

The effects of phorbol ester on mouse blastomeres: a role for protein kinase C in compaction?

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Summary

The effects of phorbol myristate acetate (PMA) and other activators of protein kinase C on the cytoskeletal organization of mouse oocytes and early embryos have been examined. The effects observed depended on the developmental stage on exposure to PMA. PMA had little effect on the cytoskeletal or microvillous organization of unfertilized oocytes. Interphase cells from embryos prior to compaction showed limited disruption and loss of microvilli when exposed to PMA and foci of polymerized actin remained visible in the cytocortex of embryos up to the early 8-cell stage. When compacted late 8-cell embryos were exposed to PMA, most microvilli were lost and little polymerized actin remained in the cytocortex. PMA also caused loss of microtubules from compact 8-cell embryos under some experimental conditions. Intercellular flattening was both prevented

and reversed. The relevance of these observations to the rearrangement of cell–cell contacts and cytoskeletal organization seen during compaction at the 8-cell stage is discussed and a possible role for protein kinase C in the generation of cell polarity proposed.

Abbreviations: conA, concanavalin A; dansyl PMA, 11-((-5-dimethylaminonaphthalene-1-sulphonyl) amino) undecanoylphorbol acetate; mPMA, phorbol 12-myristate 13-acetate 4-O-methyl ether; OAG, oleoyl acetyl glycerol; 4 α PDD, 4 α -phorbol didecanoate; 4 β PDD, 4 β -phorbol didecanoate; PIP₂, phosphatidylinositol bisphosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

Key words: compaction, cytoskeleton, mouse embryo, phorbol ester, polarization, protein kinase C.

Introduction

In the development of the mouse embryo, the first morphological transition occurs at compaction, a major rearrangement of cell structure and intercellular interactions during the 8-cell stage. Initially rounded blastomeres increase the extent of their apposition and flatten on each other (Ducibella & Anderson, 1975; Lehtonen, 1980) in a process mediated by the Ca²⁺-dependent cell–cell adhesion molecule uvomorulin (reviewed by Kemler *et al.* 1988). Accompanying these changes in surface properties, each blastomere becomes polarized along a radial axis determined by the positions of cell–cell contacts (Ziomek & Johnson, 1980; Johnson & Ziomek, 1981a). Polarized distributions of microvilli (Ducibella & Anderson, 1975; Lehtonen & Badley, 1980; Handyside, 1980; Reeve & Ziomek, 1981), cytoplasmic microfilaments (Johnson & Maro, 1984), microtubules and their organizing centres (Houliston *et al.* 1987), clathrin vesicles (Maro *et al.* 1985), endosomes (Reeve, 1981; Fleming & Pickering, 1985) and specialized junctions (Ducibella & Anderson, 1975; Magnuson *et al.* 1977) have been described. This asymmetry in 8-cell blastomeres is important for the subsequent gener-

ation of two cell types in the morula, which give rise to the trophoblast and inner cell mass of the blastocyst, respectively (Johnson & Ziomek, 1981b, 1982; Balakier & Pedersen, 1982; Fleming *et al.* 1984; Pedersen *et al.* 1986; Johnson *et al.* 1986a, 1988; Fleming & Johnson, 1988).

The mechanisms controlling cell flattening and polarization are not understood fully. It seems likely that polarity develops first in the cytocortex (reviewed by Johnson & Maro, 1986). Thus, although a polar organization is first apparent in the cytoplasm, poles can develop at the cell surface under conditions in which cytoplasmic polarization is not evident and the cytoskeleton is disrupted severely (Maro & Pickering, 1984; Johnson & Maro, 1985; Fleming *et al.* 1986a,b; Houliston *et al.* 1987, 1989). During mitosis, all evidence of cytoplasmic polarity is lost while surface poles remain (Johnson & Maro, 1985), and a cytocortical 'memory' of pole position can remain even after extreme artificial prolongation of mitosis (Johnson *et al.* 1988). The underlying mechanisms that generate and maintain blastomere polarization must therefore be largely independent of cytoplasmic organization. These mechanisms are also entirely post-translational, since inhibition

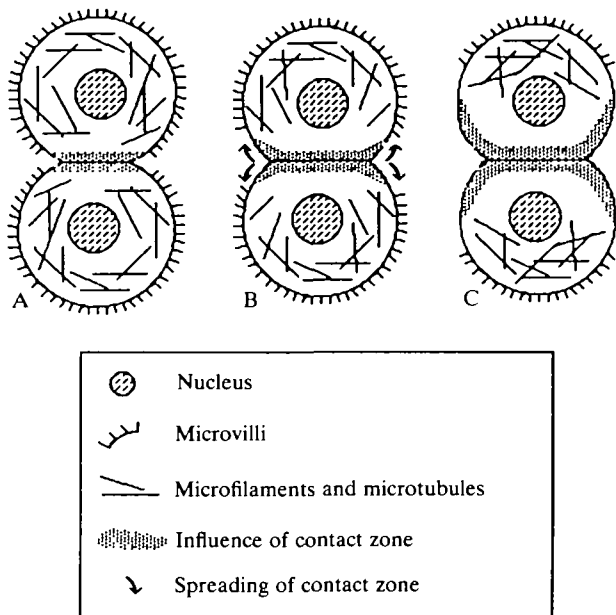


Fig. 1. Schematic illustration of the progression of cell flattening and polarization. (A) Two blastomeres from a 4-cell embryo showing localized depletion of cytoskeletal elements from the cytoplasm adjacent to cell-cell contact. Flattening of the cells onto each other is also seen at the contact region. (B) During the 8-cell stage, both cell flattening and cytoskeletal depletion radiate from the region of cell apposition. (C) A polarized and fully flattened pair of cells results.

of both RNA and protein synthesis is compatible with flattening and polarization (Kidder & McLachlin, 1985; Levy *et al.* 1986).

Cell flattening and polarization proceed together. During the 2-cell and 4-cell stages, blastomeres show only very localized flattening and depletion of cytoskeletal elements and organelles from the subcortical cytoplasm adjacent to points of contact between cells (Sobel, 1983; Johnson & Maro, 1984; Maro *et al.* 1985; Houlston *et al.* 1987). At the 8-cell stage, both flattening and subcortical depletion spread from these focal contact points to involve more of the cell surface and cytocortex, resulting in a flattened cell with an apical pole of cytoskeletal elements and organelles which becomes stabilized (Fig. 1). Postulated mechanisms underlying this transition include the flow of positive ions through the cell from apex to base (Nuccitelli & Wiley, 1985) and the propagation of a change such as a post-translational modification to cytoskeletal organizing proteins in the plane of the cytocortex itself (Johnson & Maro, 1986; Fleming & Johnson, 1988). Intracellular second messengers are obvious possible mediators of such a propagated change.

In this paper, the effect on mouse blastomeres of phorbol myristate acetate (PMA), a potent stimulator of the membrane-associated, Ca^{2+} - and phospholipid-dependent protein kinase, protein kinase C (PKC), is examined. PKC acts in the inositol phospholipid signaling pathway in a wide variety of cell types to achieve both immediate and long-term alterations to cell physi-

ology (Nishizuka, 1984, 1988). The effects of PMA on intercellular flattening and elements of the cytoskeleton and cytocortex are described and a possible mechanism for the elaboration of cell flattening and polarity is proposed.

