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Developmentally regulated cell cycle dependence of swelling-activated anion channel activity in the mouse embryo

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SUMMARY

Anion channels activated by increased cell volume are a nearly ubiquitous mechanism of cell volume regulation, including in early preimplantation mouse embryos. Here, we show that the swelling-activated anion current ($I_{Cl,swell}$) in early mouse embryos is cell-cycle dependent, and also that this dependence is developmentally regulated. $I_{Cl,swell}$ is present both in first meiotic prophase (germinal vesicle stage) mouse oocytes and in unfertilized mature oocytes in second meiotic metaphase, and it persists after fertilization though the 1-cell and 2-cell stages. $I_{Cl,swell}$ was found to remain unchanged during metaphase at the end of the 1-cell stage. However, $I_{Cl,swell}$ decreased during prophase and became nearly undetectable upon entry into metaphase at the end of the 2-cell stage. Entry into prophase/metaphase was required for the decrease in $I_{Cl,swell}$ at the end of the 2-cell stage, since it persisted indefinitely in 2-cell embryos arrested in late G_2. There is considerable evidence that the channel underlying $I_{Cl,swell}$ is not only permeable to inorganic anions, but to organic osmolytes as well. We found a similar pattern of cell cycle and developmental dependence in the 1-cell and 2-cell stages for the swelling-induced increase in permeability to the organic osmolyte glycine. Thus, entry into metaphase deactivates $I_{Cl,swell}$ in embryos, but only after developmental progression through the 2-cell stage.

Key words: Preimplantation embryo, Volume regulation, Cell cycle, Metaphase, Ion channel, Mouse

INTRODUCTION

Mammalian oocytes are naturally arrested in prophase of first meiosis in the ovary (germinal vesicle or GV stage oocytes) and then again in metaphase of second meiosis when ovulated. After activation by sperm, the fertilized oocyte proceeds through a series of synchronized cell cycles, each ending in a reductive cleavage, until the first cells differentiate at the blastocyst stage. Several cell cycle-dependent ion channels have been reported to be present in oocytes and early embryos of various species. They include a K^+ channel, Ca^{2+} channels and an inwardly rectifying Cl^- channel, with activities that differ between M phase and interphase (Block and Moody, 1990; Day et al., 1993; Day et al., 1998a; Rutledge et al., 2001; Yazaki et al., 1995). Considering that only a very few cell cycle-dependent channels have been found in any cell type, oocytes and embryos may be particularly rich in ion channels regulated by the cell cycle. Alternatively, cell cycle dependence of channel activity may be more easily identified in oocytes and early embryos, owing to the well-characterized and synchronous cell cycles.

One ion channel present in early mouse embryos is an anion channel activated by cell swelling. Cells almost universally regulate their volumes against perturbations (Hallows and Knauf, 1994; Lang et al., 1998), and increased cell volume is corrected by a regulated release of intracellular osmolytes via this swelling-activated anion channel. The ubiquitous swelling-activated anion current carried by this channel is termed $I_{Cl,swell}$. The channel is permeable to inorganic anions such as Cl^- as well as to at least some organic osmolytes, mediating osmolyte release to correct increases in cell volume (Okada, 1997; Strange et al., 1996). The swelling-activated anion channel has been the subject of intense study in somatic cells, and many of its functional characteristics and aspects of regulation by cell volume have been elucidated in detail (Okada, 1997; Strange et al., 1996). It is characteristically outwardly rectifying, more permeable to I^- than Cl^-, permeated by organic compounds such as taurine, aspartate and glycine, and has a characteristic pharmacological profile which includes being blocked not only by conventional Cl^- channel blockers but also by millimolar levels of external ATP (Okada, 1997; Strange et al., 1996). Thus far, the molecular identity of the swelling-activated anion channel underlying $I_{Cl,swell}$ remains elusive and controversial (Strange, 1998), although ClC-3, a member of the ClC Cl^- channel family, and pICln, a ubiquitous protein, have each been reported to elicit currents similar to endogenous $I_{Cl,swell}$ when exogenously expressed (Duan et al., 1997; Paulmichl et al., 1992).

The presence of $I_{Cl,swell}$ with properties indistinguishable from those in somatic cells, including blocking by Cl^- channel

blockers such as DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid), blocking by external ATP, greater permeability to I^- than Cl^- and permeability to organic compounds such as taurine and aspartate, has been directly demonstrated by patch clamp recordings in mouse 1-cell embryos (Kolajova and Baltz, 1999). Mouse embryos also exhibit increased permeability to organic osmolytes such as glycine and taurine induced by hypotonic swelling (Dawson et al., 1998; Van Winkle et al., 1994). The mouse 1-cell embryo regulates its volume against swelling, and exhibits a classic "regulatory volume decrease" which is inhibited by the same anion channel blockers as $I_{Cl,swell}$, as well as showing the same characteristic block by external ATP as $I_{Cl,swell}$, indicating that the channel functions in volume regulation in 1-cell embryos (Séguin and Baltz, 1997).

Cells exhibit regulated changes in size and shape over the course of the cell cycle, which would imply coordination between the cell cycle and cell volume-regulatory mechanisms. Thus, $I_{Cl,swell}$ is a possible target for regulation by the cell cycle. Yet nothing is known about the effect of progression through the cell cycle on the activity of $I_{Cl,swell}$, except for one recent report that quiescent cervical cancer cells have lower activity than proliferating cells (Shen et al., 2000). In addition, the unique reductive cleavages of early embryogenesis may impose particular constraints on cell volume-regulatory mechanisms not required in somatic cells, which regain their initial volumes before cytokinesis. Thus, we have examined whether $I_{Cl,swell}$ is regulated by the cell cycle in mouse oocytes and preimplantation embryos, and whether it is under developmental regulation during early embryogenesis. We have found that $I_{Cl,swell}$ is regulated by the nuclear cell cycle in mouse embryos, and that the cell cycle dependence of channel activity is itself developmentally regulated.

