

A cytoplasmic cell cycle controls the activity of a K⁺ channel in pre-implantation mouse embryos

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We previously have reported that the activity of a 240 pS K⁺ channel varies during the cell cycle in pre-implantation mouse embryos. In the present study, we show that: (i) the cycling of channel activity is not prevented by inhibiting protein synthesis and hence does not involve cyclin-dependent kinase 1 (cdk1)–cyclin B; and (ii) the cycling of channel activity continues in anucleate zygote fragments with a time course similar to that observed in nucleate fragments. We further demonstrate that: (i) persistent activation of the K⁺ channel in one-cell embryos arrested in metaphase requires the maintenance of an active cdk1–cyclin B complex; and (ii) both DNA synthesis inhibition with aphidicolin and DNA damage produced by mitomycin C prevent the down-regulation of the channel at the start of S phase by a mechanism that requires tyrosine kinase activation. Thus, the 240 pS K⁺ channel in these cells is controlled by a previously unsuspected cytoplasmic clock that functions independently of the well-known clock controlling the chromosomal cell cycle, but can interact with it.

Keywords: anucleate/cell cycle/DNA synthesis/potassium channel/tyrosine kinase

Introduction

There is an increasing amount of evidence that ion channels play important roles in cell cycle progression. Numerous studies have demonstrated the dependence of mitogenesis on the activation of ion currents (reviewed in Dubois and Rouzaine-Dubois, 1993; Strobl *et al.*, 1995; Wonderlin and Strobl, 1996). In lymphocytes, for example, a cystic fibrosis transmembrane conductance regulator (CFTR)-associated Cl⁻ conductance is up-regulated during G₁ phase (Krauss *et al.*, 1992), and cell cycle progression from the resting phase, G₀, to G₁ phase has been shown to be accompanied by K⁺ channel activation (Chandy *et al.*, 1984; Brent *et al.*, 1990). This K⁺ channel activation is believed to cause membrane hyperpolarization and subsequent opening of voltage-sensitive Ca²⁺ channels, leading to the rise in intracellular Ca²⁺ that is required for induction of cell cycle progression (Nilius and Wohlrab, 1992). The importance of channel activation during mitogenesis in several cell types has been demonstrated further by showing that application of K⁺ channel blockers

prevents cell proliferation (Leonard *et al.*, 1992; Woodfork *et al.*, 1995; Lepple-Wienhues *et al.*, 1996), and in some cell types this has been shown to be due to cell cycle arrest in early G₁ (Amigorena *et al.*, 1990; Xu *et al.*, 1996).

More recently, direct links between channel activity and particular stages of the cell cycle have been reported. For example, in HeLa cells, the size of a K⁺ current increases during M and G₁ phases (Takahashi *et al.*, 1993), and in embryos of loach (Bregestovski *et al.*, 1992), sea urchin (Yazaki *et al.*, 1995) and ascidian (Block and Moody, 1990; Villaz *et al.*, 1995), the activities of K⁺, Ca²⁺ and Cl⁻ channels, respectively, are increased during M phase. The mechanisms linking the activity of each of these channels to the cell cycle appear to be different and include regulation by cytoskeletal elements (Medina and Bregestovski, 1988; Yazaki *et al.*, 1995), changes in phosphorylation state (Medina and Bregestovski, 1991; Villaz *et al.*, 1995) and cell cycle-dependent assembly of channel proteins in the plasma membrane (Takahashi *et al.*, 1993). Furthermore, the activity of the rat *ether-a-go-go* (*R-eag*) K⁺ channel, when expressed in *Xenopus* oocytes, is down-regulated at the G₂–M transition due to the activation of cyclin-dependent kinase 1 (cdk1)–cyclin B at this time (Bruggemann *et al.*, 1997).

In a previous study, we have demonstrated in early mouse embryos that the activity of a large-conductance K⁺ channel is regulated in a cell cycle-dependent way (Day *et al.*, 1993). This channel is active in the metaphase II-arrested oocyte, remains active after fertilization during G₁ phase, becomes inactive as the cell cycle progresses through S and G₂ phases and then reactivates during mitosis. In that study, we showed that the decrease in channel activity at the end of G₁ and the increase in channel activity at the commencement of M phase of the first cell cycle do not depend on protein synthesis, suggesting that they are not dependent on the activity of cdk1–cyclin B and do not depend on the nuclear cell cycle.

In the present study, we have investigated further the relationship between the activity of the large-conductance K⁺ channel in early mouse embryos and the nuclear cell cycle. We demonstrate that the large-conductance K⁺ channel is controlled by a cytoplasmic cell cycle that can run independently of the nuclear cell cycle. We also examined the interaction between this cytoplasmic cycle and the nuclear cycle.

Results

Channel activity varies in a cell cycle-dependent manner in the absence of protein synthesis

Treatment of one-cell embryos with puromycin from G₁ phase of the first cell cycle does not prevent DNA synthesis (Table I) but blocks pronuclear membrane breakdown (Howlett, 1986; Levy *et al.*, 1986) and arrests the chromo-

Table I. Influence of drugs on the DNA content of one- and two-cell embryos

Condition		DNA content [arbitrary units \pm SD (<i>n</i>)]	Presumed C value	Stage of cell cycle arrest
Untreated				
G ₁ , one-cell		0.54 \pm 0.15 (9)	1C	
G ₂ , one-cell		0.97 \pm 0.16 (22)	2C	
G ₁ , two-cell		0.97 \pm 0.20 (36)	2C	
G ₂ , two-cell		2.05 \pm 0.28 (42)	4C	
Treated from G ₁ through to G ₂				
Puromycin (10 μ M)	one-cell	0.96 \pm 0.12 (20)	2C	G ₂
	two-cell	1.83 \pm 0.20 (60)	4C	
Aphidicolin (2 μ g/ml)	one-cell	0.52 \pm 0.19 (22)	1C	G ₁ /S
	two-cell	1.19 \pm 0.14 (10)	2C	
Mitomycin C (2 μ g/ml)	one-cell	0.89 \pm 0.11 (17)	2C	G ₂
	two-cell	2.20 \pm 0.20 (16)	4C	
Genistein (70 μ M)	one-cell	0.46 \pm 0.18 (21)	1C	G ₁ /S
Genistein (70 μ M) + aphidicolin (2 μ g/ml)	one-cell	0.42 \pm 0.16 (14)	1C	G ₁ /S

