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Polarity of the ascidian egg cortex before fertilization

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Summary

The unfertilized ascidian egg displays a visible polar organization along its animal-vegetal axis. In particular, the myoplasm, a mitochondria-rich subcortical domain inherited by the blastomeres that differentiate into muscle cells, is mainly situated in the vegetal hemisphere. We show that, in the unfertilized egg, this vegetal domain is enriched in actin and microfilaments and excludes microtubules. This polar distribution of microfilaments and microtubules persists in isolated cortices prepared by shearing eggs attached to a polylysine-coated surface. The isolated cortex is further characterized by an elaborate network of tubules and sheets of endoplasmic reticulum (ER). This cortical ER network is tethered to the plasma membrane at discrete sites, is covered with ribosomes and contains a calsequestrin-like protein. Interestingly, this ER network is distributed in a polar fashion along the animal-vegetal axis of the egg: regions with a dense network consisting mainly of sheets or tightly knit tubes are present in the vegetal hemisphere only, whereas areas characterized by a sparse tubular ER network are uniquely found in the animal hemisphere region. The stability of the polar organization of the cortex was studied by perturbing the distribution of organelles in the egg and depolymerizing microfilaments and microtubules. The polar organization of the cortical ER network persists after treatment of eggs with nocodazole, but is disrupted by treatment with cytochalasin B. In addition, we show that centrifugal forces that displace the cytoplasmic organelles do not alter the appearance and polar organization of the isolated egg cortex.

These findings taken together with our previous work suggest that the intrinsic polar distribution of cortical membranous and cytoskeletal components along the animal-vegetal axis of the egg are important for the spatial organization of calcium-dependent events and their developmental consequences.

Key words. egg cortex, polarity, endoplasmic reticulum, microtubules, microfilaments, fertilization, ascidian development

Introduction

The egg cortex can be defined as the peripheral layer of an egg cell about 1-5 μm thick that behaves as a unit during experimental micromanipulation and during the surface reorganizations that occur following fertilization and during further development (see Vacquier, 1981; Schroeder, 1986; Sardet and Chang, 1987; Dohmen and Speksnijder, 1990 for reviews). Depending on the species and the developmental stage considered, the egg cortex consists of the plasma membrane and its extracellular coat, adhering cytoskeletal elements in various states of polymerization and assembly, vesicular organelles, and a network of endoplasmic reticulum (ER). The egg cortex can be isolated by severing the ER network and the cytoskeletal elements that connect it with the rest of the egg (Sardet, 1984). In that respect, the isolated cortex can be compared to the isolated nucleus, which is itself connected to the rest of the cell's cytoplasm by strands of ER continuous with the nuclear membrane. However, unlike the nucleus, the precise structure and function of the cortex remains ill-defined. The old concept that the cortex may serve as a repository of essential developmental information is gaining interest again as important molecules and cytoskeletal structures are found to be localized in specific areas of the egg cortex (Yisraeli and Melton, 1988; Elinson and Rowning, 1988; Pondel and King, 1988; Yisraeli et al., 1990; Houliston and Elinson, 1991).

The eggs of most species display a clear polarity along their animal-vegetal axis, which is usually expressed in the position of the chromosomes and female nucleus, in the distribution of organelles, and in the distribution of

some cytoskeletal components and macromolecules (reviewed in Davidson, 1986; Elinson, 1990). Egg polarity in the cortical region of egg cells has also been analysed in various species, and includes differences in the organisation of the extracellular coat and surface microvilli (Dohmen and Van der Mey, 1977; Dohmen and Speksnijder, 1990); the composition and fluidity of the plasma membrane (Speksnijder and Dohmen, 1983; Dictus et al., 1984; Speksnijder et al., 1985a, b); the constitution and organization of the submembranous cytoskeleton (Schroeder, 1985, 1986; Elinson and Rowning, 1988; Shimizu, 1984, 1988, Klymkovsky and Maynell, 1989; Houliston and Elinson, 1991), and the density and nature of cortical organelles (Campanella and Andreuccetti, 1977; Campanella et al., 1984; Sardet and Chang, 1985; Shimizu, 1986). These differences are generally established during oogenesis and have been implicated in polarized sperm entry (Charbonneau and Grey, 1984); the trigger and propagation of a cortical contraction at fertilization and the reorganization of egg components (Shimizu, 1984, 1986; Sardet and Chang, 1987; Sawada, 1988; Houliston and Elinson, 1991) as well as in later developmental events (see Schroeder, 1986; Shimizu, 1988; Dohmen and Speksnijder, 1990).

In the eggs of ascidians, the cortical (and subcortical) layers are thought to be endowed with special properties involved in the localization of developmental potential. For example, the myoplasm, a mitochondria-rich domain that contains determinants for the development of the larval muscle cells, is mainly localized as a (sub)cortical layer in the vegetal hemisphere of the unfertilized egg (Conklin, 1905; Reverberi, 1971; Whittaker, 1987; Jeffery and Bates, 1989). Following fertilization, a wave of elevated intracellular calcium sweeps through the egg, and triggers a cortical contraction which segregates the subcortical myoplasm into a cap at a contraction pole that is close to but not always coincident with the vegetal pole (Sawada, 1983, 1988; Jeffery and Meier, 1983; Sawada and Osanai, 1984, 1985; Sardet et al., 1989, Speksnijder et al., 1989a, 1990a). This microfilament-dependent cortical contraction sets up one of the embryo's developmental axes (Bates and Jeffery, 1988; Jeffery et al., 1990; Speksnijder et al., 1990a).

We thought that at this stage it was essential to understand the polarity and structure of the egg cortex of the unfertilized egg and its reorganization following fertilization. As a first step, we have therefore carried out a thorough study of the egg cortex of the unfertilized ascidian egg. We show that besides the plasma membrane and adhering cytoskeletal elements, the isolated cortex is characterized by an extensive network of ER. In addition, the components of this network as well as the major cytoskeletal elements (microfilaments and microtubules) are distributed in a polar fashion along the animal-vegetal axis of the egg. The stability of this polar organization is examined and we discuss its possible role for the reorganization of the egg that takes place at fertilization and during early differentiation.

Materials and methods

Biological material

The tunicate *Phallusia mammillata* (Ascidiae, Tunicata) was collected in Sète (Mediterranean coast, France) and Roscoff (Brittany, France) and kept in tanks of running sea water. Gametes were obtained by dissection and handled as described previously (Sardet et al., 1989). In short, eggs were dechorionated by enzymatic digestion with 0.1% trypsin in filtered sea water buffered with 5 mM TAPS (pH 8.2) for 2 hours. They were kept in dishes and handled with pipettes coated with a layer of gelatin-formaldehyde to prevent them from sticking and lysing.

Localization of cytoskeletal components in whole eggs and sections

Microfilaments were demonstrated in whole eggs by labelling with phalloidin-TRITC. Unfertilized eggs were fixed for 10 minutes in 3.7% formaldehyde in CIM buffer (0.8 M glucose, 0.1 M KCl, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM MOPS pH 6.7; Yonemura and Mabuchi, 1987), permeabilized with 0.5% Triton X-100 in CIM buffer for 5 minutes, and incubated with 0.1 μg/ml phalloidin-TRITC (Sigma, St Louis, MS) in PBS for 45 minutes. The eggs were then rinsed extensively with PBS and labelled for 10 minutes with 10 μg/ml Hoechst 33342 in CIM buffer to visualize the female chromosomes that mark the animal pole.

For immunolocalization of actin and tubulin in sections, eggs were fixed in cold methanol (−20°C) and ethanol (−20°C) for 20 minutes each, embedded in Polyester wax (BDH), sectioned and further processed essentially as described by Nishikata et al. (1987). A polyclonal rabbit anti-actin (Sigma) and monoclonal anti-β-tubulin (Amersham) diluted in PBS + 0.5% BSA were used as primary antibodies. In addition, we used a monoclonal antibody against isolated myoplasm from the *Ciona intestinalis* egg (antibody II-G6B2; a generous gift of Dr. Nishikata, Kyoto University). As secondary antibodies, we used biotin- conjugated anti-rabbit and anti-mouse antibodies. A Streptavidin-Texas Red conjugate (Amersham) was used to visualize the antigen-antibody complex with epifluoresence optics.

Isolation of the egg cortex

The cortex isolation method originally developed by Vacquier (1975) and Schatten and Mazia (1976) based on shearing sea urchin eggs attached to a polycationic surface was applied to ascidian eggs

Dechorionated eggs were deposited into a drop of EMC (500 mM NaCl, 10 mM KCl, 2.5 mM NaHCo3, 25 mM EGTA pH 8.0; Detering et al., 1977) on a polylysine-coated glass surface. After the eggs had settled and attached to the surface (after 2-3 minutes), the medium was exchanged for an iso-osmotic, low ionic strength, low calcium buffer (buffer X: 350 mM potassium aspartate, 130 mM taurine; 170 mM betaine, 50 mM glycine; 12.9 mM $MgCl_2$; 20 mM Hepes pH 7.2, 10 mM EGTA, and 3 mM $CaCl_2$; Brady et al., 1984). The eggs were sheared by a jet of buffer X from a Pasteur pipette, and the cortices thus obtained were used immediately for further experiments as described below.