MATERIALS AND METHODS

Oocytes and embryos

Oocytes and embryos were obtained from female CF1 strain mice (Charles River, Canada) after superovulation (5 IU equine chorionic gonadotropin, eCG, followed by human chorionic gonadotropin, hCG, 47.5 hours later, except where specified), as previously described (Dawson et al., 1998; Kolajova and Baltz, 1999). First meiotic prophase-arrested germinal vesicle (GV) stage oocytes were obtained from minced ovaries 44-46 hours after stimulation by eCG alone. Ovulated unfertilized oocytes arrested in second meiotic metaphase were obtained from excised oviducts approximately 17 hours after hCG. Fertilized eggs and embryos were obtained from female mice mated with BDF strain males; fertilization was assumed to occur in vivo approximately 12 hours post-hCG. Embryos were removed from the oviducts or uterine horns at the appropriate time after mating. Oocytes and embryos were kept in KSOM embryo culture medium at 37°C in 5% CO_2 until use. For GV oocytes, 0.3 mM dibutyryl cAMP was present to maintain prophase arrest. Immediately before patch clamp measurements, the zona pellucida surrounding oocytes or embryos was removed with pronase, using the minimum time of pronase exposure required.

Solutions and chemicals

All media were based on KSOM (HCO_3^- buffered) or Hepes-KSOM (Hepes buffered) embryo culture media which have osmolarities of 230-250 mOsM (Lawitts and Biggers, 1993). For patch clamp measurements, NaCl was reduced from 95 to 65 mM to yield hypotonic 180 mOsM Hepes-KSOM, and mannitol (60 mM) was added to produce 240 mOSM medium of the same ionic composition. The internal microelectrode solution contained (in mM) 100 K gluconate, 10 KCl, 3.5 $MgCl_2$, 1 Na_2ATP, 1 EGTA, and 10 Hepes, pH 7.4, 235 mOsM (Kolajova and Baltz, 1999). Nominally Na^+-free Hepes-KSOM used for [^{3}H]glycine permeability measurements was produced by substituting 54 mM choline chloride for the normal 95 mM sodium chloride, choline bicarbonate for sodium bicarbonate, and using potassium lactate and pyruvate. Osmolarities of 185 and 285 mOsM were produced by adding raffinose. Osmolarities were measured using a vapor pressure osmometer (model 5520, Wescor, Logan, UT). Stock solutions of DIDS (Molecular Probes, Eugene, OR) in dimethylsulfoxide (DMSO), roscovitine (RBI, Natick, MA) in DMSO, monastrol (Tocris Cookson, Ballwin, MO) in DMSO, cycloheximide (Sigma, St. Louis) in Hepes-KSOM, and demecolcine (Sigma) in ethanol were used. None of the vehicles were found to affect $I_{Cl,swell}$ at their final concentrations.

Whole-cell current measurement

The method used for recording whole-cell currents from mouse zygotes has been previously described in detail (Kolajova and Baltz, 1999). Currents were measured with patch clamp microelectrodes in voltage clamp mode, using an Axopatch 200B amplifier, Digidata 1200B digitizer, and pCLAMP 6 software (Axon Instruments, Foster City, CA). Seal resistances exceeded 5 GΩ. In most experiments, whole-cell configuration was employed, but in several experiments perforated-patch recordings were made instead, using patch electrodes containing nystatin (225 μg/ml; Sigma) (Kolajova and Baltz, 1999). A 3 M KCl agar bridge reference electrode was used. Voltages were corrected for a measured liquid junction potential (LJP) of 10 mV (Kolajova and Baltz, 1999). The command voltage protocol consisted of maintaining a holding potential of –20 mV and imposing a voltage ramp (3 seconds duration) from –60 to +60 or +80 mV every 100 seconds with a sampling rate of 1 Hz during holding and 50 Hz during ramps (Kolajova and Baltz, 1999). Clampfit 6 software (Axon Instruments) was used for analysis.

The experimental protocol was as follows. Currents were continuously recorded using the protocol described above, starting with oocytes/embryos in 240 mOSM medium for 15 minutes. The medium was then changed to 180 mOsM while the total diameter of the patched oocyte/embryo was measured periodically. Since the rate of swelling varied between experiments, 180 mOsM medium was superfused until the total diameter of the oocyte/embryo had increased by about 15%. After several additional minutes, the medium was switched to 180 mOsM medium + 100 μM DIDS, and recordings continued until the current stabilized. For quantitative comparisons, current values from three representative ramps are reported for each oocyte/embryo. The first is in 240 mOsM medium immediately before switching to hypotonic medium, the second is after maximal swelling was achieved, and the third is after DIDS was introduced and current had stabilized. The currents reported are those recorded at +50 mV during a ramp (+60 mV command voltage with 10 mV LJP subtracted).

Measurement of glycine permeability

[2-^{3}H]glycine (Amersham, Arlington Heights, IL; 10-30 Ci/mmol) was added directly to the medium to a final concentration of 4 μM. Permeability of embryos to glycine was measured by determining the total [^{3}H]glycine accumulated by embryos in 15 minutes. Na^+-depleted KSOM was used during glycine incubation to eliminate transport by the robust Na^+-coupled glycine transporter in embryos (Van Winkle et al., 1994). After 15 minutes, the [^{3}H]glycine content of individual groups of 5-10 embryos was determined by scintillation counting as previously described (Dawson et al., 1998). Changes in glycine permeability upon hypotonic swelling were determined by comparing [^{3}H]glycine accumulation in 285 mOsM vs. 185 mOsM. Data were normalized to the mean amount of glycine accumulated at 285 mOsM for each experimental condition.