somes in a decondensed state in G₂. We have reported previously that inhibition of protein synthesis, by treatment with puromycin, during the first cell cycle of early mouse embryonic development fails to prevent the decrease in activity of the large-conductance K⁺ channel at the G₁-S transition or the rise in channel activity at the time the chromosomal cell cycle would have been expected to enter M phase (Day *et al.*, 1993). In the present study, we examined the effect of treatment of embryos with puromycin from late S/G₂ of the first cell cycle. Under these conditions, the nuclear cell cycle was arrested with intact pronuclei and the increase in K⁺ channel activity at the time control embryos entered M phase was not prevented (Day *et al.*, 1993). Furthermore, puromycin did not inhibit the decrease in channel activity that normally occurs at the G₁-S transition of the second cell cycle, with channel incidence falling to 0% (*n* = 7). Thus, the changes in channel activity during the first and early second cell cycles do not depend on protein synthesis, an active cdk1-cyclin B complex or on the chromosomal cell cycle.

Channel activity varies in a cell cycle-dependent manner in the absence of the nuclear cycle

The results with puromycin indicated that the activity of the 240 pS K⁺ channel varies in response to a cell cycle clock that is not tightly coupled to the chromosomal cell cycle. We thus investigated whether the changes in K⁺ channel activity that occur during the cell cycle require the presence of the nucleus. We did this by bisecting embryos within 2 h of pronuclei formation and examining K⁺ channel activity in the resulting nucleate and anucleate fragments during the first and second cell cycles (Figure 1). In nucleate fragments, the variations in the channel incidence during the first and second cell cycles resembled those observed in intact one- and two-cell embryos (Figure 1A), i.e. the channel incidence was high during G₁ and low during S and G₂ phases. Following G₂ phase, the channel incidence again rose as the cell cycle of these nucleate fragments entered M phase. It is noteworthy that the variations in channel activity in the nucleate fragments are less marked than those observed in intact embryos. This is probably due to the greater variability in the timing

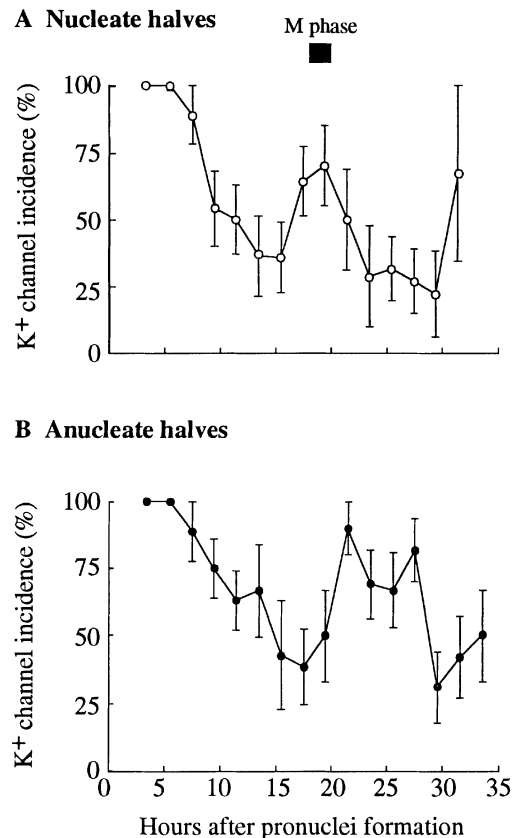


Fig. 1. Changes in channel activity in bisected embryos during the first and second cell cycle. Zygotes were bisected 0–2 h after pronuclear membrane formation. (A) Channel incidence in nucleate fragments, *n* = 3–16 cell-attached patches. The bar at the top of the panel indicates the time at which the cell cycle of nucleate fragments was in M phase, as evidenced by the disappearance of the pronuclear membranes. (B) Channel incidence in anucleate fragments, *n* = 7–19 cell-attached patches. Each point represents the mean \pm SEM.

of cell cycle progression and to the increased duration (~2–3 h longer) of the cell cycle in nucleate fragments compared with intact embryos. Consistent with this explanation was the observation that in nucleate fragments known to be in M phase, as evidenced by the disappearance of the pronuclear membranes, the channel incidence (92%,

$n = 13$) was similar to that observed in intact embryos in M phase (79%, $n = 14$). Furthermore, within 1–2 h after exit from mitosis and reformation of the nuclear membranes, the channel incidence in nucleate fragments was 26% ($n = 19$), which was not different from the incidence in intact embryos (3%, $n = 33$).

The cell cycle-dependent changes in channel incidence in anucleate fragments were very similar to those in nucleate fragments and intact embryos (Figure 1B). Progression of the cytoplasmic cell cycle was, however, even slower than in the nucleate fragments (also observed by Ciemerych, 1995), with the decline in channel incidence being completed by 17.5 h after pronuclei formation, compared with 15.5 h in nucleate fragments and 11 h in intact one-cell embryos. This slowing of the cell cycle in bisected embryos may be due to the effect of cytochalasin D (CCD) on cell cycle progression, as described below. The minimum incidence attained was, however, similar to that reached in S phase nucleate fragments. After reaching a minimum, the channel incidence in anucleate fragments rose to a level similar to that observed at the beginning of the cell cycle and then decreased as the cytoplasmic cell cycle exited an apparent M phase. Thus, changes in channel incidence occur in the anucleate fragments and are similar to those observed in nucleate fragments. These results demonstrate that K^+ channel activity is controlled by a cortical cell cycle that is not dependent on the presence of the nucleus.

