouse blastocyst formation. However, there is conflicting evidence from gene knockout studies: Wang *et al.* (1998) found that knockout of the *PLC β 3* gene causes embryonic death before the blastocyst stage whereas Xie *et al.* (1999), using a much more limited disruption of the *PLC β 3* gene, found that such embryos developed to term.

The only previous study (Kane *et al.*, 1992) on inositol uptake by mouse embryos did not examine the effect of mouse strain and the sodium dependency of uptake was examined only at the blastocyst stage; therefore, the aim of this study was to investigate [3 H]inositol uptake in crossbred (DBA $\times$ C57BL/6 $\times$ DBA) and purebred outbred (MF1) one-cell and two-cell embryos, morulae and blastocysts. These two strains were used because, in relation to the two-cell block, the crossbred is a non-blocking strain and the purebred is a blocking strain. The sodium dependency of inositol uptake in all stages was also examined, and the study extended to oocytes and two-cell parthenogenetic embryos. Parthenogenetic embryos were used to investigate whether changes in the mechanism of inositol transport at the two-cell stage were related to zygotic gene activation.

Materials and Methods

Collection and culture of embryos

Virgin female mice (6–8 weeks old) were primed with a subcutaneous injection of 5 iu eCG (Intervet, Cambridge) at 14.00 h followed 48 h later by 5 iu hCG (Intervet). Females were then placed with proven males (1:1 ratio) and mating was assumed to have occurred in the middle of the dark cycle. Crossbred embryos were produced from DBA $\times$ C57BL/6 females and DBA males. Purebred embryos were produced from MF1 females and males. Mice were maintained in an animal house with a light cycle of 07.00 h lights on, 21.00 h lights off. Oocytes and one-cell embryos were collected on day 1 after mating; two-cell embryos were collected on day 2; morulae were collected on day 3 and blastocysts on day 4. The collection medium used was Hepes-buffered medium H6 BSA (Nasr-Esfahani *et al.*, 1990). Oocytes (DBA $\times$ C57BL/6) were collected from unmated females. Oocytes and one-cell embryos were collected in a cumulus clot by oviductal puncture and the cumulus cells were then removed by incubation for 2 min in 300 U ml $^{-1}$ hyaluronidase (Type I-S; Sigma-Aldrich, Gillingham) in H6 BSA. Oviducts were flushed to recover two-cell embryos and morulae; uterine horns were flushed to recover blastocysts. Embryos were collected between 11:00 h and 13:00 h.

The composition of the basic sodium plus incubation medium for all experiments was 0.1% (w/v) BSA, 108 mmol NaCl l $^{-1}$, 4.78 mmol KCl l $^{-1}$, 1.71 mmol CaCl $_2$ $\cdot$ 2H $_2$ O l $^{-1}$, 1.19 mmol KH $_2$ PO $_4$ l $^{-1}$, 1.19 mmol MgSO $_4$ $\cdot$ 7H $_2$ O l $^{-1}$, 25 mmol NaHCO $_3$ l $^{-1}$, 0.5 mmol sodium pyruvate l $^{-1}$ and the concentrations of

amino acids, vitamins and trace elements were the same as Ham's F10 medium (Ham, 1963; Kane and Foote, 1970), with two modifications (Kane, 1989): 3 μ mol serine l $^{-1}$ and 15 μ mol inositol l $^{-1}$. The salt composition of this medium was based on the mouse embryo culture medium of Brinster (1963). The complete medium is used for rabbit embryo culture and inositol transport studies (Fahy and Kane, 1994). For the sodium minus medium, the sodium chloride and sodium bicarbonate were replaced by choline chloride and choline bicarbonate on an equimolar basis. The sodium minus medium had a low amount of residual sodium due to the presence of sodium pyruvate and some amino acids as sodium salts.

Parthenogenetic embryos were produced by incubating oocytes (DBA $\times$ C57BL/6) in modified Ca $^{2+}$ -free M16 medium with 10 mmol SrCl l $^{-1}$ and 10 $^{-3}$ mmol cytochalasin B l $^{-1}$ at 38.58°C for 3 h. After activation and classification, diploid parthenogenetic embryos were removed from the Ca $^{2+}$ -free M16 medium, washed twice in 1 ml drops of standard M16 medium and then cultured in 1 ml drops of M16 medium until they reached the two-cell stage.

