Cytoskeletal elements link calcium channel activity and the cell cycle in early sea urchin embryos

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SUMMARY

Using the whole-cell clamp technique, we show that L-type calcium channels are activated in early sea urchin blastomeres during M-phase and subsequently inactivated in S-phase. This cyclical channel behaviour occurs in the absence of the nucleus suggesting cytoplasmic regulation independent of the centrosome cycle. Puromycin at 100-400 μM does not prevent inactivation of the current showing that this phase, at least, does not require protein synthesis. Cytochalasin B at 2 μg/ml inhibits the cyclical activity in both M and S phases, while 100 μg/ml of colchicine inactivates the L-type current in M-phase and activates a large T-type calcium current in S-phase, suggesting that channel behaviour is regulated by cytoskeletal elements. Since, fragmentation experiments show the calcium channels to be clustered in the apical membrane, and some L-type calcium channel inhibitors induced a significant delay in the cell cycle, the channel may play a role in regulating cytokinesis possibly by contributing to local intracellular calcium gradients.

Key words: L-type calcium channel, cell cycle, sea urchin, blastomeres, polarization

INTRODUCTION

In the cell cycle of early embryos, S and M phases proceed in rapid succession, there is no transcription and synthesis of new plasma membrane is minimal (Davidson et al., 1982; Dan et al., 1980). A transient increase in intracellular Ca2+ accompanies many of the mitotic events in sea urchin eggs and blastomeres (Whitaker and Patel, 1990; Poenie et al., 1985; Steinhardt and Alderton, 1988), and, recently, it has been suggested that localized Ca2+ gradients regulate cytokinesis (Speksnijder et al.1989; Snow and Nuccitelli, 1993). Localized changes in calcium may be generated by a polarized distribution of intracellular stores and second messengers, or by clusters of L-type calcium channels in the plasma membrane (Silver et al., 1990).

The surface of early sea urchin blastomeres is polarized. The membrane distal to the cleavage furrow, termed the apical membrane, differs from that proximal to the furrow, the basolateral membrane, in structure (Wolpert and Mercer, 1963; Yazaki and Uemura, 1989), reactivity to antibodies (Yazaki, 1984) and cell-adhesiveness (Kuraishi and Osanai, 1989). It is not clear whether the basal membrane originates from new membrane added at cleavage or re-organization of existing membrane (Yazaki, 1991). Apart from the works of David et al. (1988) and Chambers (see ref. 1989), there is little data on the role or distribution of calcium channels in the early embryo. The aim of the present study was to characterise calcium channels in early sea urchin embryos and, in particular, to determine whether calcium channel activity was functionally linked to the cell cycle.

MATERIALS AND METHODS

Sea urchins were collected from the Bay of Naples and gametes obtained by dissection. Eggs were washed twice in natural sea water and inseminated at 106 spermatozoa/ml. The fertilization and hyaline membranes were removed in calcium- and magnesium-free sea water. Single blastomeres in natural sea water on glass slides were whole-cell voltage clamped by standard techniques. Blastomere fragments were generated using fine glass needles. Patch pipettes of 10 Mohm resistance and 1-2 μm diameter filled with an intracellular-like solution containing 200 mM KCl, 20 mM NaCl, 250 mM sucrose, 10 mM EGTA, 10 mM Heps, pH 7.4 were used. K+ currents were reduced by the addition of 10 mM Caesium chloride to the pipette solution and leak current subtracted. Currents were recorded with List EP7 amplifiers and stored on VHS tape for subsequent analysis.

For staining with anti-tubulin, blastomeres were fixed in methanol containing 10 mM EGTA at −20˚C for 15-30 minutes. After rinsing in PBS, the blastomeres were incubated for 1 hour at room temperature with a monoclonal anti-alpha tubulin (Amersham) at a dilution of 1:400. After three washes in PBS, the cells were incubated for 1 hour at room temperature with BODIPY FL goat anti-mouse IgG (H+L) conjugate (Molecular Probes Inc.), and rewashed as above before observation.

Isolated cortices were prepared by adhering blastomeres to glass slides with poly-lysine and shearing off the cytoplasm with a jet of calcium- and magnesium-free sea water for 30 minutes at room temperature with BODIPY FL goat anti-mouse IgG (H+L) conjugate (Molecular Probes Inc.), and rewashed as above before observation.
For in vitro cell cycle studies, synchronous populations of 2-cell blastomers were divided into drug-treated and control groups and an aliquot fixed every 5 minutes in formaldehyde. At each time point, 300-500 blastomers were scored for cytokinesis. Percent delay was calculated as the difference between control and drug-treated curves with respect to the control cell cycle period.