Arresting the nuclear cycle can affect channel activity

Previously we have shown that arresting the cell cycle in M phase with nocodazole, which disrupts the mitotic spindle, maintains a high incidence of the 240 pS K^+ channel (Day *et al.*, 1993). In the present study, we now show that following prolonged treatment with nocodazole the high channel incidence persists for at least 15 h after the entry into metaphase (Figure 2A). This suggests that although the cortical cell cycle can function independently of the nucleus, the nucleus does interact with the cortical cell cycle. As the arrest produced by nocodazole is due to the requirement for an intact spindle for the destruction of cyclin B (Kubiak *et al.*, 1993; Winston *et al.*, 1995), we thus examined whether the effects of nocodazole in maintaining a high channel incidence could be overcome by inhibiting synthesis of cyclin B with puromycin. We found that the addition of puromycin to one-cell embryos arrested in M phase with nocodazole reactivated the nuclear cell cycle and caused the incidence of the 240 pS K^+ channel to decline (Figure 2C). Similarly, treatment of oocytes arrested in metaphase II of meiosis with puromycin resulted in a significant decrease in channel incidence (Figure 2D). Thus, inactivation of cdk1–cyclin B is involved in the decline in channel incidence that normally occurs on exit from mitosis.

Channel activity is not dependent on cytokinesis

Since nocodazole arrests the chromosomes in metaphase it also prevents cleavage. In loach embryos, the cycling of a stretch-activated K^+ current with the cell cycle is thought to be dependent on cleavage (Medina and Bregestovsky, 1988). Thus, in order to determine whether the prolonged maintenance of channel activity by nocoda-

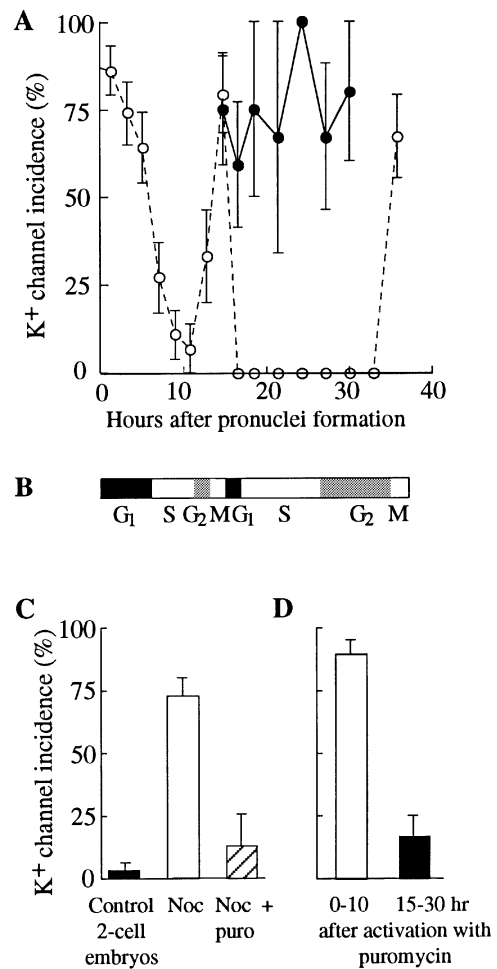


Fig. 2. Arresting the first cell cycle in metaphase maintains the activity of the K^+ channel. (A) One-cell embryos were treated with 10 μ M nocodazole from ~12 h after pronuclei formation. Channel incidence was then examined following their arrest in metaphase as evidenced by the disappearance of the pronuclear membranes. Filled circles are the incidences of the K^+ channel in embryos treated with nocodazole, and open circles are the incidences in control, untreated embryos. Each point represents the mean \pm SEM of 3–13 separate embryos. (B) Approximately corresponding stages of the first and second cell cycle. (C) Channel incidences in control two-cell embryos (black bar, $n = 29$), zygotes arrested in metaphase by treatment with 10 μ M nocodazole (Noc; open bar, $n = 41$) and zygotes arrested in metaphase with 10 μ M nocodazole and then reactivated by treatment with 10 μ M puromycin (Noc + puro; striped bar, $n = 8$). Each point represents the mean \pm SEM. (D) Channel incidence in parthenotes activated by treatment with 20 μ M puromycin.

zole described above was due to arrest of the chromosomal cycle or to inhibition of processes occurring during cleavage, G₁ phase zygotes were treated with the anti-microfilament agent CCD, to inhibit formation of the cleavage furrow and subsequent cytokinesis. Progression of the chromosomes through mitosis persisted under these conditions, resulting in the formation of a binucleate single cell. CCD did not inhibit the decrease in channel activity that occurs during S phase (Figure 3), although it did seem to delay this fall by ~2–3 h. Furthermore, CCD did not prevent the rise in channel activity during M phase of the first cell cycle and, following chromosomal separation and the formation of a binucleate cell, the channel incidence fell to a level not significantly different from that in untreated two-cell embryos (Figure 3). Thus, the cyclic

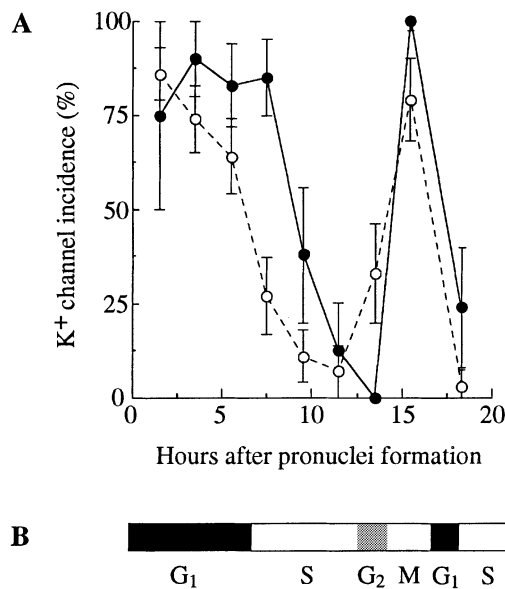


Fig. 3. The incidence of the K⁺ channel in embryos treated with cytochalasin D (CCD) during the first cell cycle and the beginning of the second. (A) Zygotes were cultured in medium containing 1 µg/ml CCD from 0.5 h after pronuclei formation and patch-clamped at known times after formation of the pronuclei (●; $n = 4-13$ patches per bin); ○, control channel incidences ($n = 7-13$). Each point represents the mean \pm SEM. (B) Approximately corresponding stages of the first and early second cell cycles.

fluctuations in channel activity do not depend on cyto-kinetic events or on changes in the cytoskeleton. This observation differs from the finding in loach (Bregestovsky *et al.*, 1992) and sea urchin embryos (Yazaki *et al.*, 1995).