Materials and methods

Recovery and culture of embryos

MF1 female mice (3–4 weeks; Central Animal Services, Cambridge, UK) were superovulated by intraperitoneal injections of 5 i.u. of pregnant mares' serum gonadotrophin (PMS) and human chorionic gonadotrophin (hCG, Intervet) 48 h apart. To obtain embryos, females were paired individually overnight with HC-CFLP males (Interfauna) and inspected for vaginal plugs the next day as an indication of successful mating.

Unfertilized eggs were recovered from unmated females at 12½–13½ h post-hCG; early 2-cell embryos were recovered at 40 h post-hCG and late 2-cell and early 4-cell embryos were recovered at 46–50 h post-hCG; 8-cell and 16-cell embryos were derived by overnight culture of 2-cell and 4-cell embryos.

Unfertilized eggs were released from oviducts into warmed (37°C) Medium 2 containing 4 mg ml⁻¹ BSA (M2 + BSA; Fulton & Whittingham, 1978) and exposed briefly to 0.1 M-hyaluronidase (Sigma) to remove cumulus cells before being returned to M2 + BSA for at least 1 h before any further manipulations. 2-cell and 4-cell embryos were flushed from oviducts into warmed M2 + BSA and cultured in drops of Medium 16 containing 4 mg ml⁻¹ BSA (M16 + BSA; Whittingham & Wales, 1969) under paraffin oil (Martindale), in Falcon tissue culture dishes, in 5% CO₂ in air.

After washing in M2 + BSA, zonae pellucidae were removed by brief exposure to acid Tyrode's solution (Nicolson *et al.* 1975) and eggs or embryos were returned to M16 + BSA for a minimum of 6 h before exposure to drugs (see Results).

All manipulations were carried out at 37°C on heated stages or in incubators.

Synchronization of embryos

4-cells

Populations of 2-cell embryos were inspected at hourly intervals and any embryos with 3 or 4 cells were selected and cultured for up to 2 h. Those that had not completed division to 4-cells in this period were discarded. All the remaining 4-cell embryos were cultured as synchronized groups and the time at which the last blastomere was seen to have cleaved was designated the time of division; times are expressed as hours postdivision to 4-cells.

8-cells

Populations of 4-cell embryos were inspected at hourly intervals and any embryos with 5–7 blastomeres were selected and cultured for up to 3 h. Those that did not complete division to 8-cells during that time were discarded. All remaining 8-cell embryos were cultured together as synchronized groups with the time of last blastomere cleavage designating the time of division; times are expressed as hours postdivision to 8-cells.

Preparation of single blastomeres and natural 2/8 pairs

The zonae pellucidae of late 4-cell embryos were removed and

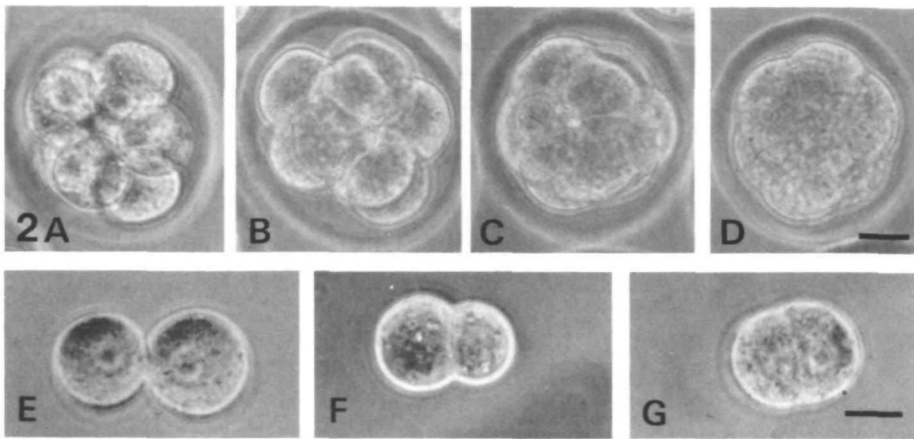


Fig. 2. Photomicrographs of control 8-cell embryos and 2/8 pairs of blastomeres, used as standards for flattening scores.

Whole embryos

- | | |
|---------------------------|----------|
| A. non-flattened, | scored 0 |
| B. partially flattened, | scored 1 |
| C. extensively flattened, | scored 2 |
| D. fully flattened, | scored 3 |

2/8 pairs of blastomeres

- | | |
|-------------------------|----------|
| E. non-flattened, | scored 0 |
| F. partially flattened, | scored 1 |
| G. fully flattened, | scored 2 |

Bar = 20 μm .

the embryos rinsed and incubated in M2 + BSA for 1 h prior to disaggregation in Ca^{2+} -free M2 + 6 mg ml⁻¹ BSA, using flame-polished micropipettes, to yield single blastomeres. Isolated blastomeres were cultured in Sterilin tissue culture dishes in drops of M16 + BSA under oil at 37°C in 5% CO₂ in air. Each hour, the cultures were inspected for evidence of division of blastomeres to 2/8 pairs. All newly formed pairs were removed and designated 0 h postdivision. Pairs were then cultured in individual drops of M16 + BSA.

Assessment of intercellular flattening

The degree of flattening of embryos was determined by examination using a Wild dissecting microscope. Embryos were compared with standards illustrated in Fig. 2 and assigned a score of 0 (A), 1 (B), 2 (C) or 3 (D) according to the degree of flattening. For a population of embryos, the extent of flattening is expressed as a percentage of the maximum possible score, were all embryos to be fully flattened. Unless otherwise indicated in the text, a score such as 33% flattening indicates that most embryos were partially flattened, rather than that one third of embryos were fully flattened.

The degree of flattening in 2/8 pairs of blastomeres was similarly assessed by comparison to standards (Fig. 2E, F, G) and assigned a value of 0, 1 or 2 (maximum).

Drugs

Stock solutions were made in dimethylsulphoxide (DMSO; BDH) of 1 mg ml⁻¹ cytochalasin D (CCD), 25 $\mu\text{g ml}^{-1}$ phorbol 12-myristate 13-acetate (PMA), 100 $\mu\text{g ml}^{-1}$ phorbol 12-myristate 13-acetate 4-O-methyl ether (mPMA), 25 $\mu\text{g ml}^{-1}$ 4 α phorbol didecanoate (4 α PDD), 25 $\mu\text{g ml}^{-1}$ 4 β phorbol didecanoate (4 β PDD), 80 mg ml⁻¹ oleoyl acetyl glycerol (OAG) all from Sigma. A stock of 40 mM-cycloheximide (Sigma) in water was prepared. All these stock solutions were stored at -70°C. 10 mM-nocodazole (Aldrich) in DMSO was stored at 4°C. DMSO was added to all incubations, including controls, to a final concentration of 0.1%. Drugs were diluted in M2 + BSA and embryos cultured in 5 ml of medium in Sterilin tissue culture dishes at 37°C in humidified air. An overlay of oil was *not* used, as many of the drugs would partition to the oil phase.

Scanning electron microscopy

The procedure used was modified from that used by Pickering *et al.* (1988). Alcohol-cleaned glass coverslips were coated with poly-L-lysine (Sigma, $M_r > 30\,000$; 1 mg ml⁻¹ in water) for at least 20 min and washed three times in 0.1 M-cacodylate buffer pH 7.3 before being placed in wells of a Nunclon 24-

well tissue culture dish containing cacodylate buffer. Cells were fixed in 3% glutaraldehyde in cacodylate buffer for 45 min at room temperature in drops under oil and then washed in cacodylate buffer and transferred to the centre of freshly prepared coverslips. Samples were dehydrated through graded alcohols (30 min each in 20%, 40%, 60%, overnight in 70%, 30 min each in 80%, 90%, 95% and dry 100%) and then critical point dried from absolute alcohol via CO₂ in a Polaron E3000 critical point drying apparatus. Coverslips were mounted on stubs with Agar silver paint (Agar Aids), left to dry and coated with a 60 nm layer of gold in a Polaron E5000 Diode sputtering system. Cells were examined in a JSM-35CF Jeol microscope under 5–20 kV.

