Changes in actin distribution during fertilization of the mouse egg

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SUMMARY

Unfertilized mouse oocytes and eggs 1–8 h after fertilization in vitro were examined at the light microscope level for structural changes, distribution of actin (as assessed by both anti-actin antibodies and NBD-phallicidin), surface binding of concanavalin A (Con A) and chromosomal distribution and condensation. The influence of cytochalasin D on these events was also assessed. Changes in actin distribution were associated with rotation of the second anaphase spindle, formation of the second polar body, the events following incorporation of the sperm nucleus, and formation and migration of the pronuclei. Cytochalasin D prevented spindle rotation, polar body formation, pronuclear migration and the restoration of Con A binding over the site of sperm entry and the site of polar body formation, but did not affect sperm fusion and entry, the loss of Con A binding at the site of sperm entry and pronuclear formation.

INTRODUCTION

The cytoskeleton occupies a central role in processes of cellular organization and movement (for review see: Organisation of the cytoplasm, 1982). It is therefore important to describe the changes in cytoskeletal organization that occur in the egg and early embryo and to understand their significance for the establishment of euploidy, the transition from maternal to embryonic control of development and the early events of cytodifferentiation. For example, abnormalities of sperm incorporation or of extrusion of the second polar body lead to aneuploidy and thus to embryo loss or malformation (Kaufman & Sachs, 1975; Magnuson, 1983). Several agents that increase the risk of aneuploidy such as anaesthetics and alcohol are believed to exert their influence via effects on the cytoskeletal system of the cell (Kaufman, 1977, 1983). Second, activation of development at fertilization involves changes in the expression of maternal mRNA and in the posttranslational modification of proteins. It is possible that changes in the association of mRNA and/or enzyme subpopulations with the cytoskeleton might provide one mechanism for this posttranscriptional regulation (Moon et al. 1983; Jeffery & Meier, 1983; Jeffery, Tomlinson & Brodeur, 1983; Showman et al. 1982). Third, the spatial segregation of components within
the egg (or within early embryonic blastomeres) appears to be important in several types of embryo (Angerer & Angerer, 1983; Gearhart, Black, Gimlich & Scharf, 1983; Johnson & Pratt, 1983). It seems reasonable to suppose that the cytoskeleton will prove to be of importance in the organization of this segregation.

Despite the potential importance of the cytoskeleton to early development, little is known of its nature or organization in early mouse embryos. Both microfilaments and microtubules (and at least some of their constituent molecules) are present at early stages of development (Abreu & Brinster, 1978; Cascio & Wassarman, 1982; Van Blerkom & Motta, 1979), and both the synthesis of intermediate filament proteins and the presence of intermediate filaments also has been reported recently (Lehtonen et al. 1983; Oshima et al. 1983), contrary to earlier findings (Jackson et al. 1980; Brulet, Babinet, Kemler & Jacob, 1980; Paulin, Babinet, Weber & Osborn, 1980). However, there is little information on the disposition of these cytoskeletal elements and their reorganization with time. In this paper, we report on the organization of actin before and after fertilization and conclude both from its disposition and from the effects of the drug cytochalasin D (CCD) that actin microfilaments play an essential role in the early postfertilization events.

**MATERIALS AND METHODS**

**Recovery and fertilization of oocytes**

A sperm suspension was prepared from the cauda epididymides of two male HC-CFLP mice (Hacking & Churchill). Two epididymides were immersed in each of two 0.5 ml drops of Whittingham’s medium (Tyrode base; Whittingham, 1971) containing 30 mg/ml Bovine Serum Albumin (BSA, Sigma) under liquid paraffin (BDH), which had been equilibrated overnight in 5 % CO₂ in air. By exerting gentle pressure with two pairs of watchmakers forceps the sperm were released into suspension and were left to capacitate for 1-5 h at 37 °C and 5 % CO₂ in air.

Oocytes were recovered from 3- to 5-week-old (C57B1.10 × CBA)F₁ mice after superovulation with 5·0 i.u. pregnant mare serum (PMS, Intervet) followed 48 h later by 5·0 i.u. human chorionic gonadotrophin (hCG, Intervet). The females were killed 12·5 h post-hCG and the ovulated oocytes released from the oviducts into pre-equilibrated drops of Whittingham’s medium containing 30 mg/ml BSA under liquid paraffin (BDH).

At time 0, the sperm suspension was mixed thoroughly by gentle pipetting. An aliquot was removed and diluted 1:9 into the drop of Whittingham’s medium containing the oocytes, giving a final sperm concentration of 1–2 × 10⁶ ml⁻¹.

The oocytes were incubated with the spermatozoa for 0, 1, 2, 3 or 4 h depending on the experimental protocol. Eggs that were to be cultured for more than
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4 h were removed from the spermatozoa at 4 h and washed two to three times in medium 16 (Whittingham & Wales, 1969), containing 4 mg/ml BSA, that had been pre-equilibrated at 37°C in 5% CO₂ in air. The eggs were then cultured further in this medium until harvesting.

Harvesting of eggs for analysis

Eggs (or oocytes) were removed from culture at the times postinsemination indicated in each experiment. Oocytes and eggs at early stages postinsemination were freed of their cumulus cells by brief exposure to 0.1 M-hyaluronidase (Sigma), and all eggs and oocytes were freed of their zona pellucidae by brief exposure to acid Tyrode's solution (Nicolson, Yanagimachi & Yanagimachi, 1975), followed by a rinse in medium 2 containing 4 mg/ml BSA (M2 + BSA, Fulton & Whittingham, 1978).

Staining of unfixed cells with Concanavalin A

Zona-free cells were incubated for 5 min at room temperature in FITC-Con A (700 μg/ml in M2 + BSA; Miles) or tetramethylrhodamine-labelled succinyl Concanavalin A (TMRTC-S-Con A, 500 μg/ml; Polysciences) and rinsed through three changes of M2 + BSA.