Channel activity is affected by agents that damage DNA or inhibit DNA synthesis

Since channel activity decreases at the G₁-S transition, we suspected that the cortical cycle may be influenced by the onset of DNA synthesis. We investigated this possibility by examining the channel incidence in zygotes treated with drugs that have been shown to affect DNA synthesis and/or DNA integrity and to affect passage through the G₁-S transition in other cell types. We used aphidicolin (2 µg/ml), which inhibits DNA synthesis (Table I) by inhibiting DNA polymerase α , and mitomycin C (MMC; 2 µg/ml), a DNA intercalator that causes strand breaks in the DNA, leading to arrest of one-cell embryos in G₂ prior to pronuclear membrane breakdown without inhibiting DNA synthesis (Table I). Despite their different mechanisms of action, both drugs resulted in the maintenance of high channel incidence during S phase when added 0.5 h after pronuclear formation (Figure 4). Thus, from these studies, K⁺ channel activity seems to be influenced by both inhibition of DNA synthesis and damage to DNA.

Channel incidence in the presence of a tyrosine kinase inhibitor

Tyrosine kinases play critical roles in many cellular processes including cell cycle regulation (Pendergast, 1996). Recent findings suggest that tyrosine kinases are involved in the signalling pathways between the nucleus and the cytoplasm, and have been hypothesized to be

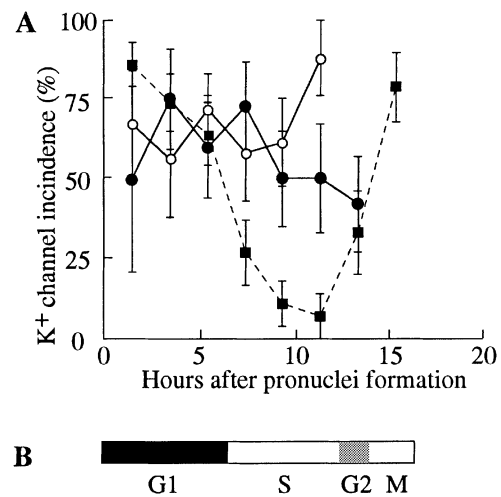


Fig. 4. Dependence of channel activity on DNA synthesis during the first cell cycle. (A) Zygotes were cultured in medium containing either 2 µg/ml mitomycin C (○, $n = 8-18$ patches per bin) or 2 µg/ml aphidicolin (●, $n = 4-12$ patches per bin); ■, control channel incidences ($n = 4-12$ patches per bin). (B) Approximately corresponding stages of the first and second cell cycle. Each point represents the mean \pm SEM.

involved in the cortical cell cycle in sea urchin oocytes (Edgecombe *et al.*, 1991). It was possible, therefore, that a tyrosine kinase was involved in driving the cortical cell cycle in mouse embryos or in controlling changes in ion channel activity in response to the cytoplasmic clock. We investigated this possibility by treating zygotes with the tyrosine kinase inhibitor genistein. When zygotes were treated with genistein (70 µM) from 0.5 h after pronuclei formation, the channel activity was high during G₁, decreased at the G₁-S transition and was low during S phase, as it is in untreated zygotes (Figure 5A). The incidence of the channel, however, did not rise at the expected time following S phase. When zygotes were treated with genistein (70 µM) after the completion of S phase, exit of the chromosomal cycle from G₂ into M phase occurred and the channel activity rose as expected (Figure 5C). These results show that the inhibition of channel activation when genistein was added from G₁ phase cannot be due to inhibition of a messenger system present at the G₂-M transition. The inhibitory effect of genistein is thus due to its presence in S phase.

Genistein prevents DNA synthesis (Table I) and has also been reported to inhibit at least some of the intracellular signalling systems by which DNA damage exerts its effects (Kharbanda *et al.*, 1994; Wei *et al.*, 1996). Thus we considered the possibility that genistein added during G₁ prevented re-activation of the channel by inhibiting DNA synthesis and that the failure of channel inactivation on entry to S phase that normally accompanies DNA synthesis inhibition (see above) is not seen with genistein because it inhibits a tyrosine kinase-based pathway that links DNA damage to inhibition of channel switch-off. We tested this theory by examining channel incidence following combined treatment with genistein and aphidicolin from G₁ of the first cell cycle. Under these conditions, DNA synthesis was arrested as expected (Table I), however, channel activity fell (Figure 5D). Thus, the mechanism linking DNA damage to sustained K⁺ channel activity