Labelling of microvillous poles

Labelling was achieved by incubation of cells for 1–2 min in 700 $\mu\text{g ml}^{-1}$ FITC-labelled concanavalin A (FITC-conA; Polysciences) in M2 + BSA at room temperature, followed by washes in M2 + BSA.

Immunocytological staining

Cells were placed in specially designed chambers (described by Maro *et al.* 1984). In most cases, the chambers were coated first with a solution of 0.1 mg ml⁻¹ conA in phosphate-buffered saline (PBS) to increase adhesion of cells to chambers. When cells had been labelled with FITC-conA, 1/10 phytohaemagglutinin (Gibco) in PBS was used instead. Chambers also contained appropriate drugs throughout processing until fixation. Chambers containing samples were centrifuged at 150 g for 10 min at 37°C (or 22°C for cells only labelled with FITC-conA). Cells were then treated in one of two ways: (i) For tubulin staining, after a recovery period of 10 min at 37°C, cells were washed quickly in PHEM buffer (10 mM-EGTA, 2 mM-MgCl₂, 60 mM-Pipes, 25 mM-Hepes, pH 6.9; described by Houlston *et al.* 1987) containing 0.6 mM-taxol (PHEM-taxol), extracted for 5 min in PHEM-taxol buffer containing 0.25% Triton X-100, washed in PHEM-taxol buffer and fixed for 45 min with 2% formaldehyde (BDH) in PHEM-taxol buffer. All these steps were carried out at 30°C. (ii) After conA staining or for visualization of actin or PMA, cells were fixed immediately after centrifugation with 4% formaldehyde in PBS.

Tubulin was visualized with a monoclonal anti- α tubulin antibody (YL1/2; Kilmartin *et al.* 1982) followed by FITC anti-rat IgG (Miles). Actin was visualized with FITC-phalloidin (Sigma) after permeabilizing cells with 0.25% Triton X-100.

PMA-binding sites were visualized using 11-((-5-dimethylaminonaphthalene-1-sulphonyl) amino) undecanoylphorbol

acetate (dansyl PMA, Molecular Probes). Embryos were incubated in medium containing 25 ng ml^{-1} dansyl PMA for 1 h prior to fixation. Chromatin was visualized by incubating fixed cells in Hoechst dye 33258 (5 mg ml^{-1} in PBS) for 45 min.

PBS containing 0.1% Tween 20 (Sigma) was used routinely in washing steps.

Photomicroscopy

The coverslips were removed from chambers and samples were mounted in 'Citifluor' (City University, London) and viewed on a Leitz Ortholux II microscope with filter sets L2 for FITC-labelled reagents and dansyl PMA, N2 for rhodamine-labelled reagents, and A for Hoechst dye. Photographs were taken on Kodak Tri-X film using a Leitz Vario-Orthomat photographic system. The three-dimensional structure of the cells is preserved in the whole mounts, but it is not possible to photograph the whole cell in the same focal plane; optical sections with only one plane through the cell in sharp focus are shown.

Scoring

For all experimental protocols, untreated controls were examined with each experimental series and similar sized groups were used for each. For fluorescence and electron microscopy, samples were scored 'blind' and data collated once scoring was completed.

Effects of PMA on cell division

Since PMA has been reported to inhibit cytokinesis (but not karyokinesis) or to reverse cytokinesis if applied before the cleavage furrow is complete (Niemerko & Komar, 1985; Sawicki & Mystkowska, 1981; Mystkowska & Sawicki, 1987), in all experiments described, embryos were examined for evidence of binucleate blastomeres (7% or less) which were not included in morphological scores.

Results

There is considerable developmental heterogeneity within a population of embryos of the same age postovulation. Throughout the experiments described below, 4-cell and 8-cell embryos were synchronized to the most recent mitotic cleavage and the age of embryos is expressed in hours postdivision.

Fluorescent localization of PMA-binding sites

In order to confirm binding of PMA to cells and to assess the localization of any binding sites, oocytes and blastomeres were cultured for 1 h in the presence of the biologically active and fluorescent derivative of PMA, dansyl PMA (Liskamp *et al.* 1985), with or without competition by an excess of unlabelled PMA, then fixed and observed by indirect immunofluorescence. Oocytes and blastomeres of all stages incubated with dansyl PMA showed bright punctate staining around the cell perimeter and patchy but diffuse staining throughout the cytoplasm but excluded from the nucleus (e.g. 4-cell embryo, Fig. 3). Preincubation for 30 min with 100- or 1000-fold excess of unlabelled PMA followed by the addition of dansyl PMA for a further 30 min prevented any detectable fluorescent binding. If both dansyl and unlabelled PMA were present throughout incubation, 1000-fold excess of unlabelled PMA prevented binding of dansyl PMA.

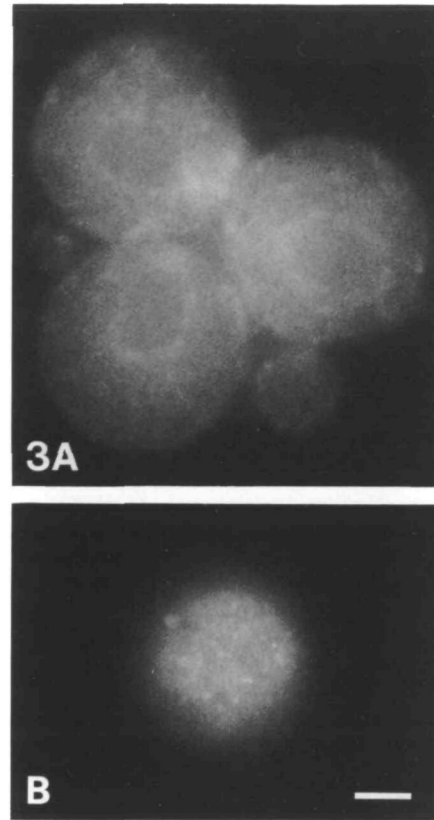


Fig. 3. Fluorescent localization of PMA-binding sites in 4-cell embryos assessed after 1 h incubation in 25 ng ml^{-1} dansyl PMA before fixation. (A) Optical section through blastomere showing patchy, diffuse staining throughout the cytoplasm but excluded from nuclei. (B) Tangential view showing bright, punctate staining at the blastomere perimeter. Bar = $10 \mu\text{m}$.

Effects of PMA on intercellular flattening

Completely nonflattened 8-cell embryos, 0 h or 3 h postdivision, were incubated in control medium or medium containing 100 ng ml^{-1} or 25 ng ml^{-1} PMA and scored hourly for evidence of flattening. Embryos were compared with the standards shown in Fig. 2 (A–D) and assigned a score according to the degree of intercellular flattening. Some embryos were left in PMA-containing medium for up to 8 h whilst others were washed and transferred to control medium after 1 h. No embryo exposed to PMA, using any schedule, flattened (12 experimental groups, 386 embryos) whereas controls flattened to 94% of the maximum possible (6 groups, 250 embryos).