RESULTS

The cell cycle in early blastomers of the mediterranean sea urchin *Paracentrotus lividus* is 35 minutes long at 22˚C. Interphase, or S-phase, is 17 minutes (Fig. 1A) and M-phase, scored from nuclear membrane breakdown (NBD, Fig. 1B) to cytokinesis (Fig. 1C) being about 18 minutes. Isolated sea urchin blastomers in natural sea water in the whole cell clamp configuration have a mean resting potential of $-48.8 \pm 9.0$ mV ($n=16$) in interphase and $-44.9 \pm 8.9$ mV ($n=13$) in M-phase. We found no significant difference between the resting potential of blastomers from 2-cell, 4-cell and 8-cell embryos. Clamping interphase 2-cell-stage blastomers at $-80$ mV and applying ramps of 10 mV depolarizing and hyperpolarizing steps generated a series of whole cell currents that had little time variance between $-120$ mV and $-20$ mV. Plotting the current at 400 msec against voltage gave an essentially linear relationship between $-120$ mV and $+50$ mV and a steady state conductance ranging from 20 to 40 nS ($n=12$). From a clamp potential of $-80$ mV, whole-cell currents at voltage steps of $-10$ mV to $+50$ mV had an inward component. These currents activate in 50 msec, and inactivate slowly reaching a plateau after 500 msec. Following leak subtraction, peak values at $+20$ mV ranged from 0 to 150 pA in interphase cells (Fig. 1A') and 1100 to 1300 pA in M-phase blastomers (Fig. 1B',C'). Current/voltage relationships of this peak inward component, up to the fourth cleavage division, showed cell cycle control of the channel behavior (Fig. 1D), with activation in M-phase and subsequent inactivation in S-phase.

Sea urchin blastomers in M-phase clamped at $-30$ mV also generated inward currents at step potentials of $+20$ mV although the initial sharp transient was reduced ($n=5$, Fig. 2A). Increasing external Ca²⁺ to 20 mM ($n=7$, Fig. 2B), or substituting Ca²⁺ with Ba²⁺ ($n=4$), generated larger inward currents indicating the Ca²⁺ dependence of this current. Finally, the inward component could be totally or partially inhibited by bath exposure to 5 mM MnCl₂ ($n=6$), 5 mM CoCl₂ ($n=8$), 10 μM calciseptine ($n=10$), 0.1 mM verapamil ($n=4$) and 0.1 mM nifedipine ($n=4$; Fig. 2C), suggesting the current corresponds...
Calcium channels and the cell cycle in blastomeres

1829

to the L-type calcium current found in ascidian oocytes (Dale et al., 1991).

2-cell-stage blastomeres in M-phase were enucleated with fine glass needles and subsequently whole-cell voltage clamped. Enucleation did not have significant effects on the resting potential or steady state conductance of the cell and, as shown in Fig. 3A,B (n=10), did not influence the cyclical behaviour of the calcium channel implying an endogenous cytoplasmic clock. Since cell cycle progression in sea urchin embryos may be blocked by inhibitors of protein synthesis (Whitaker and Patel, 1990), we investigated the effect of puromycin on calcium channel behaviour. Zygotes at the streak stage (35 minutes following insemination) and 2-cell-stage blastomeres immediately after cleavage (65 minutes following insemination) exposed to 100-400 μM Puromycin arrested in interphase of the subsequent cycle, i.e at the 2-cell and 4-cell stages, respectively.

Under these conditions, puromycin did not inhibit the activation or inactivation of the calcium current, although by definition we could not follow the subsequent cycles. Cycloheximide had a similar effect.

Since blastomeres undergo a cortical contraction with the same period as the cell cycle (Selman and Waddington, 1955; Hara et al., 1980), we studied the effects of cytoskeletal drugs on the membrane currents. Current intensity increased in M-phase blastomeres exposed to 2 μg/ml of Cytochalasin B (CB, n=6), which depolymerises actin and prevents cytokinesis, and remained elevated for up to 30 minutes, when control cells entered S-phase (Fig. 3C). In contrast, the current intensity did not alter when S-phase blastomeres were exposed to CB, and remained low for 30 minutes when the control cells entered M-phase (n=8). Colchicine, which depolymerizes tubulin and also inhibits cleavage, when applied at 100 μg/ml to M-phase blastomeres caused an immediate decrease in current intensity (n=10), while the same concentration applied to S-phase cells immediately induced a large inward current of up to 2 nA that peaked at around −15 mV and resembled the T-type calcium current observed in ascidian oocytes (Dale et al., 1991) (n=10, Fig. 3D). Tubulin and microfilaments in sea urchin blastomeres

Fig. 3. (A,B) I/V relationships of L-type calcium currents in 2-cell-stage sea urchin blastomeres in M-phase (2-M), and S-phase of the subsequent generation (4-S) to show the cyclical activation and inactivation of the channel does not require the presence of the nucleus. (C,D) I/V relationships to show that cytoskeletal elements regulate the cyclical behaviour of L-type calcium currents in sea urchin blastomeres.