Cell fixation

In order to manipulate early mouse embryos easily during fixation and immunocytological staining, we used a method which was developed first for lampbrush chromosomes (Karsenti, Gounon & Bornens, 1978) and then adapted to cellular organelles and cell cytoskeletons (Maro & Bornens, 1980).

A glass chamber (Fig. 1C) was constructed from a 25 mm external diameter (1.5 mm thick) glass washer to which a glass coverslip had been fixed by paraffin wax (Fig. 1). The coverslip was coated for 5 min at room temperature with a 1/20 dilution of Gibco stock PHA in M2, rinsed with M2 + 4 mg/ml polyvinylpyrrolidone (PVP) and the chamber filled with M2 + PVP. This procedure ensures good and immediate adhesion of the cells to the coverslip, however M2 + BSA can be used if desired, but greater care in subsequent handling is required. Oocytes and eggs were transferred into the chamber and then the chamber was closed with a second coverslip (Fig. 1D). Chambers were then stacked in a 50 ml polycarbonate centrifuge tube with a hole drilled through its base and a perspex plunger inserted in the bottom third of the tube. Up to four chambers were inserted per tube, each interleaved with a 25 mm Millipore pre-filter. The cells were sedimented onto the glass coverslip at 500 g for 10 min at 20°C by spinning the tube in a Sorvall RC-5 Centrifuge using an HB-4 rotor. After centrifugation a rod was inserted through the hole in the base of the tube, and the plunger pushed gently up until the chambers emerged and were removed. The top coverslips were removed (Fig. 1E) and the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30–45 min at
Fig. 1. Preparation of the embryo prior to the immunocytochemical staining. Note: step C can use either M2 + BSA or M2 + PVP – see Materials and Methods.

20°C, washed four times in PBS, extracted with 0.25% Triton X-100 in PBS for 10 min at 20°C and finally washed twice in PBS.

In preliminary experiments, other fixation procedures were tested (extraction by non-ionic detergent in stabilizing buffers followed by fixation with formaldehyde, glutaraldehyde or cold methanol; by methanol fixation prior to extraction) but the fixation procedure described above was adopted because it leads to a good preservation of the cells, an easy penetration of the antibodies and reduces any reorganization of intracellular proteins during extraction of the fixed cell.

**Immunocytochemical staining**

The coverslips were removed from the chambers and transferred into PBS containing 0.1% Tween-20 (PBS Tween) before any incubation with the antibodies. Immunocytochemical staining of the cells was accomplished using 8μg/ml of affinity-purified rabbit anti-actin antibodies followed by fluorescein-labelled (Cappel) or peroxidase-labelled (Institut Pasteur Production) anti-rabbit immunoglobulin antibodies. The purification and characterization of the anti-actin
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antibody used in this study is described in Gounon & Karsenti (1981). All antibodies were diluted in PBS-Tween containing 3% BSA. The second layer antibodies were incubated prior to use for 30-45 min at 4°C in PBS-Tween containing 3% BSA with fixed STO mouse cells, then centrifuged at 10000 g for 3 min at 20°C. Washing steps were performed in PBS-Tween. The peroxidase activity was revealed using the method of Graham & Karnovsky (1966). NBD-phallacidin (Molecular Probes Inc) was used at a concentration of 5 i.u./ml to stain for polymerized actin (Barak, Yocum, Nothnagel & Webb, 1980).

Nuclear staining

Fixed oocytes or eggs were incubated in Hoechst dye 33258 (50 μg/ml – gift of Dr B. Hogan) in M2 + BSA for 1 h at room temperature, rinsed and examined.

Cytochalasin D

CCD (Sigma) was dissolved as a stock solution (1 mg/ml) in dimethylsulphoxide (DMSO – Sigma) and stored at −20°C. Dilutions were made immediately prior to use. Equivalent dilutions of DMSO were used as controls.

Photomicroscopy

A Zeiss photomicroscope was used for phase, Nomarski and fluorescence observations. The following filter sets were used: for FITC-labelled probes 48 77 09, for TMRTC-labelled probes 48 77 15, and for Hoechst dye 48 77 05. Stabilization of fluorescence was achieved by use of ‘mounting medium’ (City University, London) in the mounting buffer. This reduced fading for the fluorescein label but not for NBD-phallacidin which faded rapidly.

Immunoblotting

3T3 cell nuclei were prepared either using the citric acid technique (Mirsky & Pollister, 1946) or a double detergent technique (1% NP40, 0-5% deoxycholate in 10 mM-NaCl, 5 mM-MgCl2, 1 mM-phenylmethylsulfoxide, 10 mM-Tris-HCl pH 7-4 buffer). The proteins were then separated using sodium dodecyl sulphate 7-5% polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Proteins were then transferred on nitrocellulose filters (Towlin, Staehlin & Gordon, 1979). The affinity-purified anti-actin antibody (Gounon & Karsenti, 1981) was visualized using a second antibody bound to horseradish peroxidase. The peroxidase activity was revealed using 4 chloro-1-naphthol as a substrate (Hawkes, Niday & Gordon, 1982).

RESULTS

1. The unfertilized egg

First we confirmed the mosaic nature of the surface of the unfertilized oocyte as visualized by its Concanavalin A (Con A) binding pattern (Johnson, Eager,
Muggleton-Harris & Grave, 1975), an area deficient in Con A binding sites overlying the meiotic spindle (Fig. 2A, B, D; note that the dead first polar body often binds Con A strongly e.g. 2D). The submembranous layer in this area was rich in actin as revealed by use of both anti-actin antibody (Fig. 2E) and NBD-phallicidin (which stains only polymerized actin; Fig. 2C) on whole mouse oocytes, and by examination of sectioned oocytes in which actin was visualized by antibody and a horseradish peroxidase (HRP) labelled second layer (Fig. 2F). A zone relatively free of actin (arrowed, Fig. 2E) appeared to separate the actin-rich area from the rest of the egg and to mark the boundary between the Con A negative and positive areas (more easily seen at lower powers where the depth of focus is greater Fig. 2G–I).