Observations and experiments on living cortices

The ER network and its response to osmotic stress was visualized in Differential Interference Contrast (DIC) optics in video-enhanced microscopy or epifluorescence optics after labelling with $DiOC_6(3)$ (2.5 μg/ml in buffer X for 10 seconds; see Terasaki et al., 1984, 1986) or $DiIC_{16}(3)$ (7.5 μg/ml in

buffer for 1 minute, see Terasaki et al., 1988). Microtubules could be seen in these cortex preparations after perfusing gold particles (10 nm) coated with anti-tubulin antibodies (a generous gift from M. De Brabander, Janssen Pharmaceutica foundation, Beerse, Belgium). Finally, actin filaments were visualized by labelling with phalloidin-TRITC (50 μg/ml in buffer X) for 30 minutes.

Labelling of isolated and fixed cortex components

Cortices were fixed with 3.7% paraformaldehyde with or without glutaraldehyde (0.1-1%) in CIM buffer for 10 minutes, washed with CIM buffer, and quenched with 1 M glycine Subsequent steps for immunolabelling were carried out in PBS + 0 5% BSA, using a monoclonal mouse anti-α or anti-β-tubulin (Amersham), a polyclonal rabbit anti-actin (Sigma) or a polyclonal rabbit antibody made against the calsequestrin-like protein of the sea urchin egg (Oberdorf et al., 1988, Henson et al , 1989) The latter antibody was kindly provided by Dr B. Kaminer (Boston Univ. Med. School) Secondary antibody steps and fluorescent labels (Rhodamine or Texas red) were used as described above. For microfilament labelling, fixed cortices were incubated for 30 minutes with phalloidin-TRITC in CIM or PBS (50 μg/ml). For RNA labelling, we used the dye Thiazole orange (Molecular Probes 1 mg/ml in DMSO) at a final concentration of 10 μg/ml in CIM buffer for 3 minutes. Thiazole orange (Lee et al., 1986) has been shown to stain ribosomes of sea urchin cortical ER (Terasaki unpublished results)

For simultaneous visualization of the ER network and of the cytoskeletal components, we used the dye $DiOC_6(3)$ for 10 seconds at a concentration of 2.5 μg/ml in PBS as a last step before mounting the samples for observation in a mixture of 0 5% n-propylgallate in 50% glycerol in PBS.

Polarity of the egg cortex

In order to determine the origin of the piece of cortex with respect to the animal-vegetal polarity of the egg, we first attached eggs labelled with Hoechst 33342 so as to visualize the female chromosomes that mark the animal pole. We recorded the position of each individual egg and its polarity, using scratch marks on the coverslip as reference points We then sheared the eggs, fixed the resulting cortices and labelled them with $DiOC_6(3)$ as described above. We relocated and repositioned the scratch marks on the coverslip, and matched the images of the egg and the corresponding piece of cortex with the use of an image processor. This allowed us to determine the exact position of each piece of cortex with respect to the animal pole of the egg from which it originated. We then inspected and photographed each cortex at high magnification to determine the presence and distribution of sheets and tubes of ER (see Fig 6)

Light microscopy

Living cortices on coverslips were examined using a Zeiss IM35 or Axiophot microscope and imaged through a video camera (DAGE or LHESA Newicon or SIT cameras) The image was processed through a video processing unit (Matrox card installed in an IBM PC/AT compatible personal computer and Universal imaging software; Image I) and recorded on video tape or disc (Sony UMatic recorder or Panasonic OMDR)

Epifluorescence observations were made on a Zeiss Axiophot microscope equipped with the following filters: ex 353-377, em 395; and FD 395 for Hoechst; ex 450-490, em 520-560, and FD 510 for FITC and $DiOC_6(3)$; and ex 450-490, em 520, and FD 510 for TRITC, Texas Red, and $DiIC_{16}$ (3).

We examined Phalloidin-TRITC labelled eggs with a confocal laser scanning microscope (Biorad Lasersharp MRL-500) equipped with an Argon laser (excitation 514 nm) The laserbeam was attenuated to 0.1% to 1% of its intensity to minimize photodamage Images were recorded using a slow scan rate and the Kalman integration algorithm.

Electron microscopy

Thin section electron microscopy of whole eggs was performed as described in Gualtieri and Sardet (1989). Dechorionated unfertilized eggs were fixed for 60 minutes in an ice-cold mixture of 4% glutaraldehyde, 0.2 M NaCl, 0.35 M sucrose, 5 mM $CaCl_2$, 0 2 M sodium cacodylate pH 7.2 to which 0.05% OsO_4 was added for the first 10 minutes After several rinses in cacodylate buffer, the eggs were postfixed for 60 minutes at 4°C with 1% OsO_4 in 0 2 M sodium cacodylate to which 0 8% $K_3Fe(CN)_6$ was added. The eggs were rinsed in water, dehydrated with ethanol and embedded in Spurr's resin.

To obtain deep-etched rotary shadowed replicas of cortex preparations, cortices were made as described above on small pieces of polylysine-coated glass. They were then fixed immediately in 1% glutaraldehyde in CIM buffer for 10 minutes, washed in CIM buffer and then in 0.1 M sodium cacodylate buffer pH 7.4. They were exposed for 5 minutes to ice-cold 0 5% OsO_4 in sodium cacodylate buffer, washed in distilled water and subsequently in 15% methanol After removal of excess liquid, the cortices were quick-frozen in liquid propane cooled by liquid nitrogen. They were deep-etched for 1 hour at −95°C, rotary shadowed with platinum-carbon and finally coated with carbon in a Reichert Cryofract 190 apparatus The replicas were detached from the glass with hydrofluoric acid, bleach cleaned, rinsed in distilled water, and examined in a Hitachi H-600 at 80 or 100 kV Negatives were reversed before printing.

In some preparations, microtubules were stabilized by including a tannic acid step (0 2% in CIM buffer for 15 minutes) just after glutaraldehyde fixation. Microtubules were also identified in glutaraldehyde-fixed cortices by immunogold labelling with an anti-β-tubulin mouse antibody (Amersham) followed by incubation with 5 nm gold particles coated with anti-mouse antibody (Biocell).

Finally, actin microfilaments were identified in deep-etched rotary shadowed replicas by incubating cortices before fixation with the S1 fragment of myosin (a generous gift from Dr T Pollard, John Hopkins University, Baltimore) followed by the fixation and replication techniques described above

Stratification of egg components

A 5 ml suspension of dechorionated eggs was layered on a 5 ml cushion of 40% Ficoll 400 in SW in a 12 ml centrifuge tube. The eggs were spun in a clinical centrifuge at about 2000 *g* for 5 minutes, removed from the tube, rinsed with SW and immediately processed for cortex isolation as described above

Cytochalasin and nocodazole treatment

Eggs were incubated for 1 hour in cytochalasin B (Sigma 10 μg/ml) or nocodazole (Sigma, 4 μg/ml) in sea water. The eggs were simultaneously labelled with Hoechst 33342 as described above, and cortices were prepared as indicated previously

Detachment of ribosomes from the ER

Ribosomes were detached from the ER using an isotonic, low calcium KCl-puromycin medium as described by Sardet (1984), adapted from the method of Sabatini et al. (1966) that was used to detach ribosomes from isolated rough ER microsomes. As an alternative method, buffer X was used with EDTA substituted for EGTA.

PAGE and western blotting

PAGE and western blotting were performed essentially as described in Sardet et al. 1990. Homogenates of ascidian (*Phallusia mammilata*) or sea urchin (*Paracentrotus lividus*) eggs were dissolved in SDS buffer in the presence of a cocktail of protease inhibitors (PMSF, Leupeptin, Benzamidine), deposited on polyacrylamide gel and electrophoresed. Anti-sea urchin calsequestrin (Oberdorf et al., 1988) was detected on the blot by autoradiography after labelling with I^{125}-Protein A. Preimmune serum was used as a control.

Results

The polarity of the unfertilized ascidian egg

The unfertilized egg of the ascidian *Phallusia mammillata* is arrested at metaphase of the first meiotic division. The position of the meiotic spindle defines the animal pole (Fig. 1A).

Organelles in the (sub)cortical region of the egg display a distinct animal-vegetal polarity (Sawada, 1983, 1988; Gualtieri and Sardet, 1989). As in most other species of ascidians, this is most noticeable in the distribution of mitochondria that are present as a subcortical layer in the equatorial and vegetal hemisphere region of the egg (Reverberi, 1956; Sardet et al., 1989). This vegetal subcortical region has been named the myoplasm because it is found segregated in blastomeres that will give rise to muscle cells in the tail of the tadpole larva (see Whitaker, 1987; Jeffery and Bates, 1989 for reviews). The myoplasm of the *Phallusia mammillata* egg is readily detected in its subcortical vegetal location by a monoclonal antibody raised against isolated myoplasm of the *Ciona intestinalis* egg (Fig. 1B; see Nishikata et al., 1987). Underneath the myoplasm, a thin cortical layer is labelled with an antibody against a calsequestrin-like protein isolated from sea urchin egg microsomes (Oberdorf et al. 1988; Henson et al., 1989) indicating that this region is characterized by a higher density of ER (Fig. 1 D and see further).