Data analysis

Data were expressed as mean±s.e.m., and differences between means tested for significance using *t*-tests or ANOVA with Tukey-Kramer multiple comparisons test (Instat, GraphPad Software, San Diego, CA). When comparing measures made serially on the same oocyte or embryo (i.e., patch clamp data), repeated measures *t*-test or ANOVA was used.

RESULTS

Whole-cell currents were measured in eggs and embryos using the whole-cell patch clamp technique. One-cell embryos with clear pronuclei exhibited a large increase in outwardly rectified current upon swelling, from a mean of 2.0 nA to 5.4 nA (at +50 mV) when osmolarity was changed from 240 to 180 mOsM. Swelling-activated current was inhibited with the Cl^- channel blocker, DIDS (100 μM), decreasing to a mean of 1.5 nA when DIDS was introduced in 180 mOsM medium (Fig. 1). This component of whole-cell current in mouse 1-cell embryos has previously been established to be identical to the widespread $I_{Cl,swell}$ exhibited by other cell types (Kolajova and Baltz, 1999), and thus $I_{Cl,swell}$ here is defined as a swelling-induced, outwardly rectified component of current, which is inhibitable by DIDS (Fig. 1B).

Ovulated oocytes, in second meiotic metaphase, also exhibited $I_{Cl,swell}$, showing an increase in outwardly rectified current upon cell swelling, which was DIDS-inhibitable (Fig. 1). $I_{Cl,swell}$ in oocytes was approximately half the magnitude of the swelling-activated current in 1-cell embryos for a similar extent of swelling (mean increases in diameter of 15% and 14%, respectively). GV stage, first meiotic prophase oocytes also exhibited $I_{Cl,swell}$ (Fig. 1). In the ovary, GV oocytes are connected via gap junctions to cumulus cells, which were mechanically removed. Cumulus cells patch clamped in intact cumulus-oocyte complexes did not exhibit a significant current increase upon swelling (data not shown). Thus, possible remnants of cumulus cells are unlikely to have contributed to $I_{Cl,swell}$ measured in GV oocytes, indicating that the $I_{Cl,swell}$ current arose from GV oocytes.

We also examined whether $I_{Cl,swell}$ changed over the course of preimplantation embryo development subsequent to the 1-cell stage. In 2-cell embryos, hypotonic swelling induced a large increase in current that was outwardly rectified and completely inhibited by DIDS (Fig. 1), indicating the persistence of $I_{Cl,swell}$ from the 1-cell into the mid-2-cell stage. However, when we assessed the ability of hypotonic swelling to increase current in subsequent embryonic stages, we found a sharp decrease in the amount of current that could be elicited by swelling in 4-cell, 8-cell, or compacted morula stage embryos. In each case, no significant increase in current was seen in 180 mOsM medium, nor was DIDS-inhibitable current significant, even though the extent of swelling was at least as great as in 1-cell and 2-cell embryos (Fig. 1). In some cells from the 4-cell stage onward, small swelling-activated currents were observed which were poorly DIDS-inhibitable (e.g., the 8-cell embryo example in Fig. 1B). Only in blastocyst stage embryos was swelling-activated current observed in each embryo, when the differentiated outer epithelial (trophectoderm) cells of intact blastocysts were patched (Fig. 1). We did not test for statistical significance due to the small sample, and additional recordings were not obtained owing to the substantial difficulty of recording from intact blastocysts. Thus, $I_{Cl,swell}$ in mouse embryos was found to be greatly decreased between the 2-cell and 4-cell stages, and may first reappear at the blastocyst stage.