Measurement of [3 H]myo-inositol uptake by embryos

The [3 H]inositol used for transport studies was [3 H]myo-inositol (NEN, Dupont de Nemours, Bad Homburg) with specific activities varying from 20 to 23 Ci mmol $^{-1}$. All embryos were washed in basic incubation medium before they were incubated. All incubations were carried out at 38.5°C in basic sodium plus incubation medium under mineral oil in 5% CO $_2$ in air, unless otherwise stated. A temperature of 38.5°C was used because similar studies were being carried out at about the same time in cattle and rabbit embryos for which 38.5°C is near the optimal temperature. Although this temperature is probably not optimal for mouse embryos, as mouse body temperature is about 37°C (Green, 1966), the 1.5°C difference is unlikely to have seriously affected transport. The embryos were washed through four 1 ml drops of H6 BSA after incubation. They were then placed in a scintillation vial containing 0.5 ml of 10% (w/v) trichloroacetic acid, after which 8 ml of scintillation cocktail (Ready Safe; Beckman Coulter, High Wycombe) was added. An equivalent volume of the final wash medium was treated similarly as a control. The samples were counted in a Wallac 1409 scintillation counter.

Experiment 1: effect of sodium on inositol uptake in crossbred and purebred oocytes and embryos

Oocytes, one-cell and two-cell embryos, morulae and blastocysts from crossbred (DBA $\times$ C57BL/6, oocytes; DBA $\times$ C57BL/6 $\times$ DBA, embryos) and purebred MF1 mice were incubated for 2 h in 100 μ l of either sodium plus or sodium minus incubation medium containing

Fig. 1. Effects of sodium on the uptake of inositol by oocytes and preimplantation mouse embryos. (a) Uptake in crossbred oocytes (DBA $\times$ C57BL/6) and embryos (DBA $\times$ C57BL/6 $\times$ DBA). Details of oocytes and embryos are as follows: oocytes, three replicates of 11–23 oocytes per treatment (total of 103 oocytes); one-cell embryos, six replicates of 15–29 embryos per treatment (total of 272 embryos); two-cell embryos, eight replicates of 7–38 embryos per treatment (total of 232 embryos); morulae, nine replicates of 5–11 embryos per treatment (total of 135 embryos); and blastocysts, four replicates of 3–5 embryos per treatment (total of 35 embryos). (b) Uptake in purebred MF1 oocytes and embryos. Details of oocytes and embryos are as follows: oocytes, five replicates of 12–23 oocytes per treatment (total of 177 oocytes); one-cell embryos, eight replicates of 10–21 embryos per treatment (total of 259 embryos); two-cell embryos, five replicates of 6–14 embryos per treatment (total of 103 embryos); morulae, three replicates of 5–7 embryos per treatment (total of 38 embryos); and blastocysts, five replicates of 4–7 embryos per treatment (total of 50 embryos). $\square$: sodium minus medium; $\blacksquare$: sodium plus medium. Values are means

100 μCi [^3H]inositol ml^{-1} . Inositol uptake was measured in both sodium plus and sodium minus medium. Sodium-dependent uptake was calculated as the difference between uptakes in sodium plus and sodium minus medium.

Experiment 2: effect of sodium on inositol uptake in parthenogenetically activated two-cell crossbred DBA $\times$ C57BL/6 embryos

Two-cell parthenotes were incubated for 2 h in 100 μl of either sodium plus or sodium minus medium containing 100 μCi [^3H]inositol ml^{-1} .

Experiment 3: effect of phloridzin on inositol uptake in crossbred DBA $\times$ C57BL/6 $\times$ DBA embryos

DBA $\times$ C57BL/6 $\times$ DBA one-cell and two-cell embryos and blastocysts were cultured for 2 h in 100 μl of basic incubation medium containing 100 μCi [^3H]inositol ml^{-1} and 50 μmol phloridzin l^{-1} (Sigma-Aldrich). Phloridzin is a competitive inhibitor of sodium-dependent inositol transport (Diecke *et al.*, 1995). As phloridzin was dissolved in ethanol, both the control and phloridzin media contained the same final concentration of ethanol.

Statistical analysis

Differences in inositol uptake between every two successive stages of embryo development were analysed using the Student's *t* test. It was not possible to use analysis of variance and *post hoc* tests to carry out an overall analysis because of non-homogeneity of variances related to widely different stage means. The Student's *t* test was also used to analyse differences between paired treatment means (for example, the presence and absence of sodium) at different developmental stages.