8-cell embryos (7–8 h postdivision) that were scored as fully flattened were placed in control medium or medium containing PMA at various concentrations for 1 h and scored again for flattening. The results are plotted in Fig. 4. In the concentration range over which PMA is known to activate PKC (Nishizuka, 1984), a dose-dependent reversal of intercellular flattening was seen, only 10% of the maximum possible flattening remained after 1 h of exposure to 25 ng ml^{-1} PMA. A similar result was obtained when pairs of 8-cell blasto-

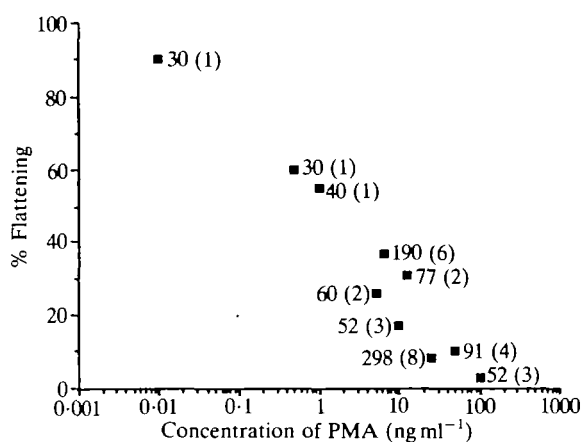


Fig. 4. Plot of the effects of various concentrations of PMA on intercellular flattening in fully flattened 8-cell embryos (7–8 h postdivision). The vertical axis expresses the degree of flattening as a percentage of the maximum possible were all embryos fully flattened. The figures beside each point indicate the number of embryos exposed to each condition and those in brackets show the number of experimental replicates. The scores from separate experiments were never more than $\pm 5\%$ different from the averages shown. Embryos placed in medium containing no PMA scored 86% (between 80% and 100% in separate experiments; 218 embryos).

meres were treated in the same way (data not shown, see Fig. 2E–G).

To determine whether the inhibition and reversal of flattening by activators of PKC was reversible, compacted 8-cell embryos (7 h postdivision) were exposed to PMA for 1 h and then washed and cultured in control medium and scored for flattening over the ensuing 24 h of culture. The results, plotted in Fig. 5A, show that the reversal of flattening persisted for 6 h and flattening was then restored gradually over a period of 12 h. This recovery of flattening was slower than that seen after exposure to medium depleted of Ca^{2+} (full flattening within 1 h of restoring Ca^{2+}) and slower than the original process of flattening during the 8-cell stage (full flattening by 7–8 h postdivision). During the period of observation, blastomeres in control embryos continued to divide and cavitation began in these embryos.

Protein synthesis is required for the restoration of intercellular flattening since embryos that were exposed to PMA for 1 h and then transferred to medium containing $400 \mu\text{M}$ -cycloheximide failed to flatten within 24 h (Fig. 5B). Cycloheximide alone had no detectable effect on intercellular flattening.

Effects of PMA on the cytoskeleton

Microtubule organization

The effect of PMA on microtubule organization depended on the sequence of treatments of the embryos. Since immunocytochemical analysis requires removal of the zona pellucida, preliminary experiments were undertaken to determine the influence of this procedure on the outcome of experiments. Some zona-intact embryos were exposed to PMA followed by

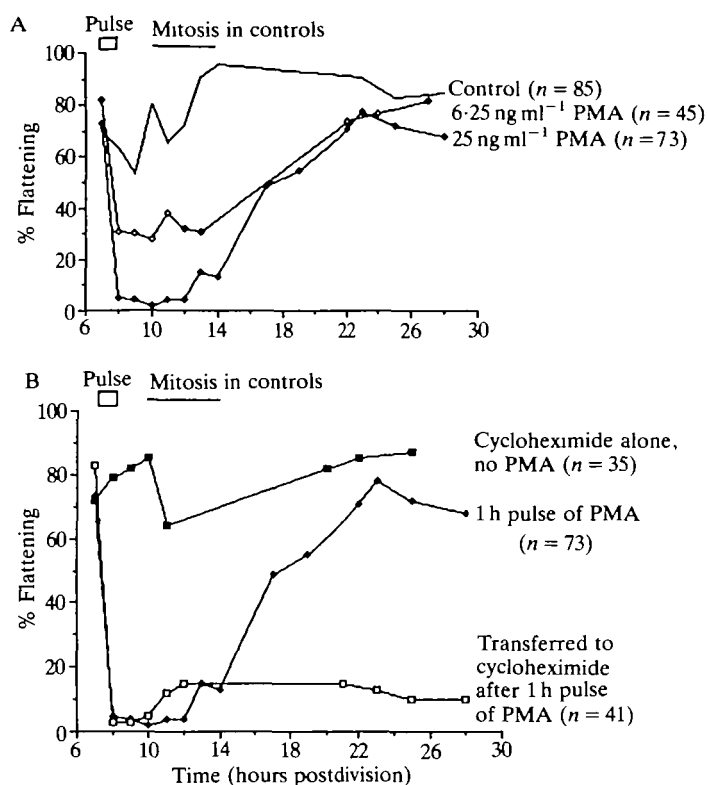


Fig. 5. Time course of intercellular flattening in 8-cell embryos exposed to 25 ng ml^{-1} PMA for a 1 h period at 7 h postdivision, washed and incubated in (A) M16 + BSA or (B) M16 + BSA containing $400 \mu\text{M}$ -cycloheximide for 24 h. The figures in brackets indicate the number of embryos examined under each condition. The period during which mitosis of 8- to 16-cell blastomeres occurred is indicated; this coincided with a transient reversal of flattening as shown (Lehtonen, 1980). Embryos incubated in PMA or cycloheximide did not cleave during observation.

removal of the zona using acid Tyrode's solution just before fixation for analysis. Other embryos had their zonae removed with acid Tyrode's solution followed by a recovery period of up to 6 h before exposure to PMA. When exposure to PMA was followed by removal of the zona prior to fixation, almost all microtubules in compact late 8-cell embryos (7 h postdivision) were destroyed. In contrast, the cortical and cytoplasmic mesh of microtubules in 1-cell, 2-cell, 4-cell or early (1 h postdivision) 8-cell embryos were not obviously affected. When microtubules were examined in embryos that had been exposed to PMA after allowing a 6 h recovery period after zona removal, the microtubule network was not detectably different from that of control embryos at any stage examined. Moreover, the incidence of a polar distribution of microtubules in these late 8-cell embryos was similar to that of controls (Table 1).

These results indicate that PMA may have some stage-dependent effect on microtubule organization in compact 8-cell embryos, but that this effect is only revealed after exposure to acid conditions (see Discussion).

Table 1. Effects of PMA and zona removal on distribution of microtubules in compact 8-cell embryos, 7–8 h postdivision

Drug	No. of cells	Incidence of microtubules (% cells)				Polarity of microtubules (%)*		mitotic/fused cells
		like controls	fewer	sparse	none	apolar	polar	
<i>(A) Zona removal after exposure to PMA, immediately prior to fixation</i>								
mPMA	225	84	0	0	0	41	43	17
Nocodazole	228	2	2	31	61	2	0	4
PMA	266	1	34	52	8	1	0	6
<i>(B) Six hour recovery period allowed after zona removal before exposure to PMA and immediate fixation</i>								
mPMA	121	94	0	0	0	74	20	6
PMA	163	94	0	0	0	51	43	6

PMA and mPMA 25 ng ml⁻¹, nocodazole 10 µM.

* Polarity is expressed only for those cells having similar numbers of microtubules to controls.