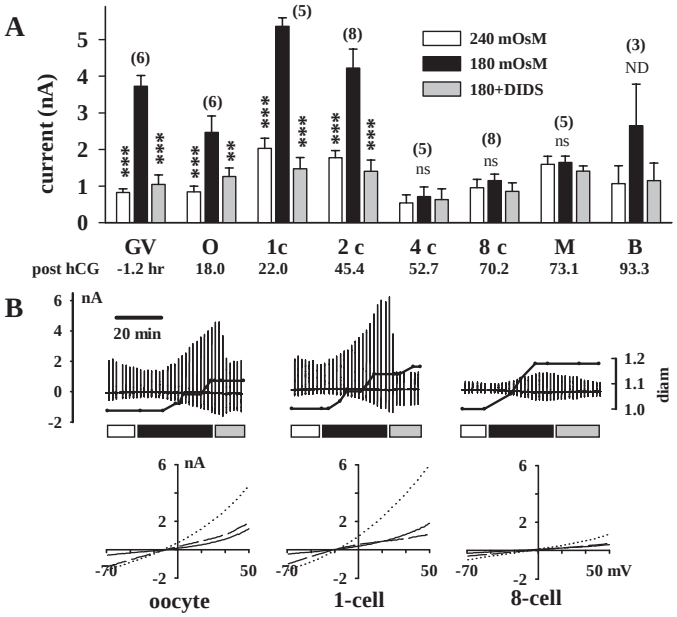

Fig. 1. $I_{Cl,swell}$ activity in mouse oocytes and embryos. (A) Magnitudes (mean ± s.e.m.) of whole-cell currents measured at +50 mV at various developmental stages of oocytes and embryos first in isotonic (240 mOsM; white bars, see key), then in hypotonic (180 mOsM; black bars), and then in hypotonic solution after the introduction of DIDS (180+DIDS; gray bars). Data are shown for oocytes in prophase of first meiosis (GV) and metaphase of second meiosis (O), as well as for 1-cell (1c), 2-cell (2c), 4-cell (4c), and 8-cell (8c) cleavage-stage embryos, morulae (M) and blastocysts (B). The time since ovulation was induced is indicated below as hours post-hCG (GV oocytes were obtained before hCG would have been administered; for embryos, fertilization occurred at about 12 hours post-hCG). Mean increase in total oocyte/embryo diameter in 180 mOsM medium at the time of the reported measurements (black bars) was 17-20%, except for oocytes and 1-cell embryos where it was 13-15%. Number of replicates (number of oocytes or embryos) is indicated above bars. Significant difference in current before swelling or after DIDS introduction relative to current at maximal swelling is indicated by asterisks (***P<0.001; **P<0.01; ns, P>0.05; ND, not determined). (B) Examples of currents elicited by swelling in ovulated (second meiotic metaphase) oocyte, 1-cell embryo, and 8-cell embryo (as indicated at bottom). At the top are traces of current over time, with each spike representing currents elicited by a voltage ramp (see text). The superimposed solid line shows total oocyte or embryo diameter relative to initial diameter before swelling (axis at right). The variable lag time between introduction of hypotonic solution and the initiation of cell swelling evident here was routinely observed. This probably arises from the time needed for the hypotonic solution to wash out the more dense and viscous 240 mOsM solution. External solutions are indicated by bars with same shadings as in the key in A. The 8-cell embryo shown here was one of the few to exhibit any significant $I_{Cl,swell}$. At the bottom are current versus voltage plots for the same oocyte/embryos (solid line, before swelling; dotted line, after swelling; dashed line, after DIDS). Voltage has been corrected for 10 mV liquid junction potential (LJP).

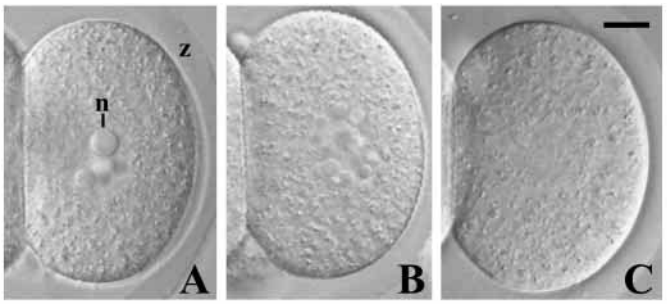

Fig. 2. Nuclear morphology of 2-cell embryos at the G_2/M border. (A) One cell of a 2-cell embryo in interphase, showing prominent nucleoli (n). Part of the zona pellucida (z) surrounding the embryo is visible on the right. The position of the nuclear membrane is marked by the transition between the clear area surrounding the nucleoli and the more granular cytoplasm. (B) Cell just before nuclear envelope breakdown, exhibiting small, more dispersed nucleoli indicative of prophase. (C) Cell in metaphase, showing absence of nucleus or nucleoli, and an even distribution of granular cytoplasm throughout. Bar, 5 μm.

In order to more precisely time the sharp decrease in $I_{Cl,swell}$ which occurred somewhere between the mid 2-cell stage and 4-cell stage (Fig. 1), we examined embryos nearer the 2-cell to 4-cell transition. Embryos in prophase at the end of the 2-cell stage could be distinguished non-invasively by their nuclear morphology, since there is a transition from the few large nucleoli characteristic of interphase (Fig. 2A) to markedly smaller, often more dispersed nucleoli (Fig. 2B) typical of prophase (Goessens, 1984; Schwarzacher and Wachtler, 1993; Van Blerkom, 1989). Entry into metaphase was then marked by complete nuclear envelope breakdown (NEBD; Fig. 2C). Timed observations indicated that cytokinesis followed approximately 1-2 hours after NEBD in a given blastomere, consistent with previous reports for the length of metaphase at the end of the 2-cell stage (Ciemerych et al., 1999). We attempted to obtain patch clamp measurements of 2-cell embryos that still possessed interphase nuclei (Fig. 2A) as close to the onset of prophase as possible, by choosing such embryos when many in the same cohort had entered prophase or metaphase; in some cases, the sister blastomere already had a prophase morphology. We found that $I_{Cl,swell}$ was present and as large in these late G_2 embryos as in earlier 2-cell embryos (Fig. 3). However, when a prophase nuclear morphology was apparent in the blastomere being patched (Fig. 2B), $I_{Cl,swell}$ was markedly decreased and sometimes absent (Fig. 3). After NEBD was complete and the blastomere had entered metaphase (Fig. 2C), $I_{Cl,swell}$ became nearly undetectable (Fig. 3). In addition, $I_{Cl,swell}$ remained small in 4-cell embryos assessed either within 1 hour after cytokinesis or 5-6 hours after cytokinesis (Fig. 3).

To test whether entry into metaphase per se caused a decrease in $I_{Cl,swell}$ in embryos, we examined whether 1-cell embryos exhibited swelling-activated current after entry into metaphase. Zygotes were thus assessed after both pronuclear membranes had disappeared. We found, however, that 1-cell embryos exhibited $I_{Cl,swell}$ after NEBD (Fig. 3) which was virtually identical to that of interphase (pronuclear) 1-cell embryos (Fig. 1), indicating that $I_{Cl,swell}$ was not affected by entry into metaphase at the end of the 1-cell stage.