Results

Experiment 1: effect of sodium on inositol uptake in crossbred and purebred oocytes and embryos

Data on the effect of mouse strain, developmental stage and sodium content of the incubation medium on inositol uptake by crossbred and purebred oocytes and embryos (Fig. 1) showed that in sodium plus medium at all stages examined, uptake in crossbred stages was higher than in purebred stages: oocytes, 2.0-fold ($P < 0.01$); one-cell embryos, 3.6-fold ($P < 0.001$); two-cell embryos, 1.3-fold ($P < 0.05$); morulae, 1.7-fold ($P < 0.05$); and blastocysts, 3.3-fold higher ($P < 0.001$).

$\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with sodium minus medium for the same embryo stage.

Inositol uptake was also markedly stage dependent. In the crossbred strain, uptake by DBA $\times$ C57BL/6 $\times$ DBA one-cell embryos in sodium plus medium (0.30 fmole per embryo per h) increased 2.2-fold ($P < 0.01$) compared with DBA $\times$ C57BL/6 oocytes (0.13 fmole per oocyte per h). The uptake per embryo increased more than 15-fold ($P < 0.001$ from the one-cell stage to the two-cell stage (4.5 fmole per embryo per h)). Uptake increased a further 1.7-fold ($P < 0.01$) at the morula stage (7.7 fmole per embryo per h) and an additional sixfold ($P < 0.001$) at the blastocyst stage (46.1 fmole per embryo per h). In the purebred strain, there was a non-significant 1.3-fold increase in uptake by MF1 one-cell embryos in sodium plus medium (0.085 fmole per embryo per h) compared with MF1 oocytes (0.066 fmole per oocyte per h). The uptake per embryo increased more than 42-fold ($P < 0.001$) from the one-cell stage to the two-cell stage (3.6 fmole per embryo per h). Uptake increased 1.3-fold ($P < 0.05$) at the morula stage (4.6 fmole per embryo per h) and increased a further threefold ($P < 0.01$) at the blastocyst stage (13.8 fmole per embryo per h).

Uptake of inositol was mainly sodium dependent in both crossbred and purebred oocytes, one-cell embryos and blastocysts. There was also significant sodium-dependent uptake in MF1 two-cell embryos and both DBA $\times$ C57BL/6 $\times$ DBA and MF1 morulae (Fig. 1). In the crossbred strain, omission of sodium from the medium decreased inositol uptake in oocytes by 85% ($P < 0.01$), one-cell embryos by 89% ($P < 0.001$), morulae by 34% ($P < 0.05$) and blastocysts by 99% ($P < 0.001$). However, inositol uptake by two-cell DBA $\times$ C57BL/6 $\times$ DBA embryos was totally independent of sodium (Fig. 1a). There was also a large proportion of sodium-independent uptake at the morula stage (66%). In purebred MF1 stages, omission of sodium decreased uptake of inositol in oocytes by 46% ($P < 0.05$), one-cell embryos by 52% ($P < 0.001$), two-cell embryos by 19% ($P < 0.01$), morulae by 61% ($P < 0.05$) and blastocysts by 96% ($P < 0.001$). However, these results also indicate that there was a large proportion of sodium-independent uptake occurring at all MF1 stages from oocyte to morula, particularly at the two-cell stage (81%).

The changes in sodium-dependent and sodium-independent inositol uptake with preimplantation embryo development are interesting. There were large increases in sodium-independent uptake from one-cell to two-cell embryos: 141-fold in DBA $\times$ C57BL/6 $\times$ DBA and 71-fold in MF1 embryos. From two-cell embryos to morulae, there was a further 12% increase in sodium-independent uptake of inositol in DBA $\times$ C57BL/6 $\times$ DBA embryos and a 38% decrease in uptake in MF1 embryos. From morulae to blastocysts, there were 92% (DBA $\times$ C57BL/6 $\times$ DBA) and 72% (MF1) decreases in sodium-independent uptake (Fig. 1).