Microvilli and microfilaments

Distribution and morphology of microvilli. Zygotes and blastomeres of 2-cell, 4-cell and early 8-cell embryos have a dense, even covering of short microvilli over the entire blastomere surface, absent only immediately adjacent to cell–cell contacts (Fig. 6B,C) and, in unfertilized oocytes, in the area overlying the meiotic chromosomes (Fig. 6A; Eager *et al.* 1976). During the 8-cell stage, microvilli are lost progressively from regions further from cell contacts until the only microvilli remaining are in an apical pole (Figs 1, 6D).

Oocytes and embryos at different developmental stages were exposed to PMA, and blastomere surfaces examined by scanning electron microscopy. Although PMA at high doses can cause activation of unfertilized oocytes and suppress formation of the second polar body (0.1–1 µg ml⁻¹; Cuthbertson & Cobbold, 1985; Niemerko & Komar, 1985), at 25 ng ml⁻¹ PMA was found to have little effect on the morphology or distribution of microvilli on oocytes (Fig. 6A, E).

In embryos, preliminary observations of microvilli by scanning electron microscopy and of microfilamentous actin by immunofluorescence revealed an interaction of the effects of phorbol ester and zona removal with acid Tyrode's solution similar to that described for microtubules. If zona removal was followed by a recovery of less than 6 h, a gross disruption of the actin cytoskeleton by PMA was detected. Hence in all the experiments described below, a minimum of 6 h recovery after zona removal was allowed.

Exposure of embryos to PMA following 6 h recovery after zona removal caused a loss of microvilli which was more severe the later the developmental stage examined, with more blastomeres having fewer visible microvilli or none at all. From the 1-cell to early 8-cell stages, a small proportion of blastomeres had no microvilli and increasing numbers had fewer microvilli than controls (Fig. 6F,G). In late 8-cell and 16-cell embryos, most blastomeres had no microvilli at all (Fig. 6H). The morphology of microvilli also changed with the developmental stage when exposed to PMA. The microvilli of oocytes exposed to PMA were indistinguishable from those of controls. 1-cell embryos exposed to PMA were

almost all covered in microvilli longer than those of control embryos and lying parallel to the cell surface rather than perpendicular as in controls (Fig. 6F). The incidence of this sort of microvilli was lower in later developmental stages which tended to have very short, sparse microvilli (Fig. 6G). The proportion of blastomeres with each type of microvilli at each stage is summarized in Fig. 7. It is clear that major changes in the response to PMA occur after fertilization and, in particular, during the 8-cell stage.

No PMA-treated blastomeres from 8-cell embryos were observed to have tightly polarized microvilli. Poles were observed in 87% ($n = 246$) of blastomeres of control embryos and 86% ($n = 219$) of blastomeres in embryos exposed to 25 ng ml⁻¹ mPMA at 8–9 h postdivision.

Fluorescent visualization of microfilamentous actin and microvilli. Microvilli may also be visualized by fluorescent labelling with FITC–phalloidin which binds to polymerized actin and reveals the microfilamentous actin cores of microvilli. Oocytes, embryos and 2/8 pairs of blastomeres, in which clearer visualization of whole cells is possible, were exposed to PMA and then stained with phalloidin. At most stages, the pattern of staining was indistinguishable from that of controls. Oocytes stained intensely in a cortical cap overlying the meiotic spindle and also in microvilli over the remainder of the surface (Fig. 8A). In zygotes, microvilli and a cortical mesh of microfilaments were apparent (Fig. 8B). In later embryos, the staining appeared only as fine spots in the region of the cell cortex and membrane, presumably reflecting microvillous cores (Fig. 8C,D). After exposure to PMA, a small proportion of blastomeres in late 4-cell embryos and early 8-cell blastomeres (1–6 h postdivision) had large patches of staining in the cell cortex and some had no staining at all, but most remained like controls (Fig. 8E,F). In later 8-cell blastomeres (8 h postdivision), 60% of cells did not stain at all with phalloidin and the remainder had large cortical patches of staining (Fig. 8G–I; summarized in Table 2). None of the PMA-treated blastomeres of any age were scored as having

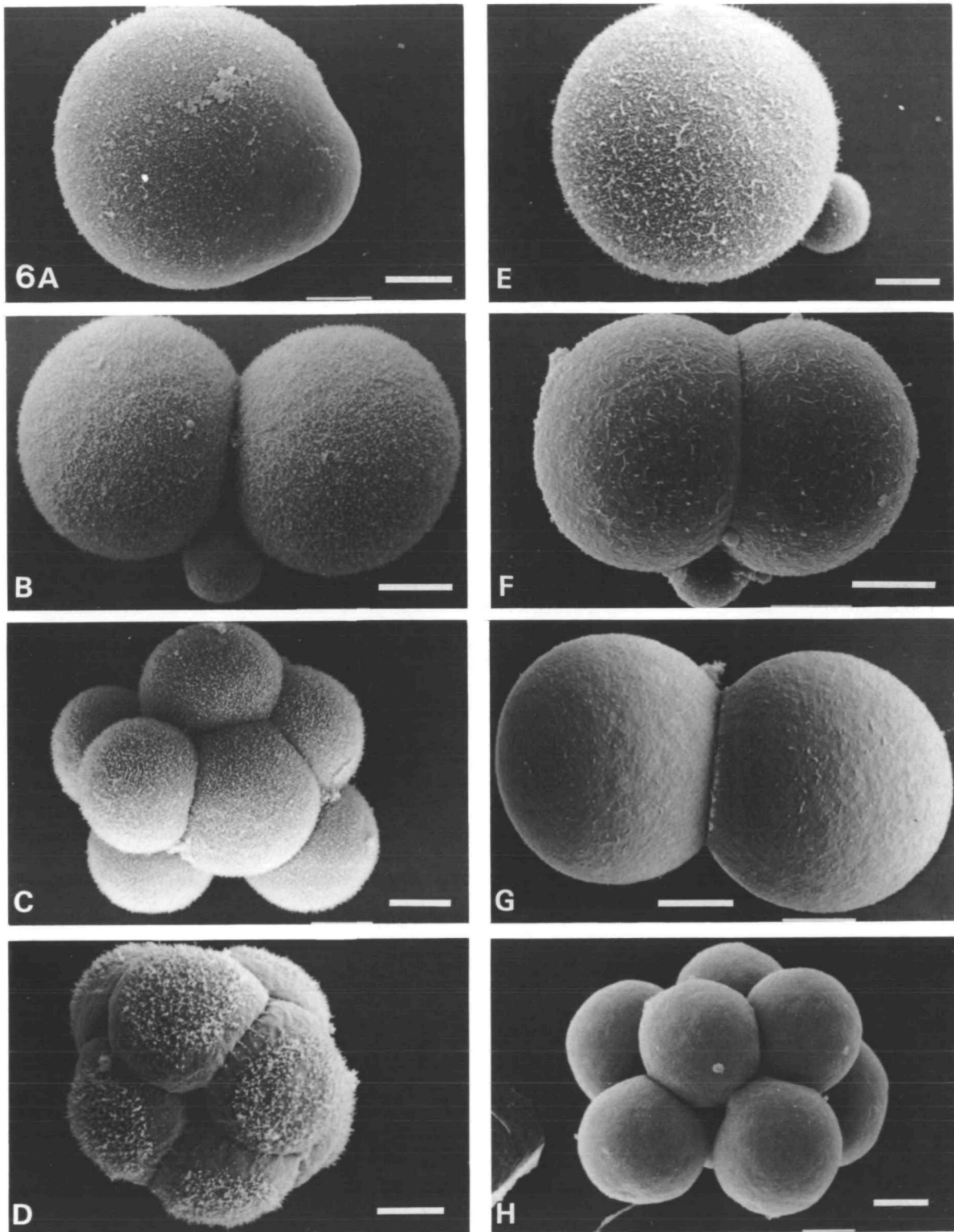


Fig. 6. Scanning electron micrographs of oocytes and embryos following 1 h incubation in control medium (A–D) or 25 ng ml⁻¹ PMA (E–H). The stages shown are oocyte (A,E), early 2-cell (B,F), late 2-cell (G), early 8-cell (C) and late 8-cell (D,H). The microvilli in (D) show a typically polar distribution, those in (F) are longer and thinner than those of controls and those in (G) are both shorter and more sparse than those of control embryos. A late 8-cell embryo exposed to PMA (H) has no detectable microvilli. Bar = 10 μm.