Because the cytoplasm is dialyzed in the whole-cell configuration, it was possible that the sharp decrease in $I_{Cl,swell}$ in 2-cell embryos after NEBD could, for example, reflect increased sensitivity to the loss of a diffusible intracellular factor ("rundown") or similar effect. We therefore used nystatin-permeablized patch recordings, which permit retention of cytoplasmic components larger than inorganic ions and provide a more physiologically normal intracellular milieu (Walz, 1995). Two-cell embryos in late G_2 (as above) assessed using permeablized patch recordings again exhibited large swelling-activated currents, while the swelling-activated current in metaphase 2-cell embryos after NEBD was approximately 10-fold smaller (Fig. 4). Four-cell embryos within 1 hour of cytokinesis and at 5-6 hours after cytokinesis again exhibited very small swelling-activated currents (Fig. 4).

To determine if entry into metaphase at the end of the 2-cell stage is required for the decrease in $I_{Cl,swell}$ at that time, we

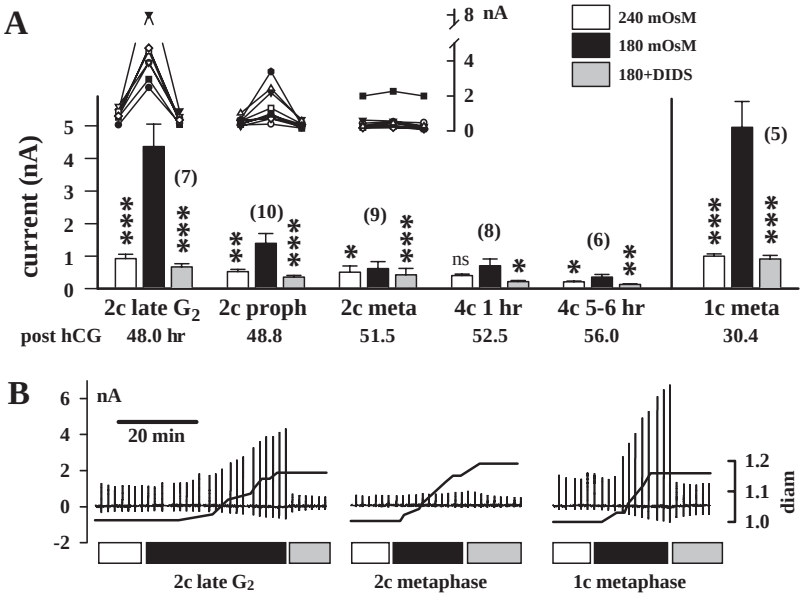

Fig. 3. Changes in $I_{Cl,swell}$ at the G2/M border. (A) Mean (±s.e.m.) currents in 2-cell stage embryos in late G_2 (2c late G_2), in prophase (2c proph), and in metaphase (2c meta), as determined by nuclear morphology (Fig. 2), and in 4-cell embryos 1 hour and 5-6 hours after cytokinesis, as indicated. Data for 1-cell embryos in metaphase (1c meta) are shown at right. Annotations are as in Fig. 1 (in addition, $*P<0.05$). The mean increase in diameter upon swelling was 16, 17 and 19% for late G_2, prophase, and metaphase 2-cell embryos respectively, 20 and 17% for 1 hour and 5-6 hours 4-cell embryos respectively, and 14% for 1-cell metaphase embryos. The inset shows currents for individual 2-cell embryos during late G_2, prophase, or metaphase, shown above the corresponding bars, with data obtained from the same embryo before swelling, after swelling, and then after introduction of DIDS connected. (B) Examples of currents in individual embryos in late G_2 of the 2-cell stage, and metaphase of the 1-cell and 2-cell stages (see Fig. 1 legend for description).

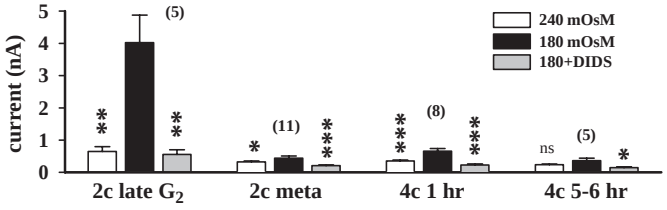

Fig. 4. $I_{Cl,swell}$ at the G_2/M border measured by nystatin-permeablized patch recordings. See Figs 1 and 3 legends for descriptions.

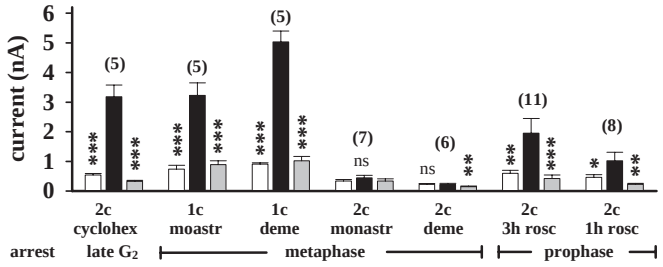

Fig. 5. Currents in embryos arrested at different points in the cell cycle. 2-cell embryos were arrested in late G_2 using cycloheximide (cyclohex); 1-cell and 2-cell embryos were arrested in metaphase using monastrol (monastr) or demecolcine (deme); 2-cell embryos were arrested in prophase using roscovitine (rosc) introduced either about 3 hours or within 1 hour before expected entry into metaphase, as indicated. Annotations are as in the legends to Figs 1 and 3.

arrested 2-cell embryos in late G_2 using cycloheximide (50 μg/ml, starting at about 4 hours before NEBD would begin) which blocks protein synthesis, including cyclin synthesis required for cell cycle progression. G_2-arrested embryos had the same appearance as normal interphase 2-cell embryos, with an intact nucleus (as in Fig. 2A). These G_2-arrested 2-cell embryos still exhibited $I_{Cl,swell}$ when assessed 20 hours after entry into metaphase would normally have occurred (Fig. 5), indicating that a sharp decrease in $I_{Cl,swell}$ did not occur without protein synthesis and progression into prophase and metaphase.