Cytoskeletal elements are found to be specially associated with or excluded from the myoplasmic area. Immunolabelling of properly oriented sections with antibodies against a or β-tubulin reveals that microtubules are present throughout most of the egg, but are excluded from the cortical and subcortical myoplasmic region (Fig. 1A). On the other hand, labelling with antibodies against actin shows that the myoplasm is characterized by a greater density of actin than the rest of the egg cytoplasm (Fig. 1C). Actin microfilaments, which were visualized by staining with phalloidin-TRITC, are most abundant near the surface of the egg (Fig. 1E,F). Confocal laser scanning microscopy demonstrates that the phalloidin-TRITC labelling is in fact punctate (Fig. 1H) which probably reflects a high concentration of microfilaments in the short microvilli that cover the egg's surface. The distribution of F-actin is also polarized along the animal-vegetal axis of the unfertilized egg with actin filaments being generally more abundant in the vegetal cortical area (Fig. 1E,F). In addition, actin filaments are most dense in a small patch in the animal pole area overlying the meiotic spindle, and are depleted in the ring-shaped zone surrounding the animal pole (Fig. 1F,G,H). This is probably due to the local surface differentiation described as the polar pit in some species of ascidian eggs (see for example Dalcq and Vandebroek, 1937).

Organization of the ER network in the isolated cortex

The cortex of the unfertilized ascidian egg can be isolated by shearing eggs attached to a polylysine-coated surface. Using this method, large fields with regularly spaced patches of cortex can be obtained (Fig. 2A). These fragments of the egg surface can be examined at both the light and electron microscope level for the presence and distribution of cytoskeletal elements or organelles. Each patch of isolated cortex represents about 10-20% of the egg surface and possesses a "comet tail" of cytoplasmic material that is attached to the polylysine-coated surface following shearing of the eggs.

The most conspicuous feature of the isolated cortex is a network of ER that can be visualized using DIC or epifluorescence optics after labelling with the cyanine dyes $DiOC_6$ (3) or $DiIC_{16}$ (3) (Fig. 2B,C). This network assumes different configurations which vary from a sparse polygonal tubular network to a tightly knit network of tubes and sheets that forms an almost continuous layer closely apposed to the plasma membrane (Fig. 2D,E). The cortical ER network is continuous with strands and sheets of ER that extend throughout the cytoplasm and are broken when the cortex is isolated (Fig. 2D, see also Fig. 4D for a stereo view).

In fast-frozen, deep-etched rotary-shadowed replicas of the isolated cortex, large particles that have the size (about 20 nm when coated with platinum) and appearance of ribosomes are seen on the tubules and sheets of the ER network (Fig. 2D,F). Furthermore, the ER network can be labelled with the RNA dye thiazole orange (Fig. 2G). Treatment of cortices with an isotonic puromycin-KCl buffer which is known to detach the ribosomes from rough ER, results in the disappearance of the particles on the ER cisternae seen in replicas (not shown), and abolishes the staining of the ER network with Thiazole orange (Fig. 2G). These observations indicate that the ER network in the ascidian egg cortex consists mainly of sheets and tubes of rough ER. The ER sheets and tubes contain a protein that cross-reacts with antibodies against a calsequestrin-like protein isolated from sea urchin egg (Fig. 2H and insert showing the relevant portion of a western blot). This protein appears to be distributed homogeneously throughout the ER network in the ascidian egg cortex in a manner similar to that previously reported in the sea urchin egg cortex (Henson et al., 1989). The specificity of the labelling for the ER is best seen outside of cortices where strands of ER to which other organelles attach form a "comet tail" (see Fig. 2A,B,C). In these "comet tails" only ER strands are labelled amongst the many organelles thrown onto the glass coverslip when eggs are sheared to make cortices.

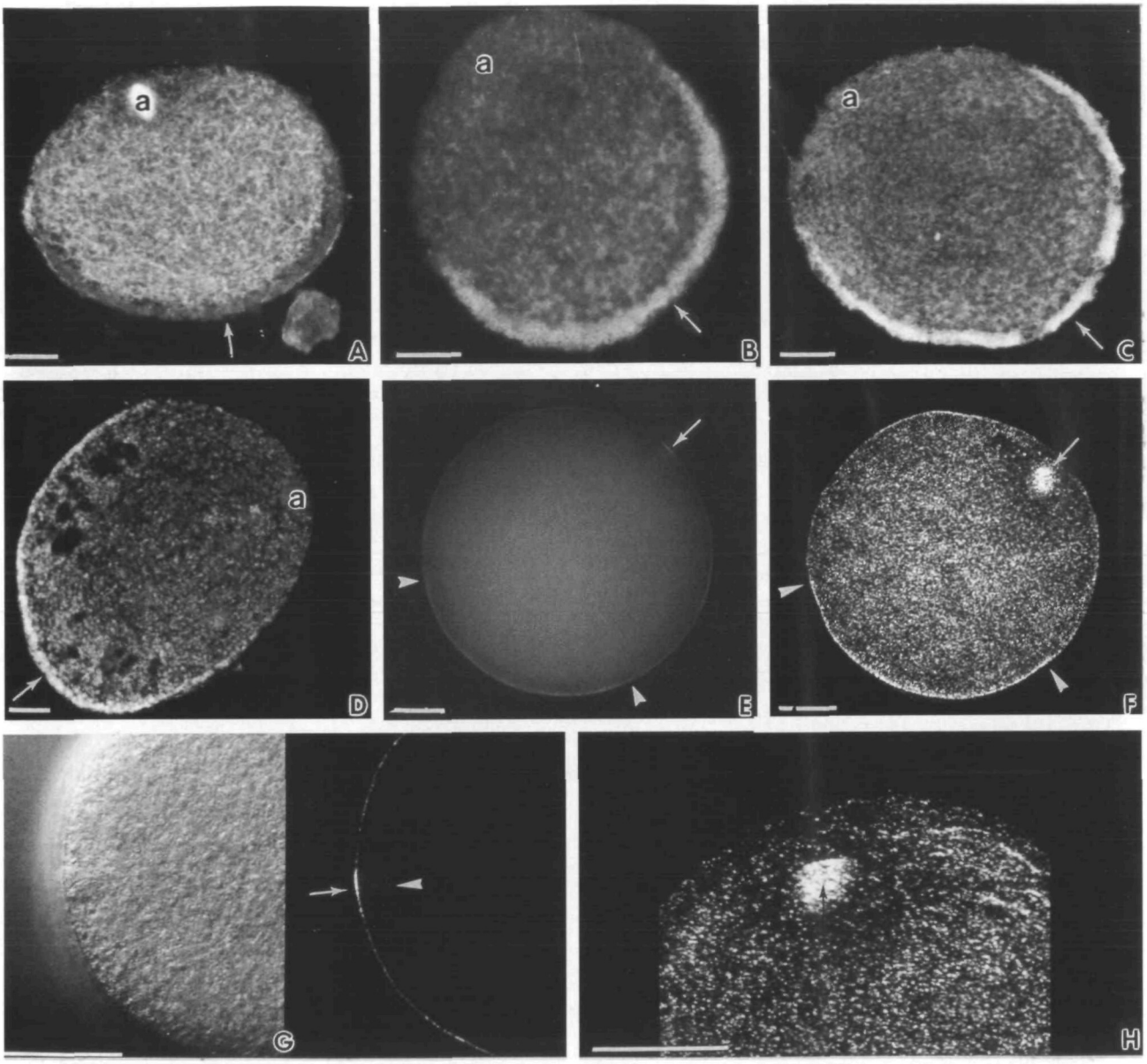


Fig. 1. Polarity of the ascidian egg (A) Anti-tubulin labelling of a thick (5 μm) section of an unfertilized egg. The position of the meiotic spindle defines the animal pole (a). Note the subcortical microtubule-poor region corresponding to the myoplasm in the vegetal hemisphere (arrow) Bar: 20 μm. (B) Anti-myoplasm antibody (IIG6B2 provided by T. Nishikata) labels the vegetal subcortical myoplasm (arrow) in a thick section. a, position of the animal pole. Bar: 20 μm. (C) Anti-actin labels the myoplasm region (arrow). a, position of the animal pole Bar: 20 μm. (D) Antibody against the calsequestrin-like protein from sea urchin eggs (provided by B Kaminer). Labelling of the thick section is most prominent in the thin cortical layer of the vegetal hemisphere (arrow), a, position of the animal pole. Bar. 20 μm. (E) Phalloidin-TRITC labelling of a whole egg. Cortical labelling is more intense above the meiotic spindle (white arrow) and slightly higher at the periphery in the vegetal cortical zone (between white arrowheads). Bar: 20 μm. (F) Confocal microscopy: projection image of a phalloidin-TRITC-labelled egg. The same regions as in E are indicated. Bar: 20 μm. (G) Confocal microscopy: pair images (DIC on the left, epifluorescence on the right) of a median section through a phalloidin-TRITC-labelled egg in the animal pole region, the surface above the meiotic apparatus has accumulated label (arrow) A very faint staining of the meiotic apparatus region is also observed (white arrowhead) Bar: 30 μm. (H) Confocal microscopy: in face view of the phalloidin-TRITC patch (arrow) and surrounding region showing the punctate nature of the labelling and the low density of punctate label surrounding the patch above the meiotic apparatus. Bar· 20 μm