Fig. 4. Effects of colchicine and cytochalasin B on cytoskeletal elements in 2-cell-stage sea urchin blastomeres.
Blastomeres were stained by a monoclonal anti-alpha tubulin and BODIPY FL goat anti-mouse IgG conjugate (A,B) while isolated blastomere cortices were stained by NBD-phallacidin for actin (C,D). (A) A control blastomere and (B) a blastomere exposed to 100 μg/ml of colchicine for 5 minutes showing depolymerization of tubulin. (C) An isolated control blastomere cortex and (D) the cortex of a blastomere at the same stage exposed to 2 μg/ml of cytochalasin B for 20 minutes. The bars in A and C represent 10 μm.
exposed to colchicine and CB at the above doses are depolymerized as shown by staining with fluorescently conjugated anti-alpha tubulin and NBD-phallacidin (Fig. 4).

Apical and basal fragments were obtained from 2- and 4-cell-stage blastomeres, according to previous criteria (Yazaki, 1984, 1991). In natural seawater, in the whole-cell clamp configuration, apical fragments have a mean resting potential of $-32\pm7$ mV ($n=7$), while basal fragments have a significantly lower resting potential of $-19\pm3$ mV ($n=5$). Irrespective of origin, the resting potential of the fragments did not vary with the cell cycle. The steady state conductance of apical and basal fragments was 27.5±4.8 nS ($n=6$) and 28.5±4.8 nS ($n=5$) respectively, while the specific conductance was 3.9±1.7 pS/cm$^2$ and 6.7±1.4 pS/cm$^2$, respectively (Table 1). From a holding potential of $-80$ mV, whole-fragment inward currents peaked around +20 mV, similar to intact blastomeres. In basal fragments, the currents at +20 mV ranged from 0 to 300 pA in both M and S phases, whereas the currents at +20 mV in apical fragments ranged from 0-300 pA in S-phase and increased to 700-1500 pA in M-phase (Fig. 5).

To determine whether the calcium channel contributes to the regulation of mitosis, we studied cell cycle progression in blastomeres exposed to a range of channel antagonists (Table 2). As seen, five of the antagonists, both inorganic and organic, induce a significant and reproducible delay in the cell cycle period of up to 20%, while 20 mM external Ca$^{2+}$ decreases the period by almost 20% ($n=5$). MnCl$_2$ did not induce a significant delay, notwithstanding being a potent calcium channel antagonist.

Table 1. Specific steady state conductance in fragments and whole blastomeres of the sea urchin Paracentrotus lividus

<table>
<thead>
<tr>
<th>Origin of cell</th>
<th>N. exp</th>
<th>Diameter (µm)</th>
<th>Conductance (pS/µm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-cell-stage</td>
<td>30</td>
<td>70</td>
<td>2.0±0.3</td>
</tr>
<tr>
<td>blastomere</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-cell-stage</td>
<td>3</td>
<td>55</td>
<td>3.4±0.7</td>
</tr>
<tr>
<td>blastomere</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apical fragments</td>
<td>6</td>
<td>39-65</td>
<td>3.9±1.7</td>
</tr>
<tr>
<td>Basal fragments</td>
<td>5</td>
<td>34-39</td>
<td>6.7±1.4</td>
</tr>
</tbody>
</table>

Fig. 5. L-type calcium currents in apical and basal fragments of 4-cell-stage blastomeres of the sea urchin Paracentrotus lividus. The bottom right blastomere was cut into an anucleate basal fragment (B) and a nucleate apical fragment (A). The sister blastomere is marked with a line to indicate the cutting plane that generated A and B. Fragment A and the remaining three blastomeres cleaved following recording (right frame). The fragments were clamped at a holding potential of $-80$ mV and step-depolarized for 400 msec to the voltages indicated. The I/V relationship of the currents from the apical (A) and the basal (B) fragments in S-phase (A-S), B-S) and in M-phase (A-M, B-M) are means of three separate experiments.