We also arrested embryos in metaphase, using the mitotic kinesin inhibitor, monastrol (75 μM; Mayer et al., 1999) added immediately after NEBD, and using demecolcine (1 μg/ml), which disrupts the spindle by depolymerizing microtubules. One-cell embryos arrested in metaphase with either monastrol or demecolcine exhibited a large $I_{Cl,swell}$ (Fig. 5), even after extended arrest in metaphase (>12 hours). In contrast, 2-cell embryos arrested in metaphase by monastrol or demecolcine did not exhibit $I_{Cl,swell}$ (Fig. 5).

The finding that $I_{Cl,swell}$ virtually disappears from 2-cell embryos during prophase/metaphase raised the possibility that activation of cyclin-dependent kinase (cdk) was required. In the presence of roscovitine (50 μM), a specific cdk inhibitor (Meijer et al., 1997), 2-cell embryos appeared to arrest in prophase indefinitely, developing characteristic small, dispersed nucleoli (Fig. 2B). This is consistent with the reported effect of roscovitine in invertebrate oocytes and embryos (Meijer et al., 1997). When assessed about 20 hours after prophase arrest was initiated, 2-cell embryos in the presence of roscovitine still exhibited some swelling-activated current which was inhibited by DIDS (Fig. 5). The currents detected were similar to those observed in 2-cell embryos normally progressing through prophase (Fig. 3). There was some difference in the observed currents depending on the timing of roscovitine introduction, although the physical appearance of the nuclei was similar. If added about 3 hours before NEBD, larger swelling-activated currents were retained than if it was added just before (within 1 hour) NEBD was expected to begin (Fig. 5). In the latter case, there were more embryos exhibiting little or no $I_{Cl,swell}$. Roscovitine had no effect on swelling-activated current in interphase 2-cell embryos with nuclei (data not shown).

Another property of the channel underlying $I_{Cl,swell}$ is its permeability to organic compounds which serve as intracellular osmolytes. Both 1-cell and 2-cell mouse embryos exhibit swelling-induced increases in permeability to glycine and taurine (Fig. 6; Dawson et al., 1998; Van Winkle et al., 1994), which are the major organic osmolytes of early mouse embryos. Increased permeability to glycine was blocked by DIDS (Fig. 6; Van Winkle et al., 1994; our data, not shown), as expected for the channel underlying $I_{Cl,swell}$. We tested whether, like $I_{Cl,swell}$, the hypotonic swelling-induced increase in permeability to glycine was altered as 2-cell embryos entered metaphase. Embryos in late G_2 exhibited the same swelling-induced increase in permeability to glycine as earlier interphase embryos, and a swelling-induced increase in glycine permeability was still evident after extended arrest (>12 hours) of 2-cell embryos in late G_2 using cycloheximide (Fig. 6). One-cell embryos arrested in metaphase with demecolcine also showed an increased permeability to glycine in hypotonic medium comparable to interphase 1-cell embryos (Fig. 6). In contrast, however, metaphase-arrested 2-cell embryos exhibited no increase in glycine permeability in hypotonic medium (Fig. 6). Because metaphase is brief, it was difficult to measure glycine permeability in metaphase 2-cell embryos without arresting them, although we were able to obtain a few such measurements. Again, no swelling-induced increase in glycine permeability was observed (Fig. 6). These measurements also revealed an unexpected effect, as permeability to glycine actually decreased upon swelling in 2-cell embryos passing through normal metaphase, unlike 2-cell embryos arrested in metaphase with demecolcine (Fig. 6). This was due to increased glycine transport at 285 mOsM via an unknown mechanism not evident in arrested embryos, while transport at 185 mOsM was essentially identical in magnitude to that in arrested embryos.

The behaviour of the swelling-induced increase in glycine permeability is similar to that of $I_{Cl,swell}$, persisting through metaphase at the end of the 1-cell stage and into late G_2 of the 2-cell stage, but then disappearing in metaphase. At the 4-cell stage, however, the results for glycine permeability diverged from the observed behaviour of $I_{Cl,swell}$. Here, a marked swelling-induced increase in glycine permeability was observed (Fig. 6), in contrast to the observed lack of $I_{Cl,swell}$, and DIDS was found to significantly inhibit this increase (data not shown).

DISCUSSION

We have found that $I_{Cl,swell}$ in mouse embryos is inactivated in metaphase at the end of the second mitotic cell cycle. The