Several observations suggest that the cortical ER network is solidly attached to the underlying plasma membrane. Their close proximity at particular sites can be visualized in stereo views of the rotary- shadowed replicas of deep-etched cortices (Fig. 3A: stereopair). Although the cortical ER is usually situated 0.25-0.50 μm away from the plasma membrane (see upper part of Fig. 3B or Fig. 2E) tight apposition of the ER and the plasma membrane can occasionally be observed in thin sections with dense regions about 0.05 μm apart at the

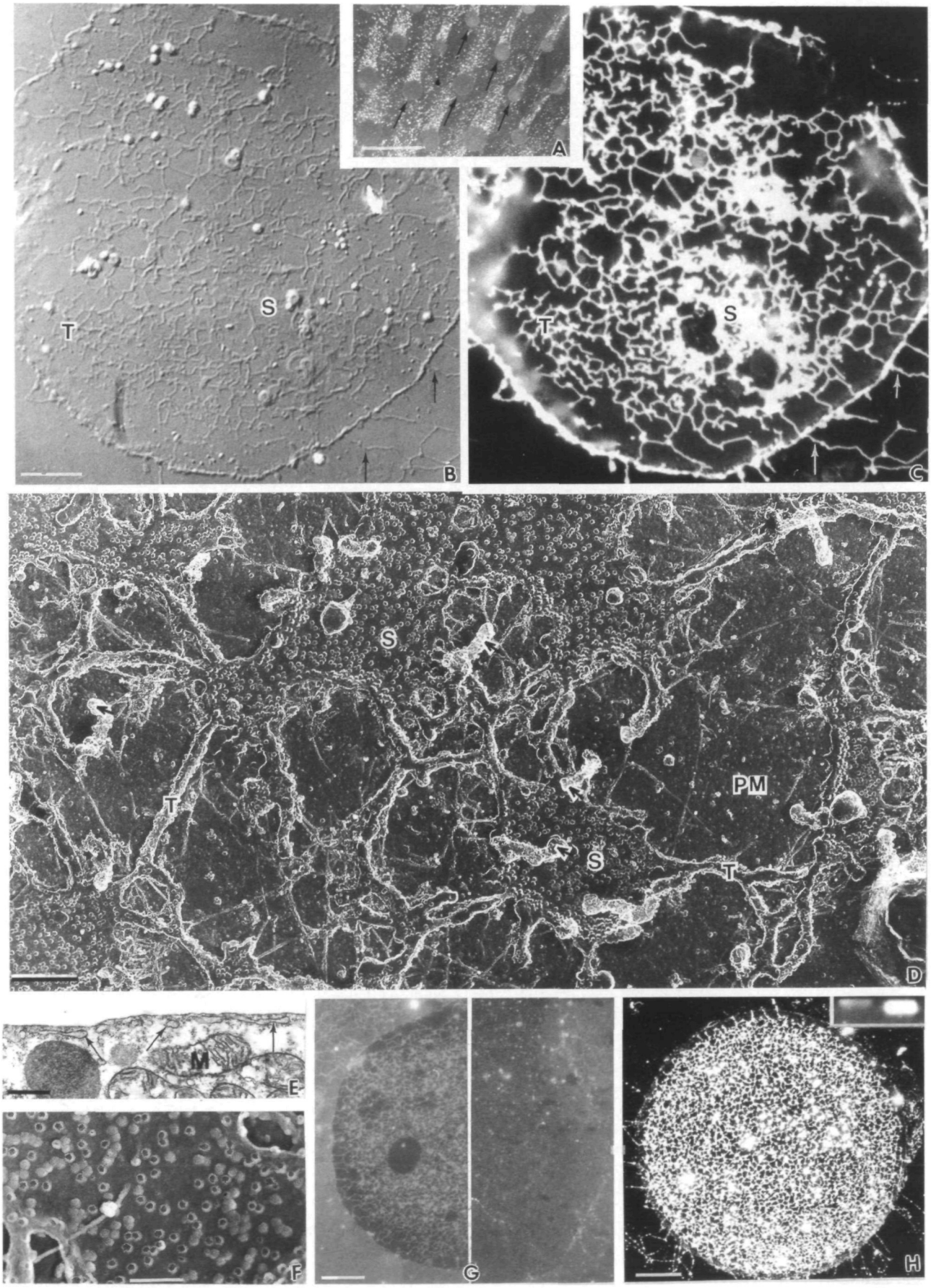


Fig. 2. For legend see p. 228

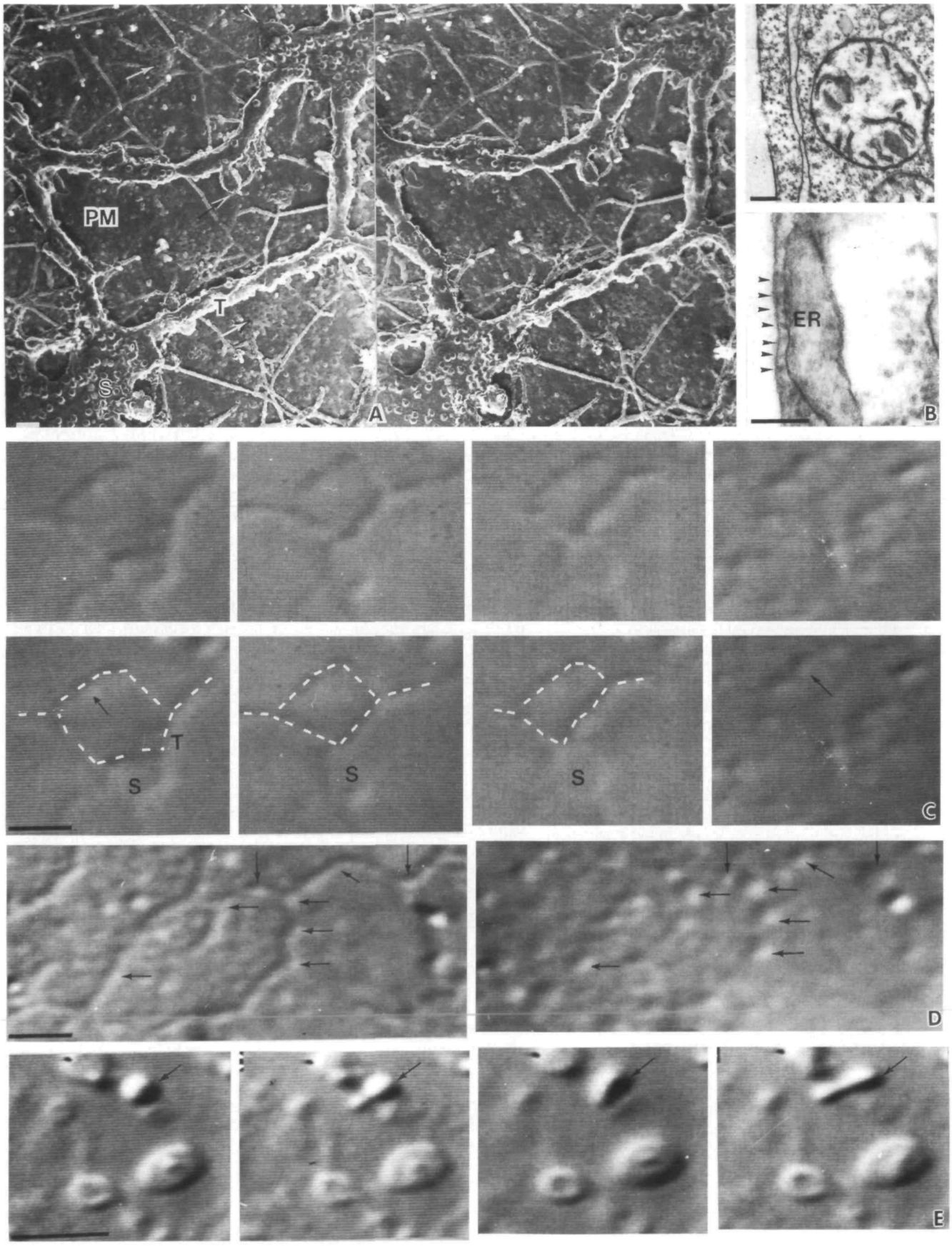


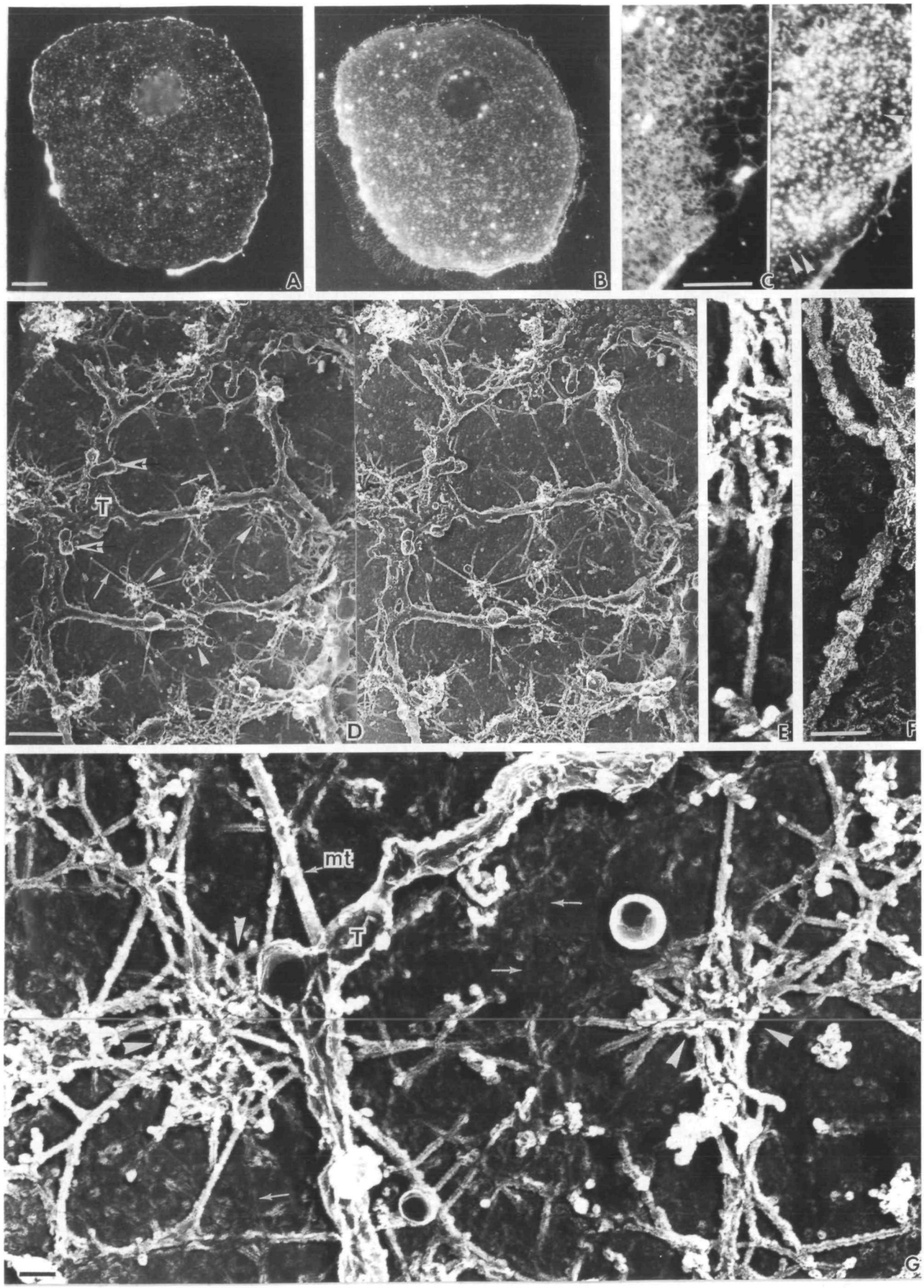
Fig. 3. For legend see p. 228

Fig. 2. The isolated cortex of the ascidian egg. (A) A field of cortices (arrows) isolated by shearing eggs attached onto a polylysine-coated coverslip. They are seen as circular patches with a "comet tail" of attached organelles (ER tubes and adhering yolk granules) in the direction of shearing (direction of arrows). Bar: 100 μm. (B, C) DIC (B) and corresponding epifluorescence (C) [$DiIC_{16}(3)$ staining] image of an isolated cortex showing the intricate network of tubes (T) and sheets (S) adhering to the plasma membrane Arrows indicate the continuity between the cortical ER and the deeper cytoplasmic ER forming the "comet tail" upon shearing. Bar: 20 μm. (D) Deep-etched rotary-shadowed replica of a cortical region showing tubes (T) and sheets of ER (S) overlying the plasma membrane (PM). The arrows show tubes of cytoplasmic ER that have been broken during the shearing process (see also the stereo pair in Fig. 4D). Bar: 0.5 μm. (E) Thin section of the vegetal cortical region of the egg showing sheets and tubes of ER beneath the surface (arrows), and mitochondria (M) that characterize the myoplasmic domain. Bar. 0.5 μm. (F) Deep-etched rotary-shadowed replica of a portion of a cortical ER sheet; note the presence of numerous ribosomal particles. These particules can be detached by KCl-puromycin treatment (not shown). Bar: 0.2 μm. (G) Thiazole orange staining of the cortical ER network (left). The staining disappears after cortices are extracted with a KCl-puromycin medium (right). Bar. 10 μm. (H) Anti-calsequestrin-like protein labelling of an isolated cortex (immunoflorescence). Bar: 10 μm The inset shows a portion of an autoradiogram of a western blot with the unique band obtained with *Phallusia mammillata* eggs (left) and sea urchin eggs (right) after SDS-PAGE, transfer and immunolabelling with anti-calsequestrin-like protein from sea urchin egg and I^{125}-Protein A. No band was detected when the corresponding preimmune serum was used.

