Control of microtubule nucleating activity in the cytoplasm of maturing mouse oocytes

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ABSTRACT   Taxol, a drug which promotes microtubule assembly, was used to assess the microtubule nucleating activity of pericentriolar material (PCM) in mouse oocytes prevented from undergoing germinal vesicle breakdown (GVBD), compared with oocytes allowed to proceed normally through GVBD and also in nucleate and anucleate oocyte fragments. Both immunofluorescence staining and ultrastructural analysis reveal that taxol induces aster formation in the cortex of oocytes undergoing GVBD, while formation of a continuous sheet of microtubule bundles parallel to the membrane is induced in metabolically GV-arrested oocytes. Since taxol also induces the formation of asters in anucleate as well as in nucleate oocyte fragments, provided they are not treated with activators of protein kinases A or C, it is concluded that microtubule nucleating activity is related to the acquisition of Maturation Promoting Factor (MPF) and does not require mixing between the nucleoplasm and cytoplasm.

KEY WORDS: *oocytes, anucleation, asters*

Introduction

Dictyate-stage mouse oocytes mechanically isolated from their follicles resume meiosis spontaneously *in vitro* (Pincus and Enzmann, 1935; Edwards, 1965). Chromatin condensation and germinal vesicle breakdown (GVBD) take place within one to three hours after the transfer of the oocyte to culture medium (Donahue, 1968; Sorensen, 1973). A spindle is assembled in the central area of the oocyte and moves centrifugally to the cortex where anaphase I, telophase I and cytokinesis take place (Longo and Chen, 1985; Alexandre and Mulnard, 1988; Alexandre et al., 1989).

Spindle formation is thus an essential feature of the G2 to M transition taking place as meiosis is reinitiated. It is therefore usually thought to be triggered by the ubiquitous «Maturation (or M-Phase) Promoting Factor» (MPF) (Masui and Markert, 1971; Murray, 1988).

Mouse oocytes lack centrioles but the poles of the spindle are composed of rims of pericentriolar material (PCM) (Szöllösi et al., 1972; Calarco-Gillam et al., 1983). This polar PCM acts as microtubule-organizing centers (MTOCs) which display a microtubule nucleating activity (Maro et al., 1985). The latter was shown recently to coincide with the appearance of a subset of PCM proteins possessing a phosphorylated epitope (Centonze and Borisy, 1990).

On the other hand, the role of a component of the germinal vesicle in the appearance of microtubule nucleating activity has been strongly suggested in amphibian oocytes (Heidemann and

Kirschner, 1975, 1978; Hanocq-Quertier et al., 1978; Heidemann and Gallas, 1980; Karsenti et al., 1984; Jessus et al., 1987, 1988). The presence of PCM displaying microtubule nucleating activity can be assessed by the use of taxol, a drug which promotes microtubule assembly by shifting the equilibrium between tubulin dimer and polymer in favor of microtubule formation (Schiff et al., 1979; Horwitz et al., 1981). Taxol thus allows the visualization of the tubulin polymerization capacity of a cell, and also the MTOCs.

In maturing mouse oocytes, not only the polar rims, but also numerous cytoplasmic PCM foci have been observed. While only the former are active as MTOCs, both display microtubule nucleating activity after the addition of taxol (Maro et al., 1985; Van Blerkom, 1991).

However, it is not known whether nuclear components are required for the activation of pericentriolar material (PCM) into MTOCs in mammalian oocytes. We therefore analyzed the effect of taxol on both mouse oocytes prevented from undergoing GVBD, and anucleate oocyte fragments. The first condition was achieved by