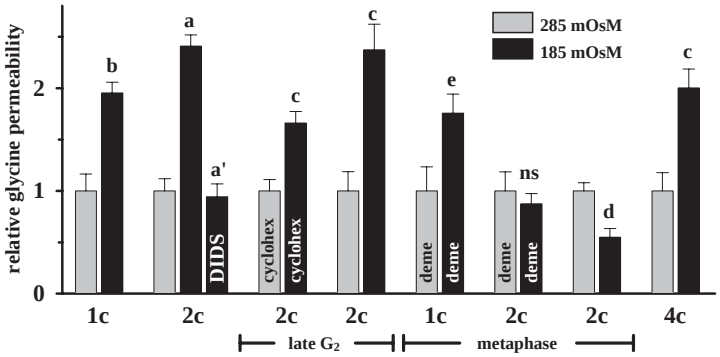

Fig. 6. Permeability to glycine induced by hypotonic swelling in embryos. Glycine permeability was determined by exposing 1-cell (1c), 2-cell (2c), or 4-cell (4c) embryos to [^{3}H]glycine for 15 minutes, and then measuring total accumulated glycine (see text). Tonicity of the solution is specified in the key. Data are normalized to the mean glycine accumulation at 285 mOsM under each condition, arbitrarily set to one. DIDS completely blocked the swelling-induced component of glycine permeability in interphase 2-cell embryos as shown by the labeled bar. Two cell embryos in late G_2 were either arrested by cycloheximide (cyclohex) or measured in embryos obtained as close to entry into prophase as possible. 1-cell and 2-cell embryos in metaphase were either arrested with demecolcine (deme) or measurements made during metaphase. Significant differences in glycine permeability between hypotonically swollen embryos versus embryos in 285 mOsM medium are indicated by letters (a, a′: $P<0.0001$; b: $P<0.0005$; c: $P<0.001$; d: $P<0.005$; e: $P=0.02$; NS: $P=0.56$; a′ indicates significant difference in the presence of DIDS versus without DIDS). The number of replicates (each replicate a group of 5-10 embryos) was 7-10 for each treatment, except only 5 replicates of 2-cell embryos in metaphase were obtained. Interphase 2-cell embryos were included in several separate experiments (total of 21 replicates at 285 mOsM and 29 at 185 mOsM); the data obtained in separate experiments were not significantly different and thus are pooled for presentation. However, statistical significance as shown was determined for differences between groups within the same experiment (e.g., for 185 mOsM versus 185+DIDS).

decrease in $I_{Cl,swell}$ appears to occur during prophase, since 2-cell embryos in late G_2 always exhibited robust $I_{Cl,swell}$, while it was markedly decreased but still present in embryos with a prophase nuclear morphology. Two-cell embryos that were blocked from initiating prophase and arrested in late G_2, using cycloheximide, retained maximal current indefinitely, indicating that entry into prophase was required for a decrease in $I_{Cl,swell}$. However, since cycloheximide inhibits all protein synthesis, it is possible that synthesis of a protein(s) other than that required for entry into metaphase could be involved in inactivation of $I_{Cl,swell}$. In the presence of roscovitine, which arrests oocytes and embryos in prophase (Meijer et al., 1997) by inhibiting the activated cyclin B/cdk complex, embryos also exhibited decreased $I_{Cl,swell}$. On average, less swelling-activated current was retained if roscovitine was introduced a short while before entry into prophase than if it were introduced several hours earlier, implying a progressive loss of $I_{Cl,swell}$ during prophase. Loss of $I_{Cl,swell}$ was apparently complete by the beginning of metaphase, as we never observed appreciable $I_{Cl,swell}$ in a metaphase 2-cell embryo. Thus, the inactivation of $I_{Cl,swell}$ would seem to be tightly coupled to the nuclear cell cycle at the end of the 2-cell stage.

The cell cycle-dependence of $I_{Cl,swell}$ was found to be developmentally regulated. While entry into metaphase at the end of the 2-cell stage led to the nearly complete loss of $I_{Cl,swell}$, there was no corresponding decrease during metaphase at the end of the 1-cell stage. One-cell embryos arrested in metaphase, with monastrol or demecolcine, retained $I_{Cl,swell}$ activity indefinitely, while 2-cell embryos arrested in metaphase had no activity. Similarly, very large currents were present in first meiotic prophase-arrested GV stage oocytes. $I_{Cl,swell}$ was also present, albeit somewhat decreased, in ovulated oocytes naturally arrested in second meiotic metaphase. Thus, $I_{Cl,swell}$ is not inactivated in prophase or metaphase during meiosis or the first mitosis, but only in the second mitotic metaphase.

The swelling-induced increase in permeability to glycine, proposed to be mediated by the same channel as $I_{Cl,swell}$, exhibited similar behavior during the 1-cell and 2-cell stages. Both 1-cell and 2-cell embryos showed similar levels of increased glycine permeability upon swelling, and swelling-induced glycine permeability was retained by 1-cell embryos arrested in metaphase and 2-cell embryos arrested in late G_2, but not by 2-cell embryos arrested in metaphase. An interesting difference was observed in 2-cell embryos that had just entered metaphase, where there appeared to be a swelling-deactivated component of glycine transport, which did not persist in 2-cell embryos arrested in metaphase.

Progression into metaphase would therefore seem to be necessary but not sufficient to inactivate $I_{Cl,swell}$, and we propose that a second signal must be present beginning in the late 2-cell stage which acts synergistically with cell cycle regulators to repress $I_{Cl,swell}$. The identity of such a second putative regulatory signal is unknown, but it may be a product of the global switch from the maternal to the embryonic genome, which occurs during the 2-cell stage in the mouse.

Following cytokinesis, substantial $I_{Cl,swell}$ did not reappear in 4-cell stage blastomeres either immediately or at the mid-point of the cell cycle (6 hours). Daughter cells in cleavage-stage embryos maintain precisely one-half the volume of the original cell and do not increase in volume prior to the next cytokinesis (Lehtonen, 1980). The expected surface area of each daughter cell is thus 63% of the original, confirmed by morphometric and electrical capacitance measurements (Block and Moody, 1987; Lehtonen, 1980). Thus, less than a 40% reduction in current would be expected at each cleavage attributable to the reduced surface area of a blastomere and any significant $I_{Cl,swell}$ would easily have been detected at the 4-cell stage. $I_{Cl,swell}$ was also undetectable in 8-cell embryos before or after compaction, and first reappeared in the differentiated outer epithelial cells of the blastocyst. Thus, the absence of $I_{Cl,swell}$ beginning at M phase of the second cell cycle appears to persist when the cells exit from metaphase and to continue through the next two to three cell cycles. It is tempting to speculate about the functional significance of the deactivation of $I_{Cl,swell}$. One possibility is that the loss of this volume-regulatory mechanism reflects the reductive cleavages unique to embryogenesis, in which there is no growth phase in the cell cycle.