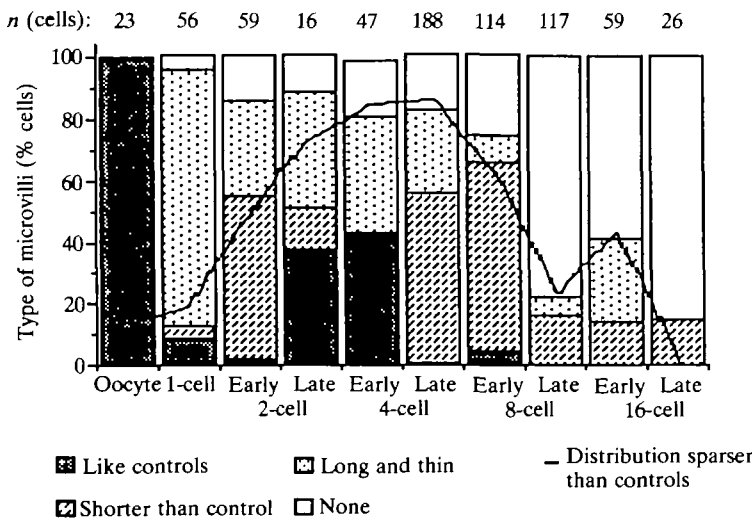


Fig. 7. Summary of effects of 1 h incubation in 25 ng ml⁻¹ PMA on distribution and morphology of microvilli of embryos of different developmental stages. The figures above each bar indicate the number of cells of each stage examined. The precise timings of the developmental stages are:

oocyte	19–20 h post hCG	late 4-cell	60 h post hCG
1-cell	25–28 h post hCG	early 8-cell	3–4 h postdivision
early 2-cell	38 h post hCG	late 8-cell	8–9 h postdivision
late 2-cell	54–58 h post hCG	early 16-cell	6 h postcompaction
early 4-cell	52–54 h post hCG	late 16-cell	12 h postcompaction

tight poles of phalloidin staining; 38% (*n* = 86) of untreated blastomeres at 8 h postdivision were scored as polarized by this method.

The final method used to visualize microvilli and surface membrane was labelling with FITC-conA which binds to receptors in the plasma membrane and therefore highlights regions of high membrane density such as microvilli (Handyside, 1980). Late 2/8 pairs (8 h postdivision) were exposed to various concentrations of PMA and mPMA before staining with conA. Control and mPMA-treated cells all had fine, punctate staining over the entire surface of the cell that was absent basolaterally, consistent with the distribution observed

for microvilli. All concentrations of PMA produced large patches of staining and reduced total staining (Fig. 9). Where any staining was evident, the incidence of polarity of conA binding was also greatly reduced in cells exposed to PMA compared to controls (Table 3).

Other activators of PKC

To determine whether the effect of PMA on intercellular flattening correlates with the activation of PKC, embryos were exposed to other biologically active and inactive phorbol esters and a synthetic diacylglycerol (OAG) and the effects on flattening scored. Only those drugs capable of activating PKC, namely PMA, 4β-phorbol-12,13-didecanoate (4βPDD) and OAG (40 μg ml⁻¹ but not 4 μg ml⁻¹), reversed flattening of previously fully flattened embryos. 4βPDD showed very similar dose–response behaviour to that of PMA while 40 μg ml⁻¹ OAG partially reversed flattening to 57% in 1 h (46 embryos). 4α-phorbol-12,13-didecanoate (4αPDD) up to 100 ng ml⁻¹ and phorbol 12-myristate 13-acetate 4-O-methyl ether (mPMA, 25 ng ml⁻¹), which do not activate PKC (Niedel *et al.* 1983), as well as the kinase inhibitor H-7 (18 μg ml⁻¹), had no apparent effect on intercellular flattening.

The effects of these biologically active and inactive phorbol esters and diacylglycerol on microvillous distribution and morphology were also assessed. Those drugs capable of activating PKC and reversing intercellular flattening, 4βPDD and 100 μM OAG, had similar effects to those shown for PMA (Figs 6, 7) on the distribution and morphology of microvilli of 8-cell embryos, while 4αPDD, mPMA, 4 μg ml⁻¹ OAG and H-7 had little obvious effect.

Discussion

The differentiation of cells in early development often involves cellular partitioning of cytoplasmic components inherited from the egg during cleavage. These components are implicated in directing or influencing cell fate (Davidson, 1986). In the mouse embryo, cell asymmetry is generated *de novo* at the 8-cell stage and is followed by one or two differentiative divisions which

Table 2. Effect of PMA on polymerized actin visualized with FITC-phalloidin

Developmental stage (as Fig. 7)	No. of cells	Staining %			% polarity in controls of same age
		like controls	*patchy	none	
oocyte	38	100	0	0	0
early 2-cell	31	100	0	0	0
late 2-cell	20	100	0	0	0
late 4-cell	57	86	4	10	0
2/8 pair:					
1 h postdivision	61	100	0	0	0
2 h postdivision	40	100	0	0	8
3 h postdivision	57	68	14	18	10
4 h postdivision	24	96	0	4	14
6 h postdivision	68	97	3	0	25
8 h postdivision	114	2	36	62	70

* See text and Fig. 8.