Fig. 3. The cortical ER network. (A) Stereo pair: Deep-etched rotary-shadowed replica of a portion of sheet (S) and polygonal tubular network (T) in contact with the underlying plasma membrane (PM) at some sites (black arrows). Filaments and coated plaques (white arrows) are observed on the cytoplasmic face of the plasma membrane. Bar: 0.1 μm. (B) Thin section of the cortical region of the vegetal hemisphere. Upper part: ER sheets or tubes are usually not closely apposed to the plasma membrane (see also Fig 2E). Lower part: in rare zones of contact with the plasma membrane electron-dense "feet" are sometimes observed. Bar: 0.25 μm. (C) DIC images of the cortical ER taken from video records. The four images are screen photos obtained from a video sequence (unlabelled images on top, labelled images below). The three pictures on the left are taken 3 seconds apart from each other. The first three images show changes in shape of a small ER sheet (S) and the polygonal tubular ER above it (T, outlined by the white stippled line). The 4th image (on the right) indicates that immobile parts of the ER network are situated in a plane of focus close to that of the plasma membrane. The area indicated by the arrow probably represents one of the knobby protrusions shown in the stereo pair (Fig 3A black arrows). Bar: 0.5 μm. (D) The cortical ER network can be ruptured by osmotic shock (perfusion of buffer X diluted 1.3 with H_2O). After perfusion, small vesicular remnants of the broken tubular ER generally appear at the branching points of the network (arrows). Taken from a video sequence before (left image) and after (right image) perfusion of the hypo-osmotic cortex medium. Bar: 0.5 μm (E) An osmotically shocked vesicles (arrow) attached to the plasma membrane and produced as described above (see Fig. 3D) is perfused again with an iso-osmotic cortex medium (buffer X). The vesicle generated by osmotic shock changes into a tubular ER section that remains attached to the plasma membrane The four images are taken 4 seconds apart from a video sequence. Bar: 0.25 μm

site of contact (see the "feet" indicated in the lower part of Fig. 3B). The dynamic properties of the cortical ER network are particularly apparent when high resolution light video microscopy is used. The ER tubules that form the polygonal network undergo typical changes in shape (Fig. 3C) that indicate that they are not affixed to the plasma membrane along their whole length, but rather adhere to it at particular sites (usually the junction points of the polygonal network). While the tubular parts move, anchoring sites that can be observed by focusing at the level of the plasma membrane, remain in a fixed position (Fig. 3C last image on the right). Furthermore, perfusion of the isolated cortex with a hypotonic buffer results in a breakdown of the ER network into vesicles that still remain attached to the plasma membrane (Fig. 3D). Upon subsequent perfusion with an isotonic buffer, reformation of short tubules can be observed from such attached vesicles (see the images from a video sequence in Fig. 3E). Finally, there is another observation that argues for a solid association between the cortical ER network and the plasma membrane: centrifugal forces that are sufficient to redistribute the cytoplasmic contents of the egg do not seem to perturb the cortical ER network. As illustrated in a later section (see Fig. 7E,F) cortices isolated from stratified eggs display a polarized ER network that cannot be distinguished from that of non-stratified eggs.