In contrast, we found that the swelling-induced increase in

glycine permeability did reappear after cytokinesis at the end of the 2-cell stage, and was evident in 4-cell embryos. It is disputed whether $I_{Cl,swell}$ and the swelling-induced permeability to organic osmolytes are in fact mediated by the same underlying channel or by separate pathways with similar properties (Junankar and Kirk, 2000). Clearly, in embryos, the two phenomena are regulated very similarly through metaphase at the end of the 2-cell stage, consistent with their being mediated by the same underlying molecular entity. Conversely, the disparity at the 4-cell stage would argue the opposite. If there are two separate transport pathways, both must be regulated by the same developmentally controlled cell cycle clock through metaphase at the end of the 2-cell stage, but then diverge in their regulation. Alternatively, there might be a single mechanism that changes from being permeable to both anions and zwitterionic osmolytes to being permeable to the latter only at the beginning of the 4-cell stage. This question could potentially be resolved by molecular identification of the $I_{Cl,swell}$ channel.

At least two candidates for the molecular identity of $I_{Cl,swell}$ – ClC-3 and pICln – have been proposed, although definitive evidence is lacking (Strange et al., 1996). We have used reverse transcription-polymerase chain reaction (RTR-PCR) with primers specific for ClC-3 and pICln to assess whether mRNA for either is present in mouse embryos, and have found that both are expressed in oocytes and embryos from the 1-cell to the blastocyst stage (data not shown). Their expression in oocytes and early embryos is also supported by reports of expressed sequence tags (in the dbEST database, accessed through LocusLink, National Library of Medicine, USA, Web Site) corresponding to ClC-3 in oocytes and 2-cell embryos, and to pICln in oocytes, 2-cell embryos and blastocysts. However, molecular details of $I_{Cl,swell}$ expression in oocytes and embryos awaits definitive determination of the molecular identity of $I_{Cl,swell}$.

Other cell cycle-dependent channels have been reported in early embryos. A 240 pS K^+ channel in mouse embryos becomes active during each metaphase from second meiosis through at least the 8-cell stage, and is inactive during each interphase (Day et al., 1993; Day et al., 1998b). Upregulation of this K^+ channel is governed by a novel cdk/cyclin B-independent cytoplasmic cell cycle, since it was activated at the normal time for the onset of metaphase even in embryos arrested in G_2 (Day et al., 1998b). In contrast, downregulation of this K^+ channel after metaphase depends on the nuclear cell cycle, as arrest in metaphase prevents loss of channel activity (Day et al., 1998b). The behavior of $I_{Cl,swell}$ in embryos thus differs in three ways from that of the K^+ channel, since $I_{Cl,swell}$ is regulated by a nuclear rather than cytoplasmic clock at the G_2/M border, it is deactivated rather than activated upon entry into metaphase, and the K^+ channel shows the same activation at M phase in every stage of oocyte and embryo examined, in contrast to the developmental regulation of $I_{Cl,swell}$. A similar cytoplasmic cell cycle also regulates Ca^{2+} currents in both mouse and sea urchin embryos (Day et al., 1998a; Yazaki et al., 1995).

The rat eag K^+ channel, heterologously expressed in *Xenopus* oocytes, provides an example of a channel whose activity is modulated by cyclin B/cdk activity and is thus tightly regulated by the nuclear cell cycle. Its conductance properties were modulated upon progression from prophase into metaphase, where outward rectification was lost via a mechanism that required cyclin B/cdk activity (Bruggemann et al., 1997).

Several anion (Cl^-) channels also have been reported to vary with the cell cycle, including examples in lymphocytes (Bubien et al., 1990) and glioma cells (Ullrich and Sontheimer, 1997), both of which have lower activity in S phase. Best-studied is a voltage-dependent, inwardly rectifying Cl^- channel in ascidian embryos that is abruptly activated at exit from metaphase of each cell cycle (Block and Moody, 1990). Entry into metaphase is required for downregulation (Coombs et al., 1992), similar to $I_{Cl,swell}$ in mouse embryos. Interestingly, this same channel is also activated by hypotonic cell swelling, but only the constitutive current, but not the swelling-activated current, varies with the cell cycle (Villaz et al., 1995). A channel with very similar properties – CLH-3, an ortholog of the mammalian ClC-2 – exists in the nematode oocyte, and is activated during meiotic maturation (Rutledge et al., 2001). Very recently, it was also reported that $I_{Cl,swell}$ itself has lower activity in cervical cancer cells arrested in G_0 by serum starvation and inhibition of DNA synthesis, and that its activity increases severalfold upon re-entry into the cell cycle (Shen et al., 2000).

Thus, we have found that $I_{Cl,swell}$ is subject to cell cycle regulation like several other channels, including several in embryos. Unlike other channels reported in mammalian embryos, however, regulation of $I_{Cl,swell}$ at the G_2/M border is coupled to the nuclear cell cycle, rather than to a putative nuclear cycle-independent cytoplasmic clock (Day et al., 1998b) proposed to govern activity of several channels in embryos. Most strikingly, regulation of $I_{Cl,swell}$ by the cell cycle is in turn regulated by development, since it is cell cycle-independent during the first mitotic cell cycle but cell cycle-dependent during the second. Such developmental regulation of cell cycle dependence has not been previously reported. Thus, there appears to be a novel developmental clock in early mouse embryos which interacts with the cell cycle and which regulates cell functions such as ion channel activity.

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