Fig. 2. Unfertilized eggs, in which the position of the equator of the second metaphase spindle is indicated throughout by the arrow head: (A) viewed with Nomarski optics; (B) with DNA of second metaphase chromosomes stained by Hoechst dye 33258; (C) viewed after staining with NBD-phallicidin; (D, E) same egg double-stained with Concanavalin A (D) and anti-actin antibody (E); large solid arrow indicates dead first polar body, small solid arrows indicate position of interface between actin-rich and actin-depleted area; (F) section of egg stained as whole mount with anti-actin antibody followed by HRP-labelled anti-rabbit Ig; scale bar throughout = 25 μm; (G, H, I) same egg stained for actin, at two focal planes (G, H) and with Con A (I) to show actin-clear zone (arrowed); scale bar = 25 μm.
2. Spindle rotation

The earliest detectable change after insemination was a furrowing of the surface membrane overlying the spindle equator (Fig. 3A, B). This furrowing, which appeared to reflect an attachment by the equator of the meiotic spindle to the membrane, created two approximately equal-sized 'shoulders' of actin (Fig. 3B), and was accompanied by an expansion of the area lacking Con A binding sites (Fig. 3C). With the spindle remaining attached to the membrane, one shoulder then decreased in size causing the spindle to rotate (Fig. 3D, E) so that its long axis became aligned along a radial axis through the egg and positioned eccentrically within the remaining shoulder which formed an actin-lined, cone-like protuberance (Fig. 3F). The remnant of the contracted shoulder remained positive for actin but the area of membrane adjacent to the equator no longer stained for actin (Fig. 3F). The whole area of spindle rotation remained Con A negative (Fig. 3G). Eggs showing the same staining patterns were also observed by use of NBD-phallicidin and in section (Fig. 3H).

3. Formation of the second polar body

Extrusion of the second polar body commenced with the formation of a short
Fig. 4. Eggs 1 to 3 h post-insemination: (A–C) same egg in each frame viewed in sequence of conditions as for 3A–C, note site of sperm entry is actin positive and Con A negative (arrow) as is remnant of contracted shoulder (arrow head) and that the spindle is almost entirely outside the egg in the nascent polar body and the tube linking it to the egg; (D–F) same egg viewed in sequence of conditions as for 3A–C, note constricted polar body and fertilization cone (arrow) that is actin lined and Con A negative; the dead first polar body stains heavily with Con A in F – upper right; (G) egg stained with anti-actin antibody plus HRP-labelled second antibody to show fertilization cone (top) and second polar body (lower right); (H, I) same egg stained with anti-actin antibody (H) and Con A (I), note remnant of contracted shoulder is actin positive and Con A negative (arrow head); the dead first polar body stains heavily with Con A in I; (J, K) same egg, viewed with phase-contrast optics (J) and labelled with NBD-phallicidin (K), note spindle position (large arrow head) and actin staining associated with proximal set of decondensing chromosomes (solid arrow head); (L) phase and (M) NBD-phallicidin stain of same egg to show fertilizing sperm tail and actin-positive fertilization cone (arrow). Scale bar = 25 μm.
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constricted tube of membrane that was deficient in submembranous actin and linked an actin-lined nascent polar body with the egg (Fig. 4A, B). The spindle lay with its equator at the interface of this tube with the nascent polar body, with its distal half in the polar body and its proximal half within the tube, the proximal set of chromosomes being aligned with the actin-positive membrane residuum of the contracted shoulder (arrowhead, Fig. 4A, B). Subsequently, the linking tube of actin-free membrane disappeared, the spindle appearing to move inwards, to yield an actin-lined polar body containing the distal set of chromosomes (Fig. 4E, H), a spindle equator at the interface of polar body and egg (Fig. 4D), and the proximal set of chromosomes within the body of the egg itself and still associated with an overlying actin-lined membrane, resulting from the remnant of the contracted shoulder (arrowhead, Fig. 4H). The membrane of the second polar body, and the adjacent membrane associated with the remains of the actin-positive shoulder, lacked Con A binding sites throughout this sequence of events (Fig. 4C, F, I). Similar results were obtained when NBD-phallicidin was used to identify microfilamentous actin (Fig. 4J, K). During the later stages of spindle rotation and polar body formation a faint actin striation associated with the spindle itself was noted (see Figs 3H, 4B, E).

4. The fertilization cone

During spindle rotation, the sperm was incorporated into the egg, and at the site of its incorporation the egg membrane ceased to bind Con A (black arrow, Fig. 4C, F). The underlying submembranous region stained strongly for actin (white arrow, Fig. 4B, E, G). This region of Con-A-negative, actin-positive membrane expanded to form the ‘fertilization cone’ (Fig. 4D, L). Similar results were obtained when NBD-phallicidin was used to stain microfilaments (Fig. 4M).