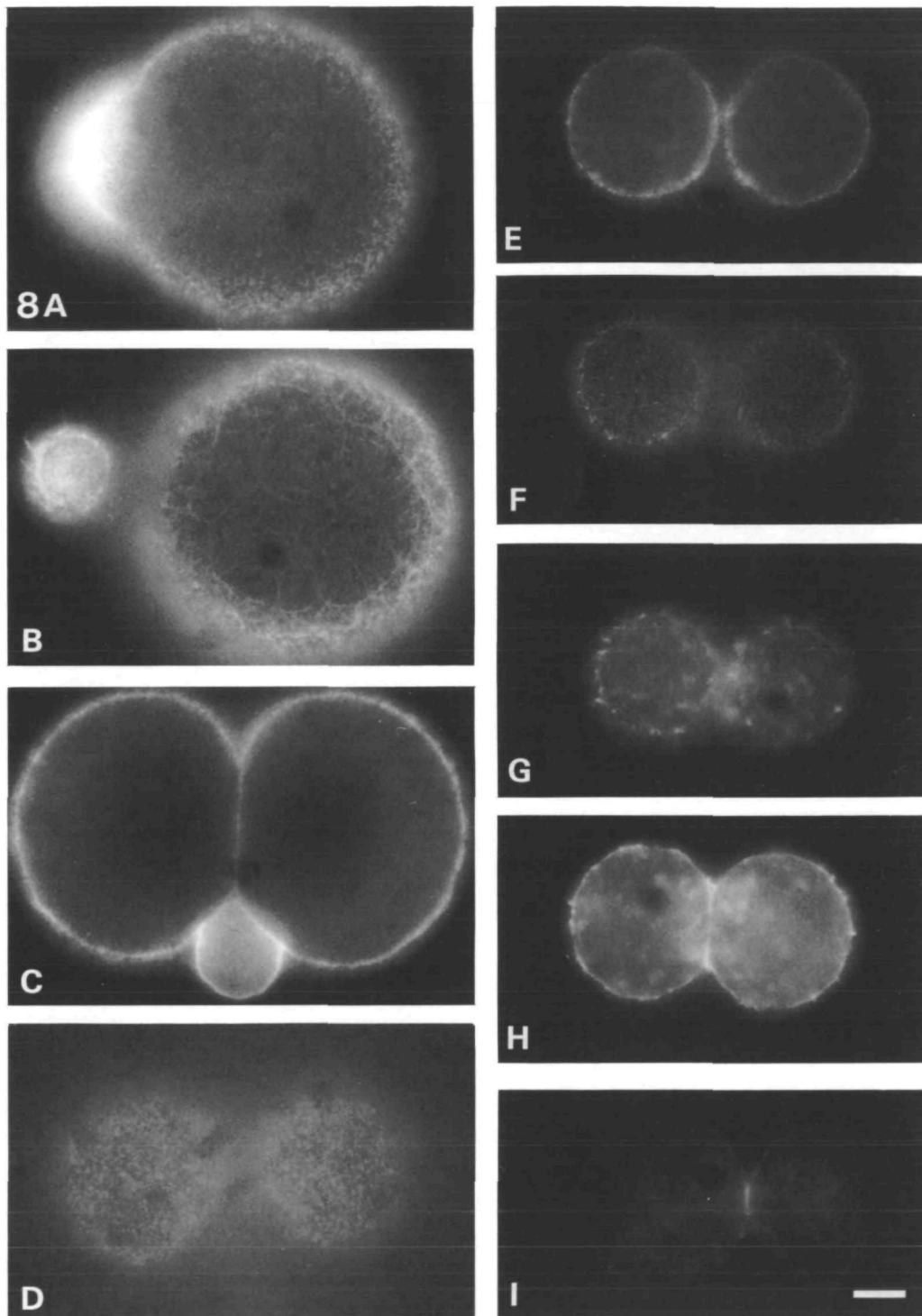


Fig. 8. Polymerized actin in untreated (A) oocyte, (B) zygote and (C,D) late 2-cell embryo, which are indistinguishable from those in PMA-treated groups. (E–I) Pairs of 8-cell blastomeres are aged (E,F) 1 h postdivision, appearing like controls, (G,H) 3 h postdivision, staining is patchy and (I) 8 h postdivision, very little staining evident. All are stained with FITC-phalloidin. Optical sections through cells (B,C,E,H,I) and tangential views (A,D,F,G) are shown. Bar = 10 μm .

lead to the formation of two cell types in the blastocyst. The production of radially polarized 8-cell blastomeres is therefore the first identifiable step in the differentiation of the mouse embryo (Johnson *et al.* 1986a).

At compaction, both blastomere polarization and the accompanying intercellular flattening appear to be associated with a change in the capacity of blastomeres to respond to uvomorulin-mediated cell–cell contact. The

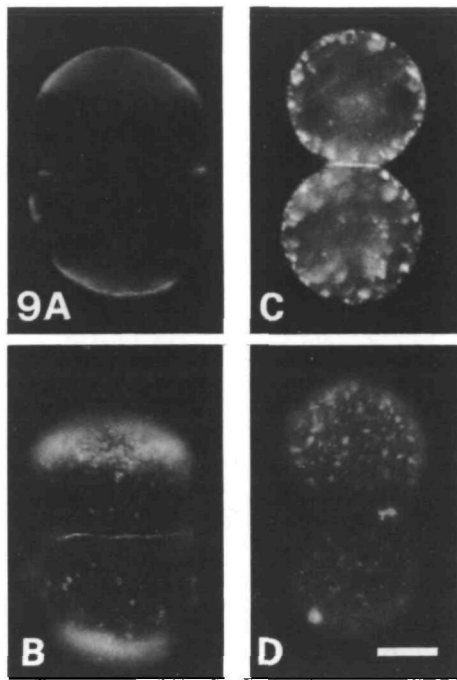


Fig. 9. Pairs of 8-cell blastomeres (8 h postdivision) examined for distribution of surface conA receptors. Pairs of cells were incubated for 1 h in control medium (A,B) or medium containing 25 ng ml^{-1} PMA (C,D) then washed and exposed to FITC-conA prior to fixation. Blastomeres are shown in section (A,C) and in tangential view (B,D).

result is a propagated cytocortical reorganization which in turn leads to cell surface and cytoskeletal changes (see Fig. 1). All the necessary proteins for this change in the response to cell contact are present prior to the 8-cell stage and the trigger for their utilization at compaction is also post-translational (Kidder & McLachlin, 1985; Levy *et al.* 1986). It seems possible that maturation of some aspect of second messenger systems, such as messenger generation, stimulation or range of action, might be involved in the changing response to cell-cell contacts. It has been reported that adenylate cyclase activity and responsiveness change during pre-implantation development, although later than compaction (Manejwala *et al.* 1986). In the experiments reported here, the possible involvement of the inositol

phospholipid signalling pathway in compaction has been investigated indirectly. Stimulation of PKC by a variety of drugs at the time when polarization and intercellular flattening normally occur leads to several changes in cell organization which do not occur if blastomeres are exposed to such drugs at other times. This suggests that such drugs reveal an otherwise hidden change in cell physiology at the 8-cell stage, which may reflect a role for PKC in the normal attainment of altered responsiveness to cell contacts.

Phorbol esters bind specifically to mouse zygotes and blastomeres at sites close to the cell membrane (Fig. 3). Their application has profound effects on cytoskeletal organization as assessed both indirectly and directly. Confirming results reported by others, cleavage furrows were prevented from forming, resulting in triploid zygotes or binucleate blastomeres (Niemerko & Komar, 1985; Sawicki & Mystkowska, 1981; Mystkowska & Sawicki, 1987). In addition, intercellular flattening was prevented and reversed when 8-cell embryos were exposed to PMA and flattening was not restored in the absence of protein synthesis (Figs 4, 5). PKC stimulation has therefore caused a change to the cell that is not simply reversed by removal of the stimulus, implying that required proteins have been destroyed or irreversibly modified in response to stimulation.

More direct evidence for effects of PMA on cytoskeletal organization has come from examination of microtubule and microfilament organization by immunofluorescence. The outcome of this analysis depended both on the developmental stage of cells examined and on the experimental protocol used. If embryos had their zonae pellucidae removed with acid Tyrode's solution immediately before fixation and after exposure to PMA, destruction of microtubules resulted at the 8-cell stage but not at earlier developmental stages. By contrast, if embryos were allowed a recovery period after zona removal before exposure to PMA, no detectable effects were seen on the number or distribution of microtubules at any of the stages examined (Table 1). An interaction between zona removal and the effects of PMA on the actin cytoskeleton was also observed. Sobel (1983) found that the distribution of myosin in mouse blastomeres is disrupted by zona removal with acid Tyrode's solution and that normal organization is

Table 3. Effect of PMA on distribution of conA receptors on 8 h 2/8 pairs of blastomeres

Drug	Concentration (ng ml^{-1})	No. of cells	Staining %			Poles %			fused/ mitotic cells
			like controls	*patchy	none	apolar	†broad pole	†tight pole	
none		125	97	2	0	19	12	67	1
mPMA	25	32	94	0	0	22	9	63	6
PMA	5	70	24	70	0	57	13	24	5
PMA	10	71	23	68	2	60	30	2	7
PMA	25	130	8	86	0	83	12	0	6
PMA	50	106	26	68	0	88	4	0	7
PMA	100	31	0	97	0	94	3	0	3

* See text and Fig. 9.