Fig. 4. Microfilaments in the isolated cortex. (A) Low magnification view of a fixed cortex labelled with phalloidin-TRITC A fine punctate pattern is present all over this large piece of cortex. Bar: 10 μm (B) Same cortex as in A; the ER network is labelled with $DiOC_6(3)$ and appears as sheets and tubes (see Figs 4, 2 and 7 for higher magnifications). (C) Higher magnification views of an unfixed cortex. The ER network is stained with $DiOC_6(3)$ (left) and the corresponding area (right) is labelled with phalloidin-TRITC (white arrowheads: punctate staining). Bar: 5 μm. (D) Stereo pair (Deep-etched rotary-shadowed replica). Actin microfilaments (white arrows) course along the plasma membrane and converge into focal aggregates (white arrowheads, see also Figs 4G and 5D). Note the deeper cytoplasmic ER tubes that were broken during shearing (black and white arrows). Bar: 0 5 μm. (E, F) Higher magnification view of actin microfilaments (E). In F, microfilaments are labelled with myosin heads. Bar: 0 1 μm. (G) Focal aggregates of microfilament (white arrowheads). Note also microtubules (mt), ER tubes (T) and smaller filaments on the cytoplasmic face of the plasma membrane (white arrows). Bar: 0.1 μm.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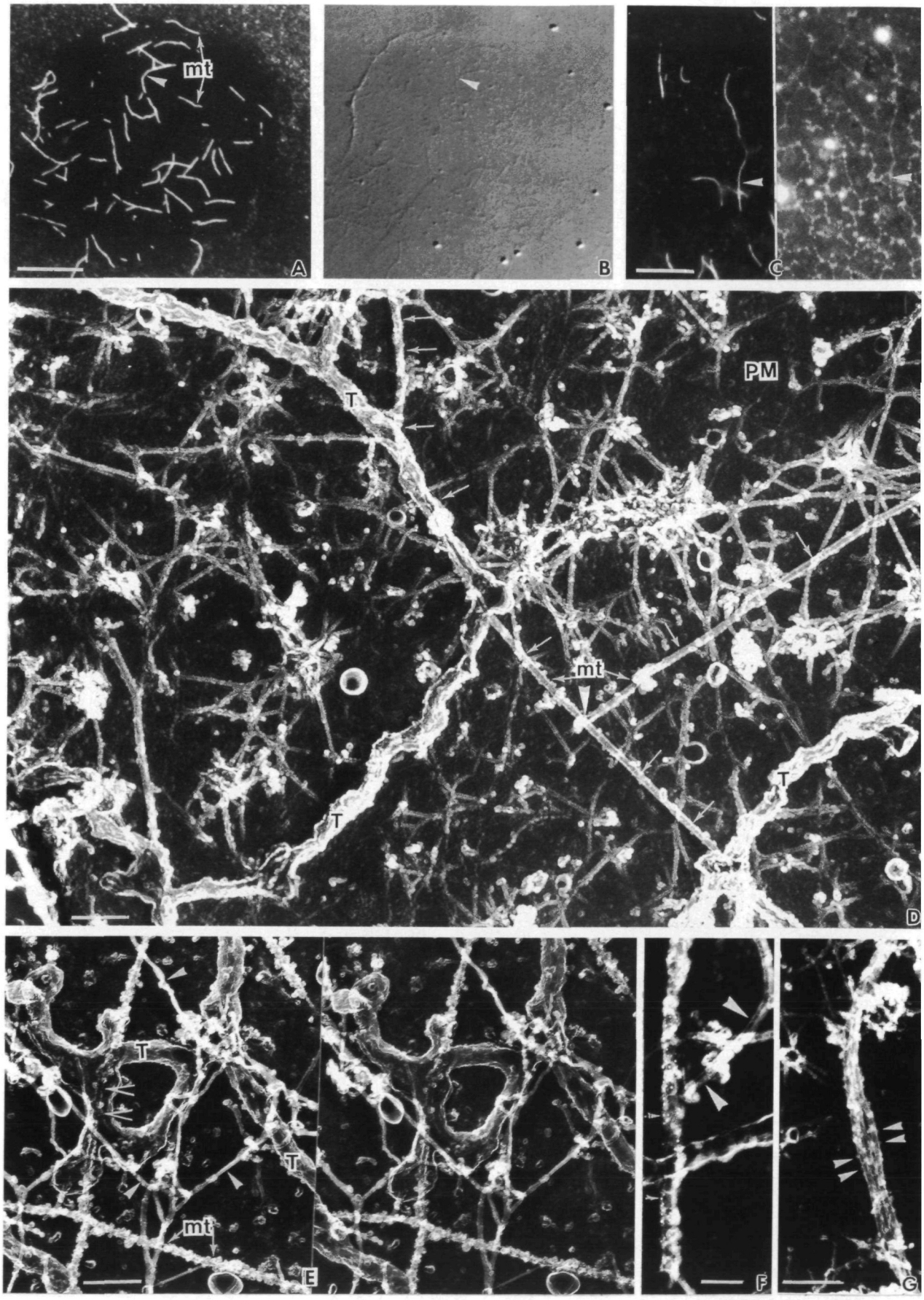
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Microfilaments, microtubules and other cytoskeletal constituents of the isolated cortex

Filamentous actin is detectable in a punctate pattern and occasionally as thin filaments in the isolated cortex using phalloidin-TRITC (Fig. 4A,B) and anti-actin antibodies (not shown) as specific probes. In replicas of fast-frozen, deep-etched and rotary-shadowed cortices, actin filaments are abundant (Fig. 4D, G). Their identity is indicated by their size (about 10 nm when coated with platinum) and their capacity to bind the S1-fragment of myosin (Fig. 4E-G). Microfilaments interact with the ER network, the plasma membrane and coated plaques (observe stereo pairs in Figs 4D and 3A), and they often form focal aggregates that most likely correspond to the punctate labelling pattern found after phalloidin-TRITC labelling of cortices or whole eggs (see Fig. 1F,H). This finding agrees with what has been previously found in the cortices of unfertilized sea urchin eggs (Sardet, 1984; Henson and Begg, 1988) where actin represents a major component (reviewed by Vacquier 1981; see also Spudich et al., 1988 and Bonder et al., 1989).

Microtubules are less numerous than microfilaments and often course along parts of the tubular ER network (Fig. 5E: stereopair) and the plasma membrane (Fig. 5D). The average length of these microtubules is 6-8 μm. The microtubules are studded with small globular particles along their length, and sometimes appear as bundles (Fig. 5D,G). These cortical microtubules are remarkably stable since they remain present in live cortices for up to an hour after incubation with an isotonic low calcium medium (buffer X) in the absence of any free tubulin. In fact it is possible to observe these microtubules in situ in unfixed cortices by decorating them with gold particles coated with an anti- tubulin antibody (not shown). We have also observed that microtubules are absent from cortices made from eggs treated with 4 μg/ml nocodazole, indicating that the cortical microtubules do not belong to a stable class of microtubules that resist drug treatment.

In addition to microtubules, we occasionally encounter thinner filaments (about 15 nm when coated with platinum). These filaments do not label with antitubulin antibodies, they bend like ropes and appear to be made up of several strands (Fig. 5E, F). Beside these rope-like filaments, very thin filaments are observed at the level of the cytoplasmic face of the plasma membrane in some of the better replicas (Figs 4G, 5D). We have not yet made any attempts to characterize these filaments but the size and appearance of the larger rope-like filaments suggest that they may correspond to intermediate filaments which have been demonstrated in the subcortical and cortical area in eggs of other species of ascidian (Swalla et al., 1991) as well as in amphibian and sea urchin eggs (Klymkovsky and Maynel, 1989; Boyle and Ernst, 1989).

Polarity of the cortex

The isolated cortex shows regions of tubes and regions of sheets (or tightly knit tubes) that form well-delineated domains that sometimes display odd shapes (see Fig. 2). By using a technique that allowed us to determine the origin of the pieces of cortex with respect to the animal pole of the egg, we found that regions of tightly knit tubes and sheets are only located in the vegetal hemisphere, whereas the extensive areas made up of sparse tubes are characteristic of the animal pole area (Fig. 6 and Table 1). These observations were confirmed on many cortices prepared from 6 different batches of eggs and examined by independent observers as described in the legend of Table 1. The quantitative results obtained from such experiments were strengthened by repeated observations of eggs in thin section electron microscopy that revealed sheets of ER located just beneath the plasma membrane. These were found only in regions with a dense concentration of subcortical mitochondria, the major component of the myoplasm mostly located in the vegetal hemisphere of the

Fig. 5. Microtubules in the isolated cortex (A, B) Anti-tubulin immunolabelling (A· epifluorescence) of microtubules (mt) in the isolated cortex Some profiles corresponding to microtubules (white arrowhead) can be seen in the corresponding DIC image in (B). Bar: 10 μm. (C) At higher magnification partial coincidence is observed (arrowheads) between some microtubules (on the left) and part of the ER network (on the right) as seen after labelling with $DiOC_6(3)$. Bar: 10 μm. (D) Deep-etched rotary-shadowed replica of the cytoplasmic face of the plasma membrane (PM). The sparse tubular ER network (T) is often followed along its length by microtubules (mt, and white arrows). Note the end interaction of one microtubule with another (arrowhead). Bar: 0.3 μm. (E) Stereo pair: A cortex area with ER tubules (T). Microtubules (mt) are identified by 5 nm gold particles along their length. Sparser labelling is observed in areas of contact with the ER tubes (black and white arrowheads) Note that other rope-like filaments (white arrowheads) are present. Bar: 0.3 μm. (F) Higher magnification view of microtubules decorated with 5 nm gold particles (white arrows). White arrowheads show a non-decorated rope-like filament Bar: 0.1 μm. (G) Bundling of microtubules studded with particles (white arrowheads) along their length. Bar: 0.3 μm.