5. Formation and migration of pronuclei

The fertilization cone was short lived, but both the absence of Con A receptors and the submembranous actin that characterized it persisted and marked the site of formation of the male pronucleus (Fig. 5A–F), adjacent to the site of sperm entry as assessed by the remains of the sperm tail penetrating the egg surface. The actin positivity diminished as the pronuclei migrated centrally (Fig. 5G–I), and the actin staining in the polar body became restricted to the apex (Fig. 5B, E, H). Male and female pronuclei and the adjacent cytoplasm stained increasingly positive for actin with the anti-actin antibody (Fig. 5H) but the polar body ‘pronucleus’, containing the jettisoned haploid set of female chromosomes, did not stain positively for actin and its chromatin remained condensed (Figs 5B, E, H and 6D, E). NBD-phallicidin did not stain the pronuclei internally, but did stain the adjacent cytoplasm (Fig. 5J–L). During pronuclear formation and migration the overall volume of the egg appeared to reduce considerably, and the areas lacking Con A receptors overlying the male pronucleus and around the
Fig. 5. Eggs at 3 to 4 h (A–F) and 8 h (G–L) post-insemination. (A–C) and (D–F) two eggs each viewed in sequence described for 3A–C showing pronuclei; actin positive and Con A negative areas associated with male pronucleus (arrow head), and second polar body; (G–I) same egg in each frame viewed in same sequence as above to show loss of actin-positive area, staining of pronuclei for actin and restoration of Con A binding over surface; (J–K) same egg viewed by phase-contrast optics and after staining with NBD-phallicidin to show pronuclear-associated staining. (L) Similar to K. Scale bar = 25 μm.

base of the second polar body diminished in extent and eventually disappeared (Fig. 5C, F, I).

6. Time course of events

Insemination in vitro with capacitated spermatozoa reduces variability in the time of fertilization to between 1 and 3 h compared with insemination in vivo (Fraser, 1983; Bolton, Oades & Johnson, 1984), and therefore yields a relatively homogeneous population of eggs. The proportion of eggs sampled at each time point showing each of the features described above is summarized in Tables 1 and 2. Spindle rotation was largely completed within an hour of insemination (confirming the recent report by Fraser, 1983), polar body formation occurred over the period 2 to 4 h postinsemination, the actin staining and loss of Con A
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receptors associated with sperm entry occurred over the first 4 h, the fertilization cone was detectable from 2 to 3 h postinsemination, pronuclear formation occurred over the period 4 to 5 h postinsemination and pronuclear migration over the period 6 to 8 h postinsemination.

7. Influence of Cytochalasin D on the events of fertilization

The changing disposition of actin observed during the first 8 h postfertilization suggested that it might play a role in various of the events observed over this period. This proposal was tested experimentally by use of cytochalasin D (CCD). The experiments reported were carried out with 0.5 \( \mu g/\text{ml} \) CCD, but eggs were also examined after insemination in medium containing 1.5 and 5.0 \( \mu g/\text{ml} \) CCD and analysed at 2, 4 or 8 h with similar results (data not included).

Oocytes were preincubated for 1 h in CCD, inseminated with spermatozoa and samples were recovered at 0, 1, 2, 3, 4 and 8 h thereafter, some were stained with Con A and all were fixed, and stained with Hoechst dye 33258 or with anti-actin antibody or with NBD-phallicidin. Controls were treated similarly but dimethylsulphoxide alone (DMSO) was substituted for DMSO containing CCD. The fertilization rate in normal medium was 76\% \( (n = 226) \), in DMSO was 89\% \( (n = 228) \), in 0.5 \( \mu g/\text{ml} \) CCD was 82\% \( (n = 564) \), in 1.5 \( \mu g/\text{ml} \) CCD was 88\% \( (n = 34) \) and in 5.0 \( \mu g/\text{ml} \) CCD was 78\% \( (n = 45) \).

The patterns of chromatin distribution and organization are summarized in Fig. 6. In all of 207 control eggs examined, spindle rotation, extrusion of the second polar body together with a progressive decondensation of the chromatin retained in the egg (but not of that extruded in the polar body) were observed (Fig. 6B–E). The sperm chromatin also decondensed, but earlier than that of the female. In contrast, polar bodies were recorded in only 2 of 169 eggs inseminated in the presence of CCD. In the remaining eggs, rotation of the spindle and extrusion of the second polar body were not observed. Instead meiosis was resumed at activation with the long axis of the spindle parallel to the overlying membrane, yielding two adjacent, subcortical chromatin masses (Fig. 6F–I), both of which decondensed. The sperm chromatin entered the egg and decondensed over the first 2 to 3 h as in the controls. However, the incidence of formation of an overt fertilization cone was reduced greatly (Table 1, column 3). At 8 h postinsemination 93\% of DMSO-treated eggs contained two pronuclei (1 male and 1 female) closely adjacent to each other at the centre of the cell (Fig. 6E). In contrast, CCD-treated eggs contained two female pronuclei which remained peripheral, and although adjacent to each other they were usually separated from the peripherally placed male pronucleus (Fig. 6F). In one experiment, eggs were examined 12 h after insemination in CCD to determine whether pronuclear migration was merely delayed. However the same result was obtained as seen at 8 h.

When CCD-treated eggs were examined for actin distribution (by either anti-actin antibody or NBD-phallicidin) several differences from controls were
Table 1. Effect of insemination of eggs in presence of cytochalasin D (0·5 μg/ml)

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<tr>
<td></td>
<td>CCD*</td>
<td>39</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* CCD was absent during removal of the zona pellucida and subsequent incubations prior to fixation.
Table 2. Effect of insemination of eggs in presence of cytochalasin D (0.5 μg/ml) on Con A and actin staining patterns