There was a striking difference between DBA $\times$ C57BL/6 $\times$ DBA and MF1 embryos in the pattern of

Fig. 2. Sodium-dependent uptake of inositol by one-cell and two-cell mouse embryos and morulae. (a) Uptake by crossbred DBA $\times$ C57BL/6 $\times$ DBA embryos. (b) Uptake by purebred MF1 embryos. Values are means $\pm$ SEM calculated from the data from Fig. 1 on the effects of sodium on the uptake of inositol by preimplantation mouse embryos. Sodium-dependent uptakes were calculated as the differences between uptake in sodium plus and sodium minus medium. The SEMs were calculated using the formula for the variance of the differences between two samples of unequal variances and means (Steel and Torie, 1960).

sodium-dependent inositol uptake (Fig. 2). In DBA $\times$ C57BL/6 $\times$ DBA embryos, sodium-dependent uptake present at the one-cell stage was abolished totally at the two-cell stage and then restored in morulae to an amount ten times that at the one-cell stage (Fig. 2). There was a further 17-fold increase at the blastocyst stage (Fig. 1). In contrast, in MF1 embryos, sodium-dependent inositol uptake increased at all development stages (Fig. 2), even though uptake at the two-cell stage was much less than sodium-independent uptake (Fig. 1b).

Experiment 2: effect of sodium on inositol uptake in parthenogenetically activated two-cell crossbred DBA $\times$ C57BL/6 embryos

Omission of sodium from the medium decreased ($P < 0.001$) inositol uptake in two-cell parthenogenetic DBA $\times$ C57BL/6 embryos by 82% (Fig. 3). Thus, there was a clear difference between the two-cell normal and parthenogenetic embryos. Sodium-dependent inositol uptake was not abolished or even inhibited in the two-cell parthenogenetic embryos; instead it increased 1.9-fold (0.52 fmol per embryo per h in two-cell-parthenogenetic DBA $\times$ C57BL/6 embryos compared with normal one-cell DBA $\times$ C57BL/6 embryos (0.27 fmol per embryo per h; Fig. 1a inset)).

Fig. 3. The effect of sodium on the uptake of inositol by parthenogenetic crossbred DBA $\times$ C57BL/6 mouse two-cell embryos. □: sodium minus medium; ■: sodium plus medium. Values are means $\pm$ SEM on the basis of three replicates of 9–17 embryos per treatment (total of 78 embryos). *** $P < 0.001$.

Fig. 4. The effects of phloridzin on the uptake of inositol by crossbred DBA $\times$ C57BL/6 $\times$ DBA mouse one-cell and two-cell mouse embryos and blastocysts. Details of embryos are as follows: one-cell, two replicates of 13–19 embryos per treatment (total of 64 embryos); two-cell, three replicates of 17–25 embryos per treatment (total of 129 embryos); and blastocysts, three replicates of 5–8 embryos per treatment (total of 36 embryos). □: no phloridzin; ■: phloridzin (50 μ mol l⁻¹). Values are means $\pm$ SEM. * $P < 0.05$, *** $P < 0.001$ compared with no phloridzin medium for the same embryo stage.

Experiment 3: effect of phloridzin on inositol uptake in crossbred DBA $\times$ C57BL/6 $\times$ DBA embryos

There was no significant effect of phloridzin, an inhibitor of sodium-dependent hexose transport, on the uptake of inositol in DBA $\times$ C57BL/6 $\times$ DBA two-cell

embryos (Fig. 4). However, phloridzin significantly inhibited inositol uptake in DBA $\times$ C57BL/6 $\times$ DBA one-cell embryos ($P < 0.05$) and blastocysts ($P < 0.001$).

Discussion

A number of conclusions emerge from the present results. First, inositol uptake by mouse oocytes and preimplantation embryos is strain dependent. Uptake of inositol at all developmental stages was higher in the crossbred DBA $\times$ C57BL/6 strain compared with the purebred MF1 strain. Second, uptake is stage dependent. When a physiological sodium-containing incubation medium was used, uptake increased at all successive stages from oocyte to blastocyst. This increase in inositol uptake with developmental stage was not related to increasing size and protein content of the mouse embryo because the early mouse blastocyst differs little in protein content and dry matter from the one-cell embryo (Brinster, 1967; Sellens *et al.*, 1981; Turner *et al.*, 1992). A similar stage-dependent pattern of inositol uptake was seen by Kane *et al.* (1992) in preimplantation C57BL/6J $\times$ CBA/Ca embryos. Third, there is clear evidence for at least two distinct inositol transport processes, one sodium dependent and one or two sodium independent, operating during mouse preimplantation development. Specific mechanisms are activated or inhibited at certain stages of development.