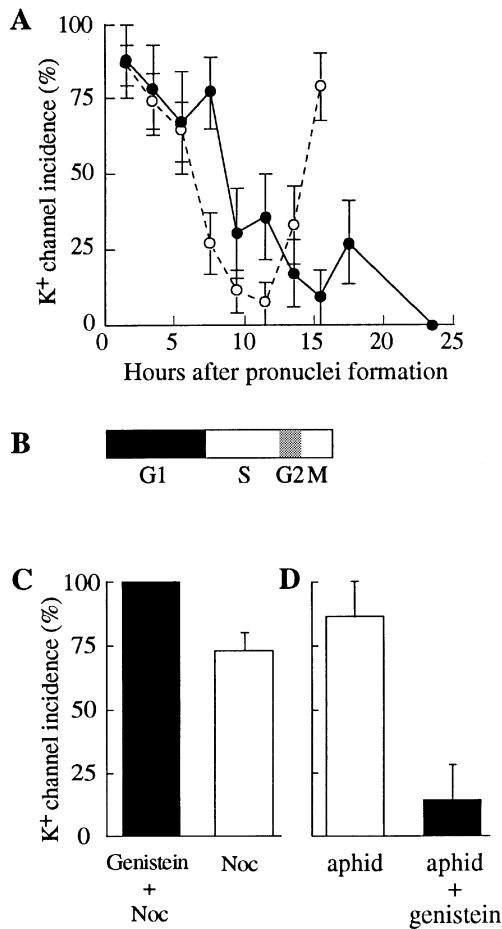


Fig. 5. Effect of genistein on channel incidence during the first cell cycle. **(A)** Incidence of the K⁺ channel in embryos cultured in 70 μM genistein from 0.5 h after pronuclei formation (●, *n* = 8–14 patches per bin); ○, control channel incidences (*n* = 7–13). **(B)** Approximately corresponding stages of the first and second cell cycle. **(C)** The channel incidence in zygotes treated with 70 μM genistein in the presence of 10 μM nocodazole (Noc) from 12 h after pronuclei formation is shown by the filled bar (*n* = 6) and that in zygotes treated with 10 μM nocodazole alone by the unfilled bar (*n* = 41). **(D)** Dependence of channel activity on DNA synthesis and tyrosine kinase activity. The channel incidence in zygotes treated with 2 μg/ml aphidicolin (aphid; open bar, *n* = 10) and 2 μg/ml aphidicolin plus 70 μM genistein (filled bar, *n* = 10). Each point represents the mean ± SEM.

is absent or overridden when protein tyrosine kinase activity is inhibited.

Correlation between K⁺ channel activity and intracellular [Ca²⁺]

It has been proposed that the mechanism by which K⁺ channels influence cell cycle progression is by changing the intracellular Ca²⁺ concentration ([Ca²⁺]) (Nilius *et al.*, 1993; Lepple-Wienhues *et al.*, 1996; Verheugen and Vijverberg, 1996), i.e. an increase in K⁺ channel activity results in hyperpolarization of the membrane potential, increased Ca²⁺ influx and, hence, increased intracellular free Ca²⁺. We thus investigated whether the activity of the large-conductance K⁺ channel is involved in regulation of intracellular Ca²⁺ in the early mouse embryo. Since we have not yet found a specific blocker for the large-conductance K⁺ channel in mouse embryos, it was not possible to use pharmacological agents to do this. Instead,

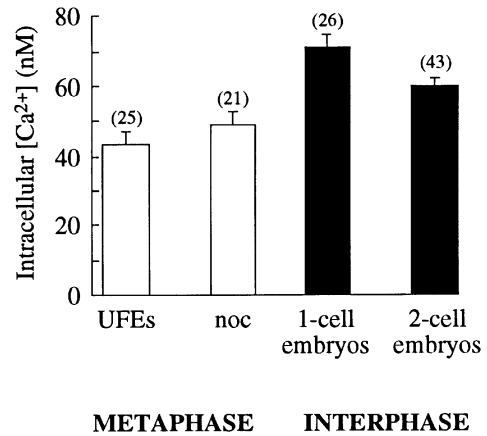


Fig. 6. Concentration of intracellular free Ca²⁺ in early mouse embryos. Open bars represent [Ca²⁺] in metaphase-arrested unfertilized eggs (UFEs) and fertilized one-cell embryos treated with nocodazole (10 μM; noc). Filled bars represent [Ca²⁺] in interphase one- and two-cell embryos. [Ca²⁺] in both UFEs and noc groups were significantly less compared with [Ca²⁺] in interphase one-cell embryos (*P* < 0.001 for both groups) and interphase two-cell embryos (*P* = 0.008 for both groups).

we first investigated the effect of manipulating the membrane potential by changing extracellular [K⁺]. We found that increasing the extracellular [K⁺] bathing metaphase embryos from 6 to 30 mM resulted in depolarization of the membrane potential by 35 ± 5 mV (*n* = 2) and a small decrease in intracellular [Ca²⁺] of 9.97 ± 1.98 nM (*n* = 36), consistent with the possibility that an increase in K⁺ channel activity would cause an increase in intracellular [Ca²⁺]. Since we have shown previously that the changes in K⁺ channel activity during the cell cycle are paralleled by changes in membrane potential (Day *et al.*, 1993), we then investigated whether intracellular [Ca²⁺] could be correlated with K⁺ channel activity. We found, however, that intracellular [Ca²⁺] was greater in embryos in interphase, when the K⁺ channel activity was low and the membrane potential was depolarized, than in embryos that were arrested in metaphase of mitosis, in which the K⁺ channel activity was high and the membrane potential was hyperpolarized (Figure 6). This finding is not consistent with the proposal that increased activity of the large-conductance K⁺ channel during M phase of the cell cycle induces cell cycle progression by increasing intracellular free [Ca²⁺].

Discussion

Progression of the cell cycle is regulated by a series of checkpoints that involves a complex process of activation and inactivation of cell cycle regulatory enzymes called cdks (reviewed in Nigg, 1995; Fisher, 1997). Ion channel activity is known to be linked to progression of the cell cycle in a variety of cell types, including early mouse embryos, in which we have shown that the activity of a large-conductance K⁺ channel is regulated by the cell cycle (Day *et al.*, 1993). This K⁺ channel is active during M and G₁ phases and inactive during S and G₂. In the present study, we have demonstrated that these cell cycle-dependent changes in K⁺ channel activity in early mouse embryos occur independently of the well-known nuclear cell cycle, since channel activity continues to cycle in