<table>
<thead>
<tr>
<th>(h) Time of observation</th>
<th>Treatment</th>
<th>No. of eggs scored</th>
<th>Con A staining</th>
<th>Actin staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1)</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2)</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>% with negative area</td>
<td>% with positive area</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>over spindle or 2nd</td>
<td>over sperm entry site</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>polar body</td>
<td>or over pronuclei</td>
</tr>
<tr>
<td>0</td>
<td>Control</td>
<td>27</td>
<td>93</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>CCD</td>
<td>19</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>21</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>40</td>
<td>ND</td>
<td>98</td>
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<tr>
<td></td>
<td>CCD</td>
<td>23</td>
<td>100</td>
<td>70</td>
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<td></td>
<td>CCD*</td>
<td>47</td>
<td>ND</td>
<td>72</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>32</td>
<td>100</td>
<td>100</td>
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<td>DMSO</td>
<td>40</td>
<td>ND</td>
<td>100</td>
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<td>CCD</td>
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<td>83</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>CCD*</td>
<td>47</td>
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<td>45</td>
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<td>3</td>
<td>Control</td>
<td>46</td>
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<td>100</td>
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<td>16</td>
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</tr>
<tr>
<td></td>
<td>CCD</td>
<td>16</td>
<td>100</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>CCD*</td>
<td>21</td>
<td>ND</td>
<td>19</td>
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<tr>
<td>4</td>
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<tr>
<td></td>
<td>CCD</td>
<td>16</td>
<td>95</td>
<td>44</td>
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<td></td>
<td>CCD*</td>
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<td>ND</td>
<td>33</td>
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<tr>
<td>5</td>
<td>Control</td>
<td>21</td>
<td>38</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>31</td>
<td>42</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CCD</td>
<td>16</td>
<td>100</td>
<td>38</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>31</td>
<td>45</td>
<td>100</td>
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<tr>
<td>8</td>
<td>Control</td>
<td>85</td>
<td>8</td>
<td>100</td>
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<tr>
<td></td>
<td>DMSO</td>
<td>57</td>
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<td>100</td>
</tr>
<tr>
<td></td>
<td>CCD</td>
<td>23</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CCD*</td>
<td>39</td>
<td>ND</td>
<td>0</td>
</tr>
</tbody>
</table>

* CCD was absent during removal of the zona pellucida and subsequent incubations prior to fixation.
observed. First, although most unfertilized oocytes examined showed an area lacking Con A receptors over the spindle (Fig. 7A), an overlying actin-rich region was observed in few, and where present it was not discretely organized as in controls (compare Figs 2E and 7B). Second, at activation, the area lacking Con A receptors persisted (Table 2, column 1), but two actin-lined shoulders did not form. Rather, a dimpling of the surface membrane overlying the spindle was observed and was associated with one of three patterns of cortical actin staining. In some eggs three local subcortical concentrations of actin overlying the equator of the spindle and each of its poles were identified (Fig. 7G–I; Table 2, column 3). In other eggs, subcortical localizations of actin were visible only over one or both poles of the spindle (Fig. 7E, F). These submembranous distinctive localizations were evident mainly at 2 h (when spindle rotation and polar body extrusion were occurring in controls), and were rare subsequently (Table 2, column 3). In addition, in most eggs, marked striations of actin associated with the juxta-equatorial region of the spindle remnant itself were evident at 2 to 4 h (Fig. 7F, I, J, L) and seemed to be an exaggeration of the striations seen in control spindle remnants (see earlier).

The site of sperm entry in CCD-treated eggs was associated with loss of Con A binding activity (Fig. 7C), and with a cortical actin localization (Fig. 7D, N, O), as in controls, but at a lower frequency at 2 and 3 h postinsemination, rising at 4 h postinsemination when the incidence of staining in the DMSO-controls had declined (Table 2, column 2 & 4). In addition, in CCD-treated eggs the site of the sperm tail was often remote from the site of the male pronucleus (Fig. 7M, N). Both CCD- and DMSO-treated eggs contained actin-positive pronuclei (Fig. 7N, O, Q). However, whereas the polar body ‘pronucleus’ of the DMSO control did not stain for actin, both the female pronuclei retained within CCD-treated eggs did (Fig. 7O, Q). Con A binding activity over the site of sperm entry and adjacent to polar body extrusion was restored in controls, but not in the presence of CCD (Fig. 7P).

8. Effects of addition of Cytochalasin D at various times in relation to insemination

Eggs were inseminated with spermatozoa in normal fertilizing medium and were transferred to medium containing 0.5 μg/ml CCD at time intervals up to 6 h thereafter. The results of analysis of these embryos at 8 h postinsemination are
recorded in Table 3 (lines h–m). Suppression of polar body formation occurs when CCD is added prior to 2 h postinsemination, but decreasingly so thereafter. This finding correlates with the observation that rotation of the spindle and extrusion of the polar body in controls takes 2 to 4 h to complete (Table 1, column 1). In some eggs, a constriction of the midbody between the eggs and polar body had occurred but this was evidently not complete as judged by the decondensation of the polar body 'pronucleus' and its reactivity with anti-actin antibody, a behaviour associated with pronuclei retained within the oocyte cytoplasm.
Pronuclear migration was suppressed totally when CCD was added prior to 4 h, but was only partially suppressed when added thereafter or as a pulse between 2 and 4 h (Table 3, line n). When CCD was added prior to insemination and removed at intervals thereafter (lines b–g, Table 3), polar body formation occurred if removal was within 1 h of insemination, but became less frequent at longer time intervals and was totally suppressed by 6 h. Pronuclear migration at 8 h was suppressed completely if CCD was present beyond 5 h, but was greatly reduced even when removed prior to this time. However, observation of eggs at 13 h revealed that recovery from the inhibitory effect of CCD was possible.