The sodium-dependent process was present in oocytes, one-cell embryos, morulae and blastocysts of both DBA $\times$ C57BL/6 and MF1 strains. The sodium-dependent process was also present in two-cell MF1 purebred embryos but not in two-cell DBA $\times$ C57BL/6 $\times$ DBA embryos. The evidence reported here on the basis of the effects of removal of sodium from the incubation medium and the effects of the sodium-dependent transport inhibitor, phloridzin, strongly supports the hypothesis that inositol transport occurs via a sodium-dependent co-transporter protein, most probably the SMIT protein, during these stages of development. There is a considerable amount of information available on SMIT, and the SMIT gene has been cloned (Kwon *et al.*, 1992; Mallee *et al.*, 1997; Porcellati *et al.*, 1998).

There are extensive regions of homology (Kwon *et al.*, 1992) between the SMIT protein and the SGLTs and thus antibodies developed to SGLTs would be expected to show crossreaction with SMIT. This possibly explains why Wiley *et al.* (1991) found that an antibody to a renal SGLT bound to an antigen on mouse preimplantation embryos, despite the fact that reports on glucose uptake by preimplantation mouse embryos, with one exception (Leppens-Luisier *et al.*, 2001), show no evidence for sodium-dependent uptake (Aghayan *et al.*, 1992; Chi *et al.*, 1993; Gardner and Kaye, 1995; Pantaleon and Kaye, 1998). The work reported by Leppens-Luisier *et al.* (2001) provides only very limited evidence for sodium-dependent glucose transport in mouse embryos

as it is based on the observation of a phloretin- and phloridzin-sensitive uptake after 6 min of incubation that was absent after 12 min. The effect of removal of sodium from the incubation medium was not investigated. It is probable that the results of Wiley *et al.* (1991) were due to the antibody binding to the SMIT protein rather than an SGLT protein. Wiley *et al.* (1991) found no binding in two-cell embryos, minimal binding in four-cell embryos and marked binding in compacted eight-cell embryos and blastocysts. This pattern of binding of the antibody is similar to the pattern of sodium-dependent inositol transport described in the present study.

The fact that inositol uptake occurred mainly via a sodium-dependent mechanism in this study is in agreement with results for many other tissues, including embryos of other species. Inositol uptake in cleavage stages of the rabbit embryo and in both cleavage and blastocyst stages of cattle embryos is mainly sodium dependent (Kane and Conlon, 1993; Hynes *et al.*, 2000). In addition to the sodium-dependent process, there was significant apparent sodium-independent uptake (taken as the uptake in the sodium minus medium) at all developmental stages examined, even when this made a minimal contribution to inositol uptake in blastocysts relative to the sodium-dependent process. Part of this apparent sodium-independent uptake may have been due to the presence of a low amount of sodium in the sodium minus medium. However, because the amount was so low ($< 1/200$ th the concentration in the sodium plus medium), it is unlikely to have accounted for all the apparent sodium-independent uptake at any developmental stage.

It is probable that there are two sodium-independent mechanisms operating. The 'lipotropic' (Holub, 1982; Wells, 1989) mechanism allows a limited diffusion across the plasma membrane because of the very low solubility of inositol in the phospholipid layers of the membrane. This lipotropic mechanism operates in almost all cells but normally only becomes important at high supra-physiological concentrations of inositol in the incubation medium. This mechanism is probably responsible for some proportion of the inositol uptake seen in all developmental stages examined in this study. However, this mechanism cannot explain the 141-fold (DBA $\times$ C57BL/6 $\times$ DBA) and 71-fold (MF1) increases in sodium-independent inositol uptake demonstrated between the one-cell and two-cell stages. There is clearly a different mechanism in operation, possibly a channel, such as a chloride channel. There is evidence in other tissues that sodium-independent inositol transport can take place via chloride channels (Gonzalez *et al.*, 1995; Reeves and Cammarata, 1996; Novak *et al.*, 2000), for example in cleavage-stage mouse embryos (Séguin and Baltz, 1997; Kolajova and Baltz, 1999; Kolajova *et al.*, 2001). Rabbit embryo uptake of inositol, although sodium dependent in cleavage stages, becomes totally sodium independent at the blastocyst stage (Kane and

Conlon, 1993; Warner and Kane, 1995). Uptake of inositol in rabbit blastocysts from day 4 to day 6 of development is sodium independent and non-saturable and may also occur by a channel mechanism.