bisected embryos in the anucleate as well as the nucleate fragments. The continuation of the cytoplasmic cell cycle in the absence of the nuclear cell cycle has been demonstrated previously by the use of enucleated cells. In eggs of *Xenopus* (Hara *et al.*, 1980; Yoneda *et al.*, 1982; Shinigawa, 1983) and mouse (Waksmundzka *et al.*, 1984; Ciemerych, 1995), surface contractions or deformations occur prior to each cleavage in nucleate fragments and at a similar time in anucleate fragments. These cortical contractions are, however, dependent on activation of cdk1 by cyclin B at the G₂-M transition since they are prevented by inhibition of protein synthesis. Similarly, circadian clock-regulated ion conductances, including K⁺ (Michel *et al.*, 1993) and non-selective cation (D'Souza and Dryer, 1996) conductances, require transcription and translation for their autonomous oscillations in activity. In contrast, the cell cycle regulation of K⁺ channel activity in mouse embryos is not inhibited by treatment with the protein synthesis inhibitor, puromycin, and so the variations in channel incidence observed in anucleate fragments cannot be due to cdk1-cyclin B activity but must be due to a cytoplasmic oscillator. A cytoplasmic clock independent of cdk1-cyclin B has been observed previously in sea urchin embryos in which the changes in intracellular [Ca²⁺] that drive the cell cycle are driven by changes in protein tyrosine phosphorylation (Edgecombe *et al.*, 1991) and in cytosolic inositol 1,4,5-trisphosphate (InsP₃) levels (Ciapa *et al.*, 1994) that are independent of cdk1-cyclin B. The endogenous mechanism controlling these cell cycle-dependent oscillations is unknown and may be similar to the mechanism controlling the cytoplasmic clock we now report in early mouse embryos.

Although the clocks controlling the chromosomal and the cytoplasmic cell cycles, represented by the changes in K⁺ channel activity in early mouse embryos, can function independently, we also show that they interact. Thus, we found that arresting the nuclear clock in metaphase of the first cell cycle, by destroying the spindle and so preventing the breakdown of cyclin B required for cdk1 inactivation (Kubiak *et al.*, 1993; Winston *et al.*, 1995), caused the K⁺ channel to remain active. It was then possible to inactivate the channel in these metaphase-arrested zygotes by inducing destruction of cyclin B by treatment with puromycin. Our results therefore show that although cycling of cdk1 activity is not required normally for the cytoplasmic clock and channel regulation during the cell cycle, preventing cdk1 inactivation does affect the cytoplasmic cycle.

Recently, the activity of a delayed-rectifying K⁺ channel, R-*eag*, has been found to decline following activation of mitosis-promoting factor (MPF; cdk1-cyclin B) when expressed in *Xenopus* oocytes (Bruggemann *et al.*, 1997). Another member of the *eag* family of K⁺ channels, H-ERG, or human *eag*-related gene, encodes a K⁺ channel that is inwardly rectifying and voltage-gated and thus, has some properties similar to the 240 pS K⁺ channel in early mouse embryos (Day *et al.*, 1993; Sanguinetti *et al.*, 1995; Trudeau *et al.*, 1995; Smith *et al.*, 1996). Furthermore, the activity of an inwardly-rectifying K⁺ channel in neuroblastoma cells with properties similar to H-ERG has been shown to be cell cycle-dependent (Arcangeli *et al.*, 1995). Although the K⁺ channel in early mouse embryos has properties similar to those of the *eag* family, its

regulation by the cell cycle differs from that of R-*eag*. The down-regulation of R-*eag* after injection of active MPF is rapid and is thought to be due to the direct phosphorylation of the channel by MPF (Bruggemann *et al.*, 1997). A direct link between the activity of the 240 pS K⁺ channel in early mouse embryos and cdk1 activity is unlikely since there is a lag of ~15 h between fertilization and the complete inactivation of the channel, whereas the activity of cdk1 has been shown to decrease within 4 h after fertilization and after the first cleavage (Moos *et al.*, 1995). Thus, it seems more likely that cdk1 affects the activity of other cell cycle regulatory mechanisms which in turn alter the activity of the K⁺ channel.

A possible intermediate between cdk1 and the K⁺ channel is mitogen-activated protein kinase (MAP kinase), a serine/threonine kinase that has been shown to be active in the metaphase II-arrested oocyte and in M phase of the first cell cycle, but is inactive during interphase (Moos *et al.*, 1995). Thus, the activity of MAP kinase parallels the changes in K⁺ channel activity during the cell cycle. Similarly to K⁺ channel inactivation, the inactivation of MAP kinase has been shown to be dependent on cyclin B destruction since arresting the cell cycle in metaphase II with nocodazole inhibits the dephosphorylation of MAP kinase that normally occurs after fertilization (Moos *et al.*, 1995). Another similarity between the regulation of K⁺ channel activity and MAP kinase is that the inactivation of MAP kinase can be separated from the inactivation of cdk1. In other words, dephosphorylation of MAP kinase can be induced in metaphase-arrested oocytes by subsequent treatment with a protein synthesis inhibitor without the destruction of cyclin B (Moos *et al.*, 1996). Furthermore, the fall in MAP kinase activity after fertilization, like the decrease in K⁺ channel activity, is significantly slower than the inactivation of cdk1. MAP kinase, however, becomes completely inactive until around the time of pronuclear membrane formation, whereas the K⁺ channel remains active for several hours after pronuclear formation. It is possible, therefore, that MAP kinase is involved in the interaction between the cytosolic and nuclear clocks.

We found that prevention of DNA synthesis in early mouse embryos by inhibiting DNA polymerase α with aphidicolin caused cell cycle arrest at the G₁-S transition as expected and also resulted in maintenance of K⁺ channel activity. Furthermore, we found that induction of DNA damage with MMC did not inhibit DNA synthesis but resulted in cell cycle arrest in G₂ phase and maintenance of high K⁺ channel activity during S phase. These results provide further evidence for the interaction between the cytoplasmic and nuclear cell cycles. These DNA damage checkpoints, at the G₁-S transition and during G₂ phase, have been shown to involve mechanisms in which the tumour suppressor gene, p53, participates and results in transcription of genes such as p21 (also referred to as *cip1* and *WAF1*), which encodes an inhibitor of cyclin-dependent protein kinases (Elledge and Harper, 1994; Hartwell and Kastan, 1994; Carr and Hoekstra, 1995; Hansen and Oren, 1997), and activation of one or more protein tyrosine kinases (Kharbanda *et al.*, 1995). We have demonstrated that the maintenance of channel activity in the absence of DNA synthesis is blocked by genistein and thus involves activation of a tyrosine kinase. However,