DISCUSSION

The distribution of filamentous cytoskeletal elements in whole-mount cells of the early mouse embryo has proved to be difficult to study, due to the large cytoplasmic volume of the blastomeres and the background of soluble monomers (Lehtonen & Badley, 1980). We have devised a convenient handling procedure for efficient processing of large numbers of eggs and have used it to evaluate a range of extraction and fixation procedures on early embryonic cells. A protocol has been developed that gives clear localization with little evidence of any major

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**Fig. 7.** Eggs preincubated in 0-5 μg/ml cytochalasin D. Throughout open arrow head indicates position of equator of spindle (or its remnant). (A, B) unfertilized egg stained with Concanavalin A and anti-actin antibody—note loss of discretely defined actin-positive area under region of low Con A binding; (C, D) egg soon after fertilization stained with Con A and anti-actin antibody—note loss of Con A binding sites (arrow) at the site of sperm entry; (E, F) egg viewed with phase-contrast optics and after staining with anti-actin antibody, note that spindle has not rotated, that two subcortical areas of actin are present over the spindle poles (solid arrow heads) and that the peri-equatorial region of spindle is positive for actin; (G, H) egg viewed en face with phase-contrast optics and after staining with anti-actin antibody with the spindle (equator indicated with hollow arrowhead) in a vertical orientation and slightly tilted; note that overlying the spindle in (H) three discrete subcortical localizations of actin are observed, two large ones associated with the spindle poles and a smaller central one associated with the equatorial regions (solid arrow heads); (I) optical section through an egg similar to that shown en face in (H), in which the three subcortical actin localizations can be observed, as can the spindle-associated actin seen in higher power in (J); (K, L) egg viewed with phase-contrast optics or after staining with NBD-phallicidin—note the actin staining associated with the spindle equator and adjacent subcortical regions; (M, N) egg viewed with phase-contrast optics and after staining with anti-actin antibody to reveal separation of sperm tail (arrowed) from male pronucleus and overlying subcortical actin (arrow head); (O) two female and one larger male pronucleus all stained for actin, including concentrated perinuclear speckles, and the male pronucleus also associated with subcortical actin (arrow head); (P, Q) egg 8 h postinsemination stained with Con A and anti-actin antibody to reveal continuing presence of Con A negative areas overlying original position of spindle now occupied by two female pronuclei and site of sperm entry now occupied by male pronucleus—note failure of pronuclei to migrate centrally.
Table 3. Effect of cytochalasin D (CCD; 0.5 μg/ml) on polar body formation and pronuclear migration

<table>
<thead>
<tr>
<th>Period of exposure to CCD in relation to insemination time (=0 h)</th>
<th>No. of eggs examined (Columns 3 &amp; 4)</th>
<th>% with 2nd polar body and two chromatin masses at 8 h</th>
<th>% with adjacent and centrally located pronuclei at 8 h</th>
<th>% with adjacent and centrally located pronuclei at 13 h (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) DMSO only</td>
<td>57</td>
<td>100</td>
<td>93</td>
<td>100 (36)</td>
</tr>
<tr>
<td>(b) -1 to 2</td>
<td>33</td>
<td>94</td>
<td>46</td>
<td>100 (41)</td>
</tr>
<tr>
<td>(c) -1 to 3</td>
<td>17</td>
<td>70</td>
<td>41</td>
<td>ND</td>
</tr>
<tr>
<td>(d) -1 to 4</td>
<td>20</td>
<td>30</td>
<td>35</td>
<td>ND</td>
</tr>
<tr>
<td>(e) -1 to 5</td>
<td>17</td>
<td>18</td>
<td>0</td>
<td>93 (45)</td>
</tr>
<tr>
<td>(f) -1 to 6</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>(g) -1 to 8</td>
<td>95</td>
<td>2</td>
<td>0</td>
<td>85 (34)</td>
</tr>
<tr>
<td>(h) 1 to 8</td>
<td>65</td>
<td>6</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>(i) 2 to 8</td>
<td>77</td>
<td>26*</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>(j) 3 to 8</td>
<td>19</td>
<td>58</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>(k) 4 to 8</td>
<td>75</td>
<td>88</td>
<td>29</td>
<td>ND</td>
</tr>
<tr>
<td>(l) 5 to 8</td>
<td>75</td>
<td>91</td>
<td>27</td>
<td>100 (35)</td>
</tr>
<tr>
<td>(m) 6 to 8</td>
<td>77</td>
<td>99</td>
<td>48</td>
<td>ND</td>
</tr>
<tr>
<td>(n) 2 to 4</td>
<td>19</td>
<td>21</td>
<td>32</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Borsuk (1982) reported a value of 8–10 % on addition of CCB at 2 h after insemination of Swiss or CBA-T6T6 oocytes. Niemierko (1975) using in vivo fertilized eggs also induced triploidy by addition of CCB to eggs recovered 2.5 to 3.5 h postcopulation; it is difficult to relate this timing precisely to time postinsemination of the eggs.

distortion of cell organization. Both specific anti-actin antibodies and NBD-phallicidin have been used to examine the changes in distribution of G-actin (soluble) and F-actin (polymerized in microfilaments) during the events of mouse egg fertilization (summarized in Fig. 8). The two types of reagents gave similar staining patterns, the only observed differences being: (i) a higher background with use of anti-actin antibodies presumably corresponding to the pool of soluble G-actin which is not detected by NBD-phallicidin, (ii) a perinuclear area rich in microfilaments that was more easily observed with NBD-phallicidin than with anti-actin antibodies where the background of soluble cytoplasmic actin reduced the contrast, and (iii) an intranuclear staining of the pronuclei observed when using anti-actin antibodies but not with NBD-phallicidin; this point will be discussed below. In addition, we have used CCD to investigate the role of actin microfilaments during the fertilization process. CCD inhibits gelation of both cytoplasmic extracts and reconstituted systems in a substoichiometric manner (Tannenbaum, 1978; Hartwig & Stossel, 1976, 1979; MacLean-Fletcher & Pollard, 1980) and reduces the rate of actin filament growth.
Fig. 8. Schematic summary and interpretation of events occurring in the first 8 h postinsemination. In (A) the changing distribution of the principle areas of actin staining are indicated in cross hatching, and in (B) the larger cross hatching represents patterns of Con A binding.