DISCUSSION

In somatic cells, it has been shown that clusters of L-type calcium channels in the plasma membrane may contribute to intracellular calcium gradients by generating cytosolic hotspots of calcium (Silver et al., 1990). Few reports have studied the behaviour or role of calcium channels in the early embryo (David et al., 1988; Chambers, 1989). Here we have shown for the first time, using voltage regimes and pharmacological agents, that L-type calcium channels are expressed in early sea urchin blastomeres with comparable characteristics to those found in somatic cells (Bean, 1985) and invertebrate oocytes (Dale et al., 1991). Furthermore, we show, by cutting the blastomeres into fragments, that the channels are predominantly expressed in the apical membrane, that is the surface distal to the cleavage furrow. This supports previous work showing the polarized distribution of other cellular components in early cleavage stages of the sea urchin (Wolpert and Mercer, 1963; Yazaki, 1984; Yazaki and Uemura, 1989). A polarized distribution of the Na/K pump has been reported in trophoderm cells of the mammalian blastocyst (Robinson et al., 1991), while polarized efflux of Na$^+$ and K$^+$ has been observed in the blastomeres of amphibia and mouse embryos using an extracellular vibrating probe (Kline et al., 1983; Nuccitelli and Wiley, 1985).

Although at present we have no direct evidence that polarized calcium channels in sea urchin blastomeres generate localized intracellular calcium gradients, or that such gradients regulate cytokinesis, as suggested by Speksnijder et al. (1989) and Snow and Nuccitelli (1993), the significant delay in the cell cycle period induced by some calcium channel antagonists
suggests a functional link between the cell cycle and calcium channels.

The L-type calcium channel in sea urchin blastomeres is activated in M-phase and is quiet in S-phase. To our knowledge, the only other example of cell cycle control of an ion channel in early embryos is a large conductance K+ channel expressed in the mouse embryo (Day et al., 1993). Encelulation experiments show that the cyclical behaviour of the calcium channel is not directly controlled by the nucleus or by the centrosome cycle (Maniatis and Schliwa, 1991). Cell cycle progression is regulated by cyclin-dependent kinases (Murray et al., 1989; Solomon et al., 1990) and inhibitors of protein synthesis block cyclin synthesis and prevent cell cycle progression in sea urchin embryos (Whitaker and Patel, 1990; Edgecombe et al., 1991). Although exit from mitosis is known to be independent of protein synthesis, we show here that calcium channel inactivation is also independent of protein synthesis.

Since blastomeres are known to go through a cortical contraction sequence with the same period as the cell cycle (Selman and Waddington, 1955; Hara et al., 1980), and actin filaments are localized at the apical cortex of blastomeres (Yazaki, 1991; Robinson et al., 1991), we investigated the effect of cytoskeletal drugs on channel behaviour. Cytodysasin B, which depolymerises actin, and vinblastine, inactivated the L-type current in M-phase and prevented the T-type calcium current (Dale et al., 1991) in S-phase. Since, sea urchin blastomeres divide in the absence of extracellular calcium (Chambers, 1980; Schmidt et al., 1982) the plasma membrane calcium channel is not essential for mitosis. Microinjection of Ca2+ buffers blocks the cell cycle (Whitaker and Patel, 1990; Poenie et al., 1985; Steinhardt and Alderton, 1988), however total elimination of intracellular or extracellular Ca2+ induces multiple deleterious cellular effects. In fact, deletion of any cellular component involved in the cell cycle, either by mutation, or by microinjection, will of course be deleterious, if not lethal, to the cell; however, if we consider the cell cycle to be an endogenous oscillatory process (Novak and Tyson, 1993), then protocols to alter, rather than block, progression will be more informative. Although we have not identified the molecules linking cell cycle progression and calcium channel activity, we have excluded nuclear and centrosomal participation and identified a new regulatory mechanism at the level of the cytoskeleton.

**REFERENCES**


**Table 2. Effect of Ca2+ channel antagonists on cell cycle progression in sea urchin blastomeres**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>N. exp</th>
<th>Concentration (nM)</th>
<th>% delay (X±s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnCl2</td>
<td>8</td>
<td>1-20</td>
<td>1.0±1.6</td>
</tr>
<tr>
<td>NiCl2</td>
<td>4</td>
<td>0.1-5</td>
<td>7.0±10.0</td>
</tr>
<tr>
<td>CoCl2</td>
<td>7</td>
<td>1-10</td>
<td>13.0±9.0</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>4</td>
<td>0.1</td>
<td>21.0±7.0</td>
</tr>
<tr>
<td>Verapamil</td>
<td>3</td>
<td>0.1</td>
<td>18.0±3.0</td>
</tr>
<tr>
<td>Gallamipil</td>
<td>2</td>
<td>0.1</td>
<td>10.0±5.0</td>
</tr>
</tbody>
</table>


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