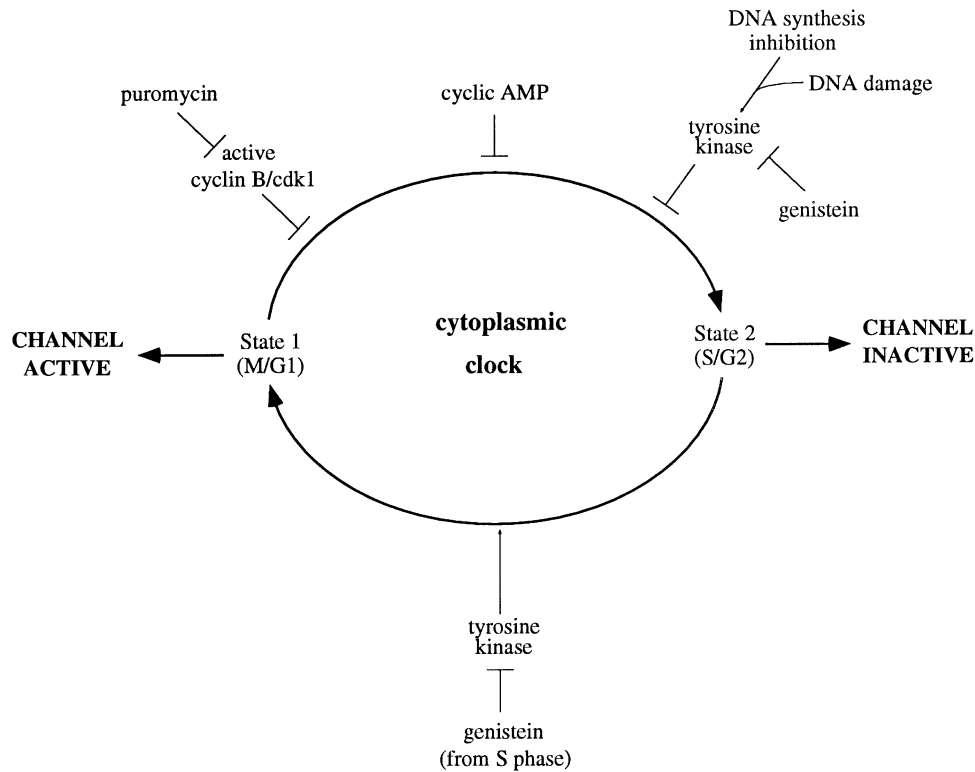


Fig. 7. Mechanisms involved in regulation of K^+ channel activity by the cytoplasmic clock in early mouse embryos.

the complete signalling pathway linking the DNA checkpoints in mouse embryos to the cytoplasmic cell cycle is not yet fully understood.

We have also demonstrated that a tyrosine kinase is involved in normal progression of the cytoplasmic clock. Transition from G_1 to S phase in early mouse embryos requires the activation of a tyrosine kinase, since we have shown that genistein inhibits the nuclear cell cycle at this time and prevents DNA synthesis. Inhibition of the nuclear cell cycle with genistein does not affect the cytoplasmic cell cycle at the G_1 -S transition since channel activity decreases at this time as it does in control embryos. The inhibition of tyrosine kinase, however, did prevent the activation of the K^+ channel at the G_2 -M transition, but only when genistein was present from G_1 phase. Endogenous cycling of phosphotyrosine levels has been observed previously in embryos of the sea urchin in the absence of the nuclear clock (Edgecombe *et al.*, 1991). The results of our present study suggest that tyrosine phosphorylation is involved in the cytoplasmic clock in early mouse embryos.

Some studies have suggested that cell cycle control of ion channels during mitosis is involved in volume regulation during cytokinesis (Yazaki *et al.*, 1995). In ascidian eggs, for example, the activity of a Cl^- channel is sensitive to changes in cell volume due to a swelling-induced dephosphorylation of the channel which causes it to be activated (Villaz *et al.*, 1995). Similarly, the changes in activity of a K^+ channel in loach embryos during cleavage is thought to be caused by cAMP-dependent phosphorylation of the channel caused by changes in the cytoskeleton (Bregestovsky *et al.*, 1992). Direct interactions between actin microfilaments and ion channels have also been demonstrated (Berdiev *et al.*, 1996; Ismailov *et al.*, 1997).

In the present study, we demonstrate that K^+ channel activity still oscillates when embryo cleavage is inhibited with CCD, suggesting that the incidence of the channel is not dependent on changes in cell volume or the cytoskeleton.

In summary, we have identified an autonomous cytoplasmic oscillator in early mouse embryos represented by cell cycle-dependent changes in the activity of a large-conductance K^+ channel (Figure 7). This cytoplasmic clock can operate independently of the nuclear cell cycle since we show that the K^+ channel activity still oscillates in the absence of the nucleus and of protein synthesis. However, we also demonstrate that the cytoplasmic and nuclear clocks interact since arresting the nuclear clock (i) with an active cyclin B-cdk1 complex, (ii) with high levels of intracellular cAMP (Day *et al.*, 1993) and (iii) by DNA synthesis inhibition or DNA damage blocks the cytoplasmic clock in the active state. Furthermore, we show that active tyrosine kinase during S phase is required for the cytoplasmic clock to go from the inactive to the active state.

Materials and methods

Preparation of embryos

Oocytes and embryos were recovered after superovulation of 3- to 4-week-old QS mice (Lab Animal Services, University of Sydney, Australia) or MF1 mice (OLAC, Bicester, UK) by intraperitoneal injections of 10 IU of pregnant mares' serum gonadotrophin followed 48 \pm 2 h later by 10 IU of human chorionic gonadotrophin (hCG; both from Intervet, Sydney, Australia). Female mice were placed with mature male QS mice immediately after hCG injection for mating.