by blocking monomer addition at the growing end (Brown & Spudich, 1979; MacLean-Fletcher & Pollard, 1980). Under physiological conditions inhibition of network formation seems to be the most important effect. In intact cells, CCD inhibits two types of motile process: (i) changes in interaction between actin filaments (Morris & Tannenbaum, 1980), which can lead to the energy-dependent generation of star-like aggregates (Miranda, Godman, Deich & Tannenbaum, 1974; Weber, Rathke, Osborn & Franke, 1976); and (ii) polymerization or turnover of actin filaments (Casella, Flanagan & Lin, 1981). There is no effect
on stabilized microfilaments such as are observed in striated muscle and brush border microvilli (Sanger, Holtzer & Holtzer, 1971; Manaseh, Burnside & Shoman, 1972; Mak, Trier, Serfilippi & Donaldson, 1974).

1. The unfertilized oocyte

A clearly delineated area rich in submembranous microfilamentous actin overlies the metaphase spindle. This area of cytoplasm is also deficient in organelles and its membrane lacks microvilli, is relatively deficient in Con A receptors and contains a higher proportion of readily diffusible membrane proteins than other regions (Johnson et al. 1975; Eager, Johnson & Thurley, 1976; Nicosia et al. 1977; Wolf & Ziomek, 1983). Whereas the clearly delineated area of submembranous actin is sensitive to the action of CCD, the zone relatively free of Con A receptors (and by implication microvilli) persists in the presence of the drug. Taken together these results indicate that the modulation of both the spatial distribution and the fractional mobility of surface receptors in this region is not related in any simple way to the number of underlying microfilaments as has been suggested by some studies (Tank, Wu & Webb, 1982).

2. Fertilization

Changes in the apparent distribution of actin at and after fertilization could result from focal losses or gains of actin or from a combination of both. It is our impression from the qualitative studies undertaken here that the discrete foci of submembranous actin observed after fertilization, and centred around the sites of sperm entry and polar body extrusion, arise by the relative reduction and turnover of actin in the rest of the egg. We will consider the evidence for this conclusion for each of the various changes observed.

3. Spindle rotation and polar body extrusion

The sequence of structural changes that take place in the live mouse egg after fusion with the spermatozoon were described by Sato & Blandau (1979) using a polarizing microscope. They observed first the anaphase II of meiosis followed by the initial appearance of the cleavage furrow as a dimple at the equator, the rotation of the spindle by differential reduction of one ‘shoulder’ and then polar body formation. We have confirmed this sequence by sampling fixed, extracted eggs and have identified associated modifications in the localization of actin during the three later events (Fig. 8A). A causal role for actin in these latter events is suggested strongly from the effects observed after adding CCD prior to insemination. Although a dimpling of the egg surface still occurs, spindle rotation, furrow constriction and polar body extrusion are prevented; moreover, if CCD is removed prior to completion of this sequence in the controls, or is first added part way through the sequence, then the expected partial deficiencies of rotation and extrusion occur (Table 3). The precise mechanism by which actin is causally involved is less clear, but some clues may be obtained
by comparing the distribution of actin in control and CCD-treated eggs.

A discontinuity in the submembranous actin overlying the spindle develops after fertilization of control oocytes, the region over the equator and distal pole becoming distinct from that over the proximal pole, and separated from it by an intervening ‘tube’ of actin-free membrane containing the proximal half of the spindle and linking the nascent polar body to the egg. Subsequently, the whole spindle moves towards the egg until the equator is aligned with the egg cortex and it is possible that the equatorial striations of actin observed in the late meiotic spindle and its postmeiotic remnant (Fig. 7J) are involved in this movement. We suspect, but have not demonstrated, that as the spindle moves the actin-negative ‘tube’ of membrane collapses to form the convoluted mass of midbody membrane described previously between the definitive polar body and the egg (Eager et al. 1976 and Fig. 8A). Subsequently, the submembranous regions overlying the remnants of first the equator and then the proximal pole (now the site of female pronuclear formation) also become actin negative leaving a localization of actin exclusively over the remnant of the distal pole in the polar body itself.

In the light of these observations it is of interest that CCD treatment over the first 1 to 4 h postinsemination yields eggs in which one, two or three foci of submembranous actin are readily visible, presumably because they represent relatively stable foci for aggregation of filamentous actin revealed by the relative loss of background effect of adjacent unstable microfilaments. These foci overlie each pole of the spindle and (in fewer eggs) the equator itself. Taken together, these observations suggest that the spindle poles and equator (or some feature associated with them) represent stabilization foci for submembranous actin, that change in their efficiency according to a programmed time table determined by the underlying meiotic events. The nature of our studies precludes comment on whether new stabilizing foci for actin develop, on the extent or importance of filamentous actin turnover at non-stable foci, and on the involvement of cytoskeletal components other than actin, and a full account of the mechanism of rotation and polar body extrusion requires such information.

4. The site of sperm entry

The fertilization cone, which has been shown previously to be devoid of microvilli (Shalgi, Phillips & Kraicer, 1978), is also characterized by the absence of Con A binding sites, by a layer of submembranous actin and by a sensitivity to CCD. In contrast, the loss of Con A binding sites at fertilization is not blocked by addition of CCD, which is not surprising as it is probably a depolymerization event simply reflecting the disassembly of microvilli (Shalgi et al. 1979). CCD also fails to block the occurrence of submembranous actin associated with the developing sperm pronucleus which may reflect simply a stabilizing influence of that nucleus (or an associated structure) at a time when adjacent actin is depolymerized or turning over rather than an active induction of submembranous actin at the fertilization site. This view is strengthened by the observation that
CCD does not block fusion or incorporation of the sperm nucleus (unlike the situation in the sea urchin in which incorporation is blocked; Schatten & Schatten, 1981) and that in many CCD-treated eggs the male pronucleus with its associated submembranous actin may be remote from the site of sperm entry at which the sperm tail remains (e.g. 32% of eggs at 4 h – see Fig. 4L, M).