† Poles were scored as 'broad' if more than 1/3 cell was stained.

only restored some six hours later. It therefore seems that zona removal has a profound effect on the cytoskeleton, presumably mediated *via* the associated pH change. PMA allows this change to be observed selectively at the 8-cell stage resulting in severe cytoskeletal disruption.

In addition to these observations, in the absence of any pH-mediated effects on the cytoskeleton, exposure to PMA had increasingly severe effects on the microfilament system with increasing developmental age. Thus microvilli of zygotes and early 2-cell embryos collapsed, becoming longer and thinner than in controls and less densely distributed (Figs 6, 7). With further development up to the 8-cell stage blastomeres tended to have fewer microvilli, a phenomenon probably due to their retraction since actin cores were still evident cytochemically although the microvilli appeared extremely short when examined by scanning electron microscopy (Figs 6, 8). At the 8-cell stage the numbers of microvilli declined much further; there were no microvilli on many cells after exposure to PMA and polymerized actin cores were no longer visible. Although a relatively crude approach, taken together these results do suggest a change in the response of the cytoskeleton to PKC stimulation at the time during development at which cell flattening and polarization normally occur.

PMA has been reported to have a variety of effects on the actin cytoskeleton of various cultured cell lines (Rifkin *et al.* 1979; Schliwa *et al.* 1984; Meigs & Wang, 1986; Hedberg *et al.* 1987; Burn *et al.* 1988) and to cause changes in cell shape (Robinson *et al.* 1987; Phaire-Washington *et al.* 1980; Croop *et al.* 1980). Increasing evidence is now available from studies of the red blood cell membrane and cytoskeleton that the phosphorylation of constituent proteins can profoundly affect cell shape (reviewed by Backman, 1988). Just as the response to PMA in the early embryo shows stage-dependent changes, it seems likely that in other systems the particular effects of PMA are highly specific to cell type or differentiated state (Toutant & Sobel, 1987), reflecting the normal range of physiological response available to each cell type.

The change in response to PMA of mouse blastomeres during the 8-cell stage may reflect an altered physiological state which is relevant to the events of compaction. Both compaction and exposure to PMA are associated with the dismantling of microtubules, microfilaments and microvilli. At compaction these changes are spatially restricted in relation to the positions of intercellular contact. By contrast, following PKC stimulation with PMA, dismantling of the cytoskeleton occurs throughout the cell. Thus it is possible that the exposure of cells to PMA is equivalent to symmetrical and universal cell contact (see Fig. 10). Indeed, if 8-cell blastomeres are surrounded by contacts on all sides, nonpolar, nonmicrovillous cells which may be analogous to PMA-stimulated blastomeres result (Ziomek & Johnson, 1981). Attempts to mimic the effects of localized cell-cell contact by delivery of PMA to only a small portion of the cell surface have failed

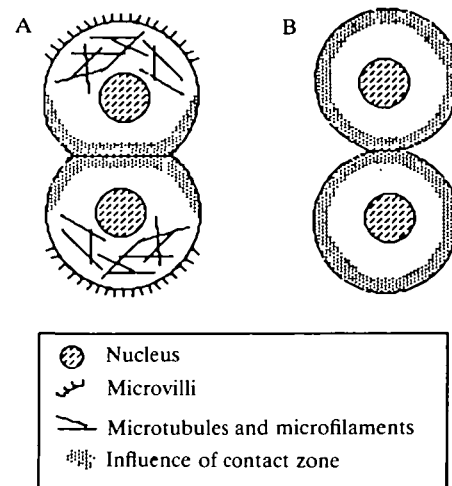


Fig. 10. Schematic representation of a model for the effects of PMA on 8-cell blastomeres. (A) As Fig. 1C, compacted blastomeres. (B) The cytocortex is competent to generate a widespread change in adhesive properties and cytoplasmic organization at this stage of development. PMA acts over the entire plasma membrane resulting in the whole cell behaving like the contact zone with maximal adhesivity and cytoskeletal depletion.

since PMA distributes extremely rapidly all around the cell surface from the initial point of application (as visualized with dansyl PMA, data not shown).

A large number of studies of the physiological activation of PKC has not revealed any means of stimulation other than by diacylglycerol, which is most commonly produced by increased phosphoinositide turnover following occupation of ligand receptors in the plasma membrane (Nishizuka, 1984; Kikkawa & Nishizuka, 1986; Bell, 1986). However, there are reports of hydrolysis stimulated by such diverse triggers as the action of light at photoreceptors (Fein, 1986) and the arrival of sperm at the egg plasma membrane (Ciapa & Whittaker, 1986; Bloom *et al.* 1988). In translating patterns of cell contacts into an intracellular response, it is possible that material concentrated at cell contacts can activate PKC either directly or indirectly. Potential candidate molecules include uvomorulin (Kemler *et al.* 1988), components of the cytoskeleton (Sobel & Alliegro, 1985; Reima & Lehtonen, 1985; Lehtonen & Reima, 1985; Damjanov *et al.* 1986; Sobel *et al.* 1988) or plasma membrane (Pratt, 1985; Ziomek, 1987) which are known to be localized asymmetrically. However, such mechanisms of PKC activation have not been demonstrated previously.

The nature of the postulated permissive change in cell physiology that results in altered effects of PMA on blastomeres at the 8-cell stage remains unknown. The early embryonic cells described here only acquire the features of mature cells gradually, during successive cell division cycles (reviewed by Fleming & Johnson, 1988). Elements of the inositol phospholipid second messenger system may also be acquired progressively during this period. It is unlikely that availability of an activatable protein kinase C is the limiting factor, since

phorbol esters both bind to, and can have effects on, oocytes and earlier embryos. It is possible that the pathways for diacylglycerol generation and response are only mature at the 8-cell stage; the response to phorbol esters at earlier stages does not yield any information about the availability of phosphatidylinositol bisphosphate (PIP₂), the substrate from which diacylglycerol is normally produced, at those earlier stages, or of the functioning of phospholipase C, the enzyme responsible for cleaving PIP₂. Alternatively a new PKC isotype with different stimulus, and/or substrate, specificity may be activated at the 8-cell stage (Nishizuka, 1988). Perhaps the most likely explanation is that specific substrate(s) for phosphorylation by PKC become accessible at the 8-cell stage and not earlier. Experiments aimed at distinguishing these possibilities are currently in progress. From the available evidence, a tentative model for the elaboration of polarity (see Fig. 10) in previously apolar blastomeres could be (i) nonpolar cells respond to cell-cell contacts with localized flattening and depolymerization of the cytoskeleton, possibly mediated by local PKC activity, (ii) at the 8-cell stage this restriction of PKC response (or stimulation), and hence of cytoskeletal depolymerization, to the immediate zone of contact no longer exists, (iii) this allows propagation of the depolymerization and cell flattening around the cytocortex.

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