To obtain one-cell embryos just before pronuclear formation, female mice were killed by cervical dislocation 16-18 h post-hCG injection. Zygotes were released from the oviducts into M2 medium (Fulton and Whittingham, 1978) containing 4 mg/ml bovine serum albumin (M2 +

BSA, Sigma). Cumulus cells were removed by brief treatment with hyaluronidase (0.01%). Embryos were then cultured in medium CZB (Chatot *et al.*, 1989) containing 4 mg/ml BSA under oil (BDH Chemicals, Poole, UK) in 5% CO₂ at 37°C. The zonae were removed from embryos by brief treatment with 10 mg/ml pronase (CalBiochem; Alexandria, Australia) immediately before patch-clamping.

Embryo bisection

Zygotes were bisected between 0 and 2 h after pronuclear formation. The bisection was performed in M2 + BSA at 37°C on an agar-coated Petri dish (1% agar in 0.9% NaCl) as modified from Tarkowski (1977). The cutting was accomplished by pressing a fine glass pipette down onto individual zona-free zygotes at a plane such that the two pronuclei were pushed to one side and the fragments were then separated by gentle pipetting. Thus, it was possible to produce a nucleate fragment, which contained both pronuclei, and an anucleate fragment, which contained only cytoplasm. Fragments were cultured individually in microdrops of CZB + BSA containing 1 µg/ml CCD, to prevent fragmentation (Waksmundzka *et al.*, 1984), in 5% CO₂ at 37°C until required for patch-clamping.

Electrophysiology

Zona-free oocytes and embryos were patch-clamped at known times after pronuclei formation. Membrane currents were recorded in the cell-attached patch-clamp configuration using a LIST EPC-7 amplifier (List, Darmstadt, Germany). Pipettes with a tip resistance between 2 and 8 MΩ were used. Medium M2 (without BSA) was used as the bathing solution, and a KCl-rich pipette solution was used, containing (in mM) KCl (140), HEPES (10), EGTA (0.5), glucose (10) and EDTA (0.01), pH 7.2. All data are represented as channel incidences (Day *et al.*, 1993), which is the number of cells in which the 240 pS K⁺ channel was observed as a percentage of the total number of cell-attached patches obtained. Standard errors have been calculated assuming a binomial distribution. Only seals with resistances >10 GΩ were used. Each patch was voltage-clamped at transmembrane potentials between 200 and 80 mV for ~30 s at 20 mV intervals to promote channel opening and to ensure the validity of channel incidence as a measure of channel activity.

Measurement of DNA content

Untreated embryos were analysed at the G₁ and G₂ stage of the first (i.e. at 2–3 and 10–11 h after pronuclei formation) and second cell cycle (i.e. at 0 and 11–14 h after cleavage) to provide the relative fluorescent levels for 1C, 2C and 4C levels of chromosomes. All treated embryos were exposed to drugs from G₁ phase through to G₂ phase of either the first or the second cell cycle and then analysed for their DNA content at the time equivalent to the G₂ phase in controls. Embryos were then fixed in 2% formaldehyde + 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 30 min at room temperature and then washed in PBS. The DNA was stained by incubating embryos for 15 min at room temperature in the DNA chromophore 4',6-diamidino-2-phenylindole (DAPI) at 0.05 µg/ml in H6 + BSA. Embryos were then rinsed through three drops of H6 + BSA, followed by two incubations of 5 and 10 min in H6 + BSA, before being placed in a drop of Citifluor (City University, London) on an ethanol-cleaned slide and sealed under a coverslip.

Discrete nuclei in DAPI-stained oocytes and embryos were assessed photometrically using a Leitz Ortholux II microscope previously described in detail (Winston *et al.*, 1993). The photometer was zeroed against the background of each oocyte and embryo measured. The diaphragm within the phototube was set at an aperture large enough to surround the most expanded nuclei. Conversion of each reading to an equivalent scale was enabled by the inclusion of standard controls of known DNA content, such as unfertilized oocytes (2C).

Measurement of intracellular Ca²⁺

Embryos were loaded with fura-2-AM (1 µM; Molecular Probes, OR) in M2 + BSA for 30 min and washed through two drops of M2 + BSA. The embryos were then placed on a concanavalin A- (Con A; 0.2 mg/ml in M2) coated coverslip attached to the base of a Perspex perfusion chamber which was mounted on the stage of a Nikon inverted microscope. The chamber was perfused immediately with M2 at a rate of ~1 ml/min at room temperature. Intracellular free Ca²⁺ measurements were made using a 20× fluor objective (Nikon) and an intensified CCD camera (Videoscope International, Washington, DC). The ratio of fura-2 fluorescence at 520 nm, excited by UV light alternately at 340 and 380 nm at 20 s intervals, was calculated. Excitation wavelengths were alternated using a computer-controlled filter wheel (Sutter Instruments, Novato, CA), and image acquisition was controlled by the Ionvision

package (Improvision, Coventry, UK) running on a Mac-IIci computer (Gibb *et al.*, 1997). *In vivo* calibration of fura-2 was performed using ionomycin (1 µM) to equilibrate external and internal Ca²⁺ concentrations (Dinudom *et al.*, 1993). The minimum ratio was obtained using an M2 solution containing 10 mM EGTA and 1 µM ionomycin, and the maximum ratio was obtained using M2 solution containing 20 mM Ca²⁺ and 1 µM ionomycin. Intracellular Ca²⁺ concentration was calculated using the formula of Grynkiewicz *et al.* (1985).

Chemicals

Puromycin (Sigma) was prepared as a stock solution of 10 mM in water. All other chemicals were dissolved in dimethylsulfoxide (DMSO) at the following stock concentrations: nocodazole (Sigma; 10 mM), CCD (Sigma; 1 mg/ml), aphidicolin (Sigma; 2 mg/ml), MMC (Sigma; 2 mg/ml), genistein (CalBiochem, Alexandria, Australia; 70 mM) and DAPI (Sigma; 2 mg/ml). Where DMSO was used as a solvent, control solutions contained equivalent DMSO concentrations.

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