The linearity of inositol uptake with time was not examined in the present study. Possible reasons for non-linearity include metabolism after uptake affecting transport and also back transport of inositol. Inositol uptake is linear in mouse blastocysts from 2 to 8 h (Kane *et al.*, 1992). Because of the very low rate of uptake of inositol in cleavage-stage mouse embryos (one-cell stage, 0.3 fmole per embryo per h; two-cell stage, 4.5 fmole per embryo per h) examination of linearity in the time period 0–10 min would require large numbers of embryos and mice. The linearity of uptake of inositol in cleavage-stage rabbit embryos and blastocysts in which uptake due mainly to embryo size is much greater than in mouse embryos, has been examined: uptake by cleavage-stage rabbit embryos in which uptake is mainly sodium dependent is linear from 5 min to 4 h and uptake by blastocysts in which uptake is mainly sodium independent is linear from 5 min to 2 h (S. M. Warner, F. V. Conlon and M. T. Kane, unpublished). It is unlikely that metabolism after uptake affects inositol transport in oocytes, one-cell embryos and blastocysts, otherwise treatment with phloridzin and sodium removal would not have markedly reduced inositol uptake. It is also highly unlikely that inositol back transport is significant for these stages because of the dependence of sodium-dependent transport on the direction of the sodium gradient. In addition, leakage of inositol from cleavage-stage rabbit embryos is negligible (S. M. Warner, F. V. Conlon and M. T. Kane, unpublished). However, in the two-cell mouse embryo, where uptake may have taken place by a channel mechanism, it is possible that the results of the present study underestimate the true rate of transport due to back diffusion through channels.

The marked change in the mechanism of inositol uptake between the one-cell and the two-cell stage of crossbred DBA $\times$ C57BL/6 $\times$ DBA embryos (and to a lesser extent between the same two stages in purebred MF1 embryos) can perhaps be explained by the process of zygotic gene activation occurring at about the two-cell stage. Zygotic gene activation involves inactivation of maternal or oogenetic gene transcription and activation of zygotic gene transcription (for reviews, see Nothias *et al.*, 1995; Schultz *et al.*, 1995; Latham and Schultz, 2001). The oogenetic sodium-dependent inositol transporter gene may be inactivated between the one-cell and two-cell stages while a zygotic sodium-independent transport gene(s) of some kind, possibly a channel gene, is activated. The fact that the increase in sodium-independent transport and the abolition of sodium-dependent transport does not take place in two-cell parthenogenetic embryos is consistent with the hypothesis that these changes are related to zygotic

gene activation. However, it is less easy to explain why, by the blastocyst stage, the gene(s) responsible for sodium-independent transport is inactivated in turn to be replaced by expression of a zygotic sodium-dependent transporter gene.

Because the trophectoderm constitutes the entire outer layer of the blastocyst and thus completely encloses the inner cell mass, blastocyst uptake must reflect trophectoderm transport and therefore transport of inositol by the trophectoderm must also be sodium dependent. However, mouse embryonic stem cells, which can be regarded as a model system for the study of inner cell mass cells, also transport inositol by a sodium-dependent system (B. D. Higgins and M. T. Kane, unpublished). The large increases in inositol uptake during preimplantation development in the mouse and the changes in transport mechanisms to allow this increased uptake to take place are surprising in view of the fact that the presence of inositol in the culture medium is not essential for development of one-cell or two-cell embryos to blastocysts. The fact that preimplantation mouse embryos can develop *in vitro* without exogenous inositol, despite the fact that the PtdIns system appears to be essential for blastocyst formation (Stachecki and Armant, 1996a,b), may reflect the well-known ability of early embryos to adapt to their environment, for example, in this case by recycling endogenous inositol or by converting glucose to inositol. However, it is also possible that, *in vivo*, inositol uptake at the preimplantation stage promotes improved embryonic viability after implantation. There is clear evidence (Cockcroft, 1991; Cockcroft *et al.*, 1992) that exogenous inositol is required for postimplantation development in mice, as a dietary deficiency of inositol causes serious problems of organogenesis, particularly neural tube defects. Increasing uptake during the preimplantation and very early postimplantation stages may be providing a safety factor for later development.

The switching on of a sodium-independent mechanism of inositol transport at the two-cell stage and its switching off before the blastocyst stage is still surprising. It is possible that this process is not specific to the inositol transport system but merely an artifact of another system, for instance transport through an ion channel. It may reflect general changes to ion and metabolite transport taking place at about the two-cell stage about the time of zygotic gene activation, with some of these changes being reversed as the embryonic genome becomes fully activated.

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