5. The pronuclei and their migration

In addition to submembranous actin associated with the nascent male and female pronuclei (at the fertilization cone and ‘contracted shoulder’ respectively) actin is also detectable within and around the pronuclei.

The intranuclear staining of both the male and female pronuclei was observed with anti-actin antibody. Nucleoli were not stained but bright dots of fluorescence were present at their periphery. This pattern of staining may be related to the decondensation of the chromatin since the polar body nucleus, which does not decondense, was not stained by the anti-actin antibodies. The intranuclear staining was only observed when anti-actin antibodies were used, whatever the origin of the second antibody. It was absent when other rabbit first antibodies (anti-clathrin, anti-endoplasmic reticulum: data not shown) or the second antibody alone were used. The anti-actin antibodies we used were affinity purified on an actin immunoadsorbant after elimination of the non-specific binding activity by preliminary filtration of the serum on a myosin immunoadsorbant (Gounon & Karsenti, 1981). The antibodies reacted only with actin in Western blots using either a Triton X-100 soluble or insoluble cell extract as antigen although a faint second band at $40 \times 10^3$ (relative molecular mass) was observed when immuno blots were made on nuclear extracts (Fig. 9). Although the presence of a nuclear protein sharing an antigenic determinant with actin cannot be ruled out, other studies, in which both poly- and mono-clonal antibodies have been used, have

![Fig. 9. Mouse nuclear proteins were fractionated by SDS-PAGE and immunoblotted using affinity-purified anti-actin antibody which was visualized using a second antibody bound to horseradish peroxidase followed by the peroxidase reaction. Proteins coming from $10^6$ nuclei were fractionated in each lane. Lane A: control, second antibody alone; lane B: anti-actin antibody (12 μg/ml); lane C: anti-actin antibody (24 μg/ml).](image-url)
also demonstrated a nuclear staining that was abolished by pre-absorption of the antibodies with actin (Lazarides, 1975; Fagreus, Orvell, Norberg & Norby, 1983). Furthermore, biochemical studies (Jockush, Brown & Rusch, 1971; Les-tourgeon, Totten & Forrer, 1974; Paulin et al. 1976) have indicated the presence of actin in nuclei. Although these studies were originally criticized on the grounds that actin might be detected in the nucleus for trivial reasons since it seemed to be freely exchangeable between the cytoplasm and the nucleus (Goldstein, Rubin & Ko, 1977), the latter authors modified their position subsequently after demonstrating that nuclear actin was less soluble than cytoplasmic actin (Rubin, Goldstein & Ko, 1978). Moreover, the presence of actin in the germinal vesicle of amphibian oocytes has been demonstrated clearly (Clark & Merriam, 1977; Clark & Rosenbaum, 1979; Gounon & Karsenti, 1981), and microfilaments associated with RNP particles have been observed by electron microscopy. It has also been shown that anti-actin antibodies injected into the nucleus of Xenopus oocytes inhibit specifically the condensation of the chromosomes (Rungger, Rungger-Brandle, Chaponnier & Gabbiani, 1979). However, we would not claim on present evidence that the pronuclear staining observed by us can be unequivocally assigned to actin.

The migration of both male and female pronuclei occurs between 6 and 8 h postinsemination (Table 1 and Fig. 5). Actin microfilaments may be involved both during and prior to this migration, since (i) both pronuclei are overlain by submembranous actin during their early formation, (ii) microfilaments are found in large amounts around the formed pronuclei (Fig. 5), and (iii) unlike the situation in the sea urchin (Schatten & Schatten, 1981), CCD blocked the movement of pronuclei whether present prior to or during migration (Table 1 & Table 3, column 4 and Figs 6I and 7O, Q; a similar blocking effect of CCB added 2 h postinsemination may be inferred from figure 1 of Borsuk, 1982). However, recovery from the CCD effect is possible, a delayed pronuclear migration having occurred when the analysis was made at 13 h rather than 8 h postinsemination (Table 3, column 5).

Migration of male and female pronuclei away from the cortex is accompanied by the loss of their associated subcortical actin foci and the restoration of overlying Con A binding. Not surprisingly the former (a depolymerization event) is not inhibited by CCD whereas the latter (requiring elaboration of actin filaments in the microvilli) is inhibited.

6. Speculation

A feature central to our description of the changes in actin distribution after fertilization is the association of stable foci of actin with the male nucleus, the poles and equator of the meiotic spindle and the female pronucleus. Microtubule-organizing centres are not only observed at the pole of spindles, but they are also associated with nuclei (Bornens, 1977; Maro & Bornens, 1980; Kallenbach & Mazia, 1982; Tassin, Maro, Fardeau & Bornens, 1983). We are
therefore currently investigating the possibility that microtubule-associated structures are involved in controlling actin distribution and turnover in the mouse egg.

We wish to acknowledge gratefully the patient assistance of Tim Crane and Roger Liles with the many photographic reproductions, Caroline Hunt for preparing the manuscript and Raith Overhill for the preparation of diagrams. We thank Dr E. Karsenti and P. Gounon for the generous gift of anti-actin antibodies and Ms M. C. Marty for the help in the western blot experiments. The work was supported by grants from the Medical Research Council and Cancer Research Campaign to M.H.J. B.M. is in receipt of an EMBO Fellowship.

REFERENCES


Actin during mouse egg fertilization


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(Accepted 11 January 1984)