Table 1. *Polarity of ascidian egg cortices*

	Control		Cytochalasin B treated	
Eggs	Animal	Vegetal	Animal	Vegetal
Number of cortices examined	28	22	4	11
with sparse tubes	28 (100%)		4 (100%)	5 (55%)
with tightly knit tubes or sheets		22 (100%)		6 (45%)

Living, Hoechst-stained eggs were attached to a polylysine-coated coverslip, and the location of the female chromosomes which mark the animal pole was recorded on an image processor as shown in Fig 6 The eggs were then sheared to produce cortices that were fixed and labelled They were subsequently scored by the first observer for the presence of either sparse tubes or tightly knit tubes and sheets. The second observer then independently determined the origin of the cortices on the basis of the recorded video images of the Hoechst stained eggs

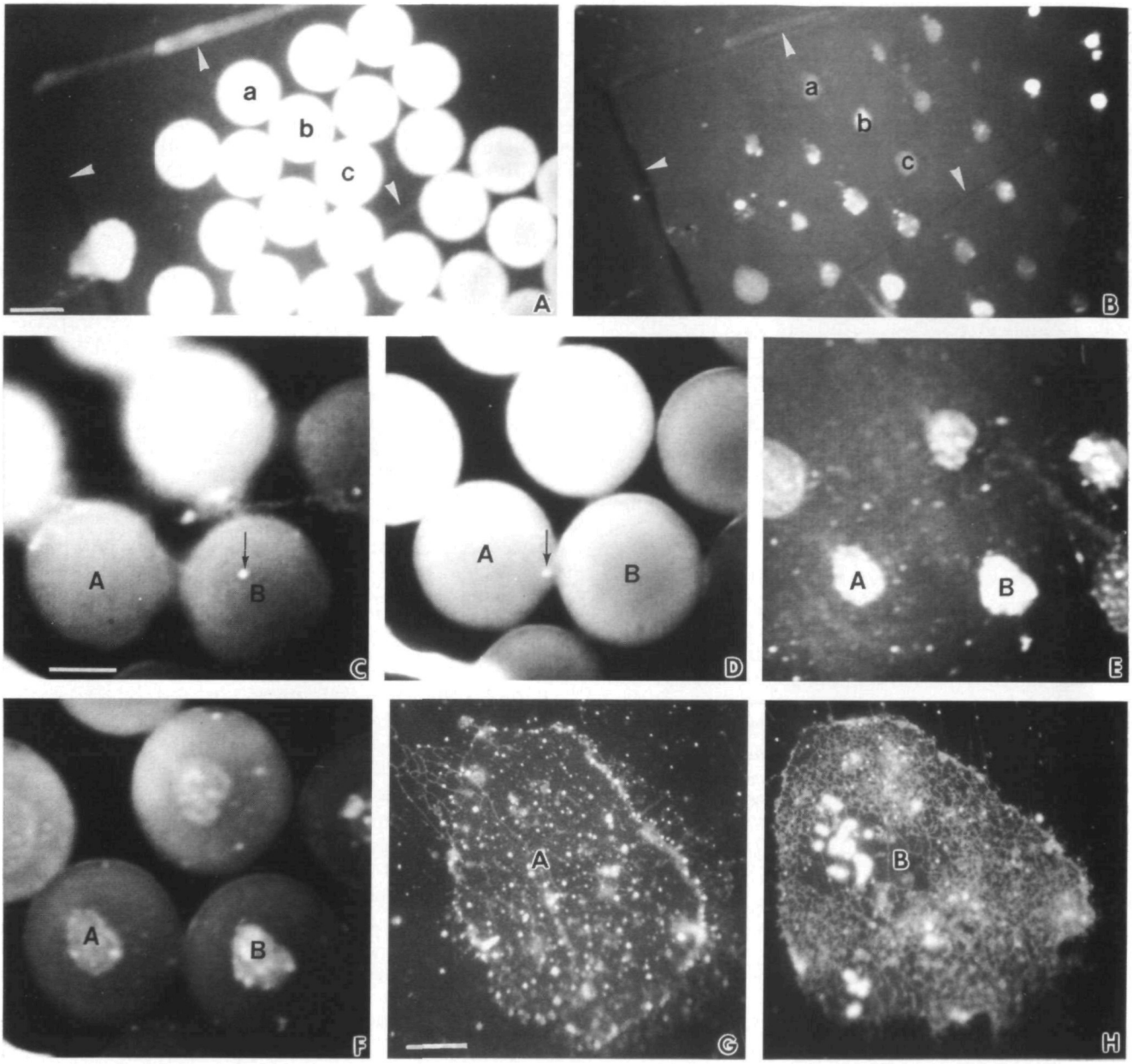

Fig. 6. Polarity of the isolated egg cortex. (A, B) The position of living eggs (left image) attached to a polylysine-coated glass coverslip are recorded with respect to scratch marks made on the glass (white arrowheads) After shearing (right image), the corresponding fixed cortices can be found again after labelling with $DiOC_6(3)$. The cortices are then repositioned and matched with the eggs from which they originated using the scratch marks (see for example the eggs a, b, c and corresponding cortices). Bar: 100 μm. (C-E) The position of the chromosomes in two eggs (A and B) stained with Hoechst 33342 at the animal pole (black arrow) are recorded by focusing near the glass surface (image D) or away from it (image C). The orientation of the animal-vegetal axis of the egg is thus determined and related to the corresponding piece of cortex attached to the glass surface (seen in image E). Bar: 50 μm (F) Super imposition of image D and E; the origin of the piece of cortex can then be determined. (G, H) Micrographs of cortices corresponding to eggs A and B A is a piece of cortex derived from the animal hemisphere. It displays vesicles and a low density of tubes. B is a piece of cortex originating from the vegetal pole region It contains a high density of tightly knit tubes. Bar: 10 μm.

egg (Figs 2E, 3B, see also Gualtieri and Sardet, 1989). Thick sections of the eggs labelled with antibodies against the anti-calsequestrin-like protein from sea urchin eggs, which serves as a marker of the ER, also indicate that a high density of ER is present in the cortical region of the vegetal hemisphere (Fig. 1D).

We repeatedly observed that microfilaments and microtubules are distributed unevenly in the isolated cortex with respect to the polarized ER network. In the vegetal areas rich in ER sheets, microtubules are rare whereas microfilaments are numerous (Fig. 7A-D). The opposite is found in areas where only sparse ER tubes are present; i.e. the density of stable microtubules is the highest in the animal hemisphere regions. These

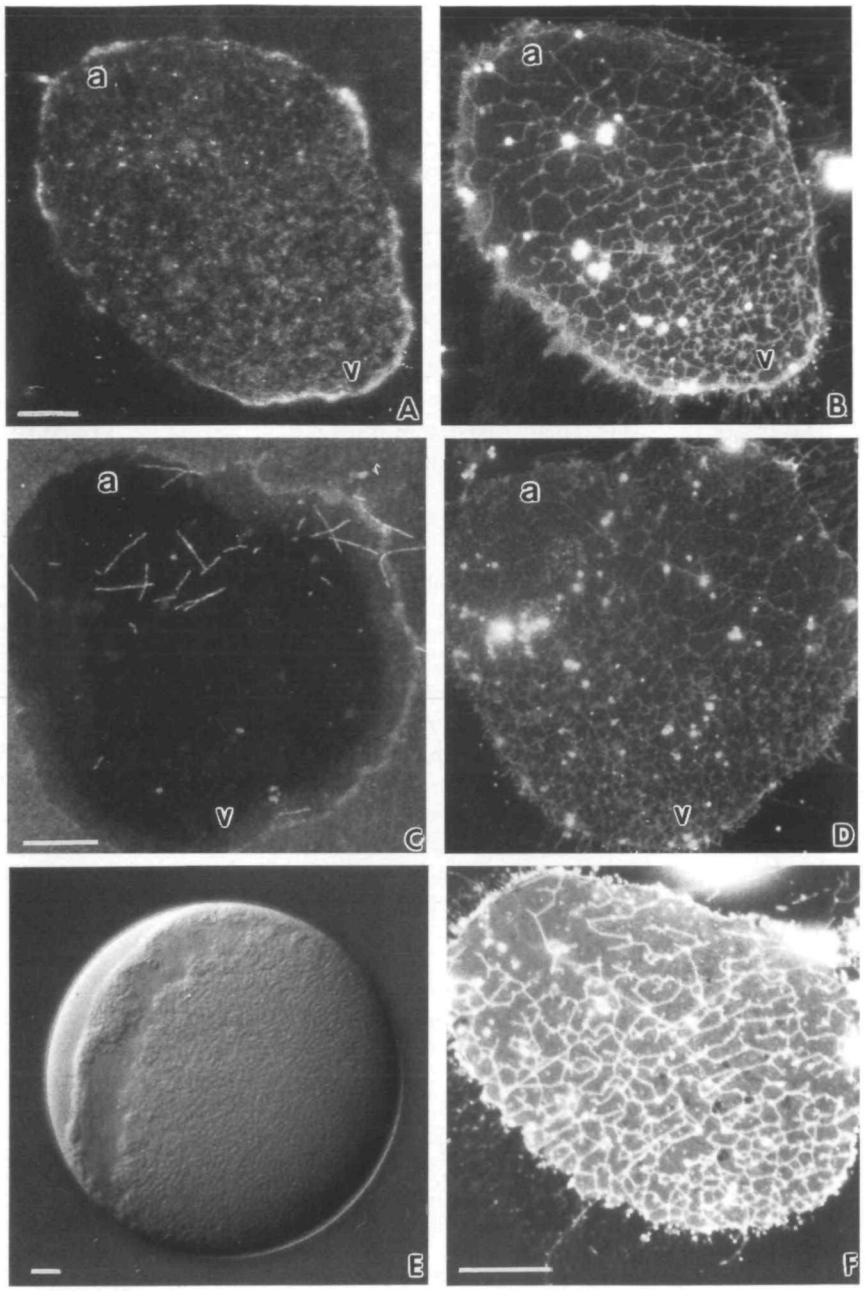

Fig. 7. Polarity and stability of the cortex. (A, B) Isolated cortex (corresponding to an equatorial region of the egg) labelled with phalloidin-TRITC to visualize microfilaments (A) and $DiOC_6(3)$ to observe the ER network (B). The orientation of the animal vegetal axis is indicated (a, v). Bar: 10 μm. (C, D) Isolated cortex (equatorial region) labelled for microtubules (C) and the ER network (D). Microtubules are only present in the area with the sparse ER network (animal hemisphere). (E) Ascidian egg with its organelles stratified by centrifugation. (F) A cortex obtained from such eggs. The ER network is labelled with $DiOC_6(3)$. Bar: 10 μm.

findings reflect what is visible in sections of eggs labelled with antibodies against tubulin and actin (Fig. 1A, C). Therefore, before fertilization, the cortex of the ascidian egg is clearly polarized with respect to its membranous and cytoskeletal elements.

The stability of the isolated cortex

The appearance of the cortical ER network is preserved through displacement of the cytoplasmic organelles by centrifugation of the egg (Fig. 7E,F). In contrast, incubation of the eggs in micromolar concentrations of cytochalasin B for periods that are sufficient to affect the manifestation of the egg's functional polarity (Speksnijder et al., 1989b, 1990b) disrupt the clear separation of sheets and tubes into distinct areas. This effect of cytochalasin can be best characterized as a disruption of the high density zone of sheets and tubes in the vegetal pole area (Table 1). In contrast, cortices isolated from eggs treated with nocodazole still showed clearly delineated areas of sparse tubes or tightly knit tubes or sheets indistinguishable from controls (not shown). This suggests that, although microtubules are disrupted by nocodazole treatment of the egg, the cortical ER network remains in place probably via its adhesion to the plasma membrane.

Discussion

Polarity of cytoskeletal elements in the unfertilized ascidian egg and its cortex

The remarkable polarization of the unfertilized ascidian

egg was first detailed by Conklin in an epoch-making contribution (Conklin, 1905). Since then, the polarized distribution of mitochondria, vesicular organelles, specific proteins and mRNAs have been documented. Most of these differences reflect the presence of a subcortical, mitochondria-rich layer in the equatorial and vegetal regions of the egg inherited by precursor muscle cells, which is called the myoplasmic domain. (Reverberi, 1956; Sawada, 1983, 1988; Jeffery and Meier, 1983, 1984; Jeffery et al., 1984, 1990; Nishikata et al., 1987; Gualtieri and Sardet, 1989; Swalla et al., 1991). This domain as well as adjacent regions are rearranged after fertilization as a consequence of an early microfilament-mediated cortical contraction toward the future posterior pole area (Sawada and Osanai, 1985). Later the bulk of the myoplasm moves toward the future posterior pole of the embryo as a result of the growth of a microtubular aster in the male pronuclear region (Sawada and Schatten, 1988, 1989). There is however very little information on the distribution of microfilaments and microtubules in the unfertilized egg (except for the abstract by Sawada, 1986).

Our observations clearly show for the first time that, in the unfertilized egg, the myoplasmic domain is different from the rest of the cytoplasm with respect to microtubules and actin microfilaments and these differences extend to the cortical layer between the myoplasmic region and the plasma membrane. Microtubules are excluded from the myoplasm and from the corresponding vegetal cortex area, but in the animal hemisphere region they reach all the way to the plasma membrane and course along its internal surface. The opposite is true of microfilaments, although in this case, the difference between animal and vegetal hemisphere is more subtle and suggests a gradient-like distribution (see Fig. 7).

Since, in several other species of ascidians, intermediate filaments have been shown to be concentrated in the myoplasmic region (Swalla et al., 1991), it appears that already before fertilization the myoplasm has a different composition from the rest of the egg cytoplasm with respect to all the major cytoskeletal elements.

The cortical ER network and its function

The presence of abundant ER vesicles and tubules in the cortex was first noted in amphibian eggs (Campanella and Andreucetti, 1977; Gardiner and Grey, 1983; Campanella et al., 1984; Charbonneau and Grey, 1984). The latter authors suggested that junctions between the ER and the plasma membrane that resemble diads of muscle cells may play a role in calcium release at fertilization. The presence of abundant ER has since then been demonstrated in the egg cortex of several species (Sardet, 1984; Sardet and Chang, 1985; Luttmer and Longo, 1985; Speknijder et al., 1989c). In addition, we have shown that the cortex of the sea urchin egg possesses a continuous polygonal network of ER studded with ribosomes (Sardet, 1984) and it is now known that this network extends throughout the egg (Terasaki and Jaffe, 1991). Physiological experiments have suggested that the ER cortical network is able to pump and release calcium (Oberdorf et al., 1986; Payan et al., 1986; Terasaki and Sardet, 1991). In addition, the presence of a calsequestrin-like protein, the high concentration of calcium measured within the ER of ascidian and sea urchin eggs and experiments with calcium dyes (Terasaki and Sardet, 1991) support the notion that the ER network plays a major role in the regulation of the free cytosolic calcium concentration (Oberdorf et al., 1988; Henson et al., 1989; Gualtieri and Sardet, 1989; Terasaki and Sardet, 1991). Thus, there exists just beneath the plasma membrane a network of tubes and sheets of rough ER tethered to the plasma membrane at particular attachment sites that is capable of calcium pumping and release. Although this description of cortical egg ER is reminiscent of the muscle cell sarcoplasmic reticulum (SR) there are significant differences between the two. The cortical ER of the egg is studded with ribosomes, and unlike muscle SR where calsequestrin is concentrated in terminal cisternae, the calsequestrin-like protein of ascidian and sea urchin eggs appears homogeneously distributed throughout the entire cortical and cytoplasmic ER network. A major point that needs clarification is the presence in the cortex of plasma membrane-ER junctions that resemble, in structure and function the terminal cisternae-membrane junctions in muscle cells (Gardiner and Grey, 1983). We now know that in muscle cells, specific molecules (ryanodine receptors) constitute the feet that characterize the SR-plasma membrane junctions. More importantly, ryanodine receptors form the calcium channels that are probably responsible for the localized calcium- induced calcium release during muscle contraction (reviewed in Fleisher and Inui, 1989). Early experiments (reviewed in Jaffe, 1983) and recent observations using inhibitors (Whitaker and Crossley, 1990) suggest that such a mechanism may be at work in eggs and that the cortical ER network may be the primary site of such calcium release. It will now be essential to characterize the molecules involved and determine whether in eggs as in muscle cells some calcium is released at specific sites (such as for example ER-plasma membrane junctions).

The polarity of the egg's cortical ER network

Undoubtedly the difference in the configuration of the ER network between the animal and vegetal hemisphere of the ascidian egg is the most interesting finding reported here. At present, it is not clear how such a polarity may arise. The recently discovered close relationship between the ER network and microtubules may provide the necessary mechanisms (Terasaki et al., 1986; Dabora and Sheetz, 1988; Lee et al., 1989). On the other hand, interactions with the plasma membrane, to which the ER network is anchored may also provide adequate spatial cues. The presence of a polarized cortical ER network along the animal-vegetal axis of the egg could of course have important functional implications. We have previously demon-

strated that there is a preferential entry of sperm in the animal hemisphere region of the egg of the ascidian *Phallusia mamillata* (Speksnijder et al., 1989b). Such polar sperm entry may be linked to the differential stabilization of the sperm-egg fusion step in different regions of the egg, since in sea urchins reversible fusion is known to be possible (McCullough and Chambers, 1986). In this regard, the density of cortical ER or of its junction sites with the plasma membrane could set up the proper local conditions to stabilize or destabilize fusion; for example, by controlling the concentration of calcium within the immediate proximity of the plasma membrane. This idea is not entirely new: in the egg of the amphibian *Xenopus* a difference in the number of ER-plasma membrane junctions between the animal and vegetal hemispheres has been reported and assumed to be linked to the preferential entry of sperm at the animal pole region via their putative role in calcium regulation (Charbonneau and Grey, 1984). We proposed that, in ascidians egg, the presence of large areas of tighly knit tubes or sheets of ER positioned behind the membrane in the vegetal pole area and which contain up to 30 mM calcium (Gualtieri and Sardet, 1989) may provide conditions that suppress sperm entry. In that respect our observations that cytochalasin B disrupts the ER network and disperses the ER sheets and tubes in the vegetal area is important, since we have shown previously that treatment of the ascidian egg with this drug randomized sperm entry (Speksnijder et al., 1989b).

Thus, we suggest that the polar distribution of the egg cortical ER could establish the conditions necessary for the maintenance of stable gradients in the region situated just beneath the plasma membrane. This may take the form of a local gradient of calcium concentration or be linked to the involvement of the ER in the synthesis and processing of particular proteins since numerous ribosomes (and presumably attached messages) are present on its surface.

It has been known for years that stratification of egg's organelles by low-speed centrifugation does not perturb normal development (for ascidians see Jeffery and Meier, 1984; Sawada and Osanai, 1984). Our present observations, in conjunction with others to be reported (Speksnijder, in preparation) show that the cortex maintains the polar organization of its ER network through centrifugation. It therefore seems that the cortex is endowed with a stable structural and functional polarity that could provide essential spatial coordinates for normal development as suggested by classical embryologists.

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