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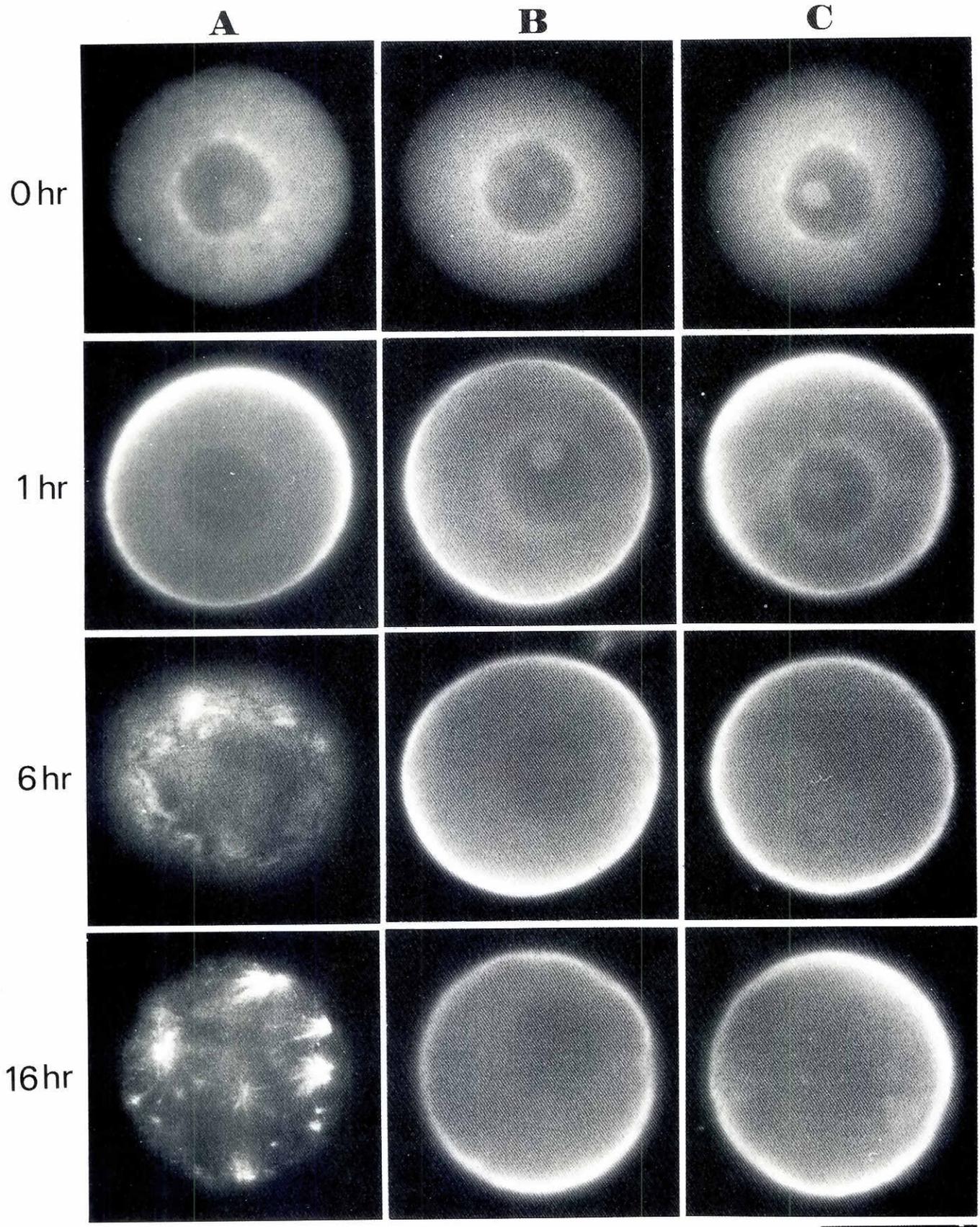
treatment with activators of either protein kinase A (3-isobutyl-1-methylxanthine, IBMX), or protein kinase C (12-O-tetradecanoylphorbol-13-acetate, TPA), which are known to inhibit MPF activation (Bornslaeger *et al.*, 1986a,b). The effects of anucleation were also investigated as, in contrast to IBMX- or TPA-treated oocytes, anucleate fragments undergo certain of the cytoplasmic features of meiotic maturation (Schultz *et al.*, 1978).

Our results suggest that cytoplasmic maturation, and presumably MPF accumulation, rather than a nuclear component, are required for the development of MTOCs.

Results

Taxol induces formation of cytasters

Taxol was used to assess the microtubule nucleating activity of the PCM in maturing mouse oocytes. Competent taxol-treated oocytes were analyzed by anti alpha tubulin immunofluorescence staining.

In agreement with our previous report (Alexandre *et al.*, 1989), oocytes display a microtubular crown associated with the GV at the time of isolation from the follicle. This crown disappears shortly before GVBD (Fig. 1A). No fluorescence is seen in the nucleoplasm. After one hour of culture with taxol, before GVBD, a bright fluorescence appears in the cortex. This submembranous fluorescence is no longer homogeneous after GVBD has taken place (Fig. 1A, 6 h). Microtubules are subsequently organized into numerous cytoplasmic asters from nucleating centers scattered throughout the cortex (Fig. 1A, 16 h). Despite the massive capacity of the cytoplasm to organize asters, no spindle is assembled.

Double fluorescence staining for tubulin and chromosomes of taxol-treated oocytes shows that chromosomes remain more or less clustered (Fig. 2A) and not associated with the asters (Fig. 2B). This confirms our previous cytological observations on taxol-treated oocytes (Alexandre *et al.*, 1989).

Taxol fails to induce formation of cytasters in metabolically-GV-arrested oocytes

As expected, GVBD is prevented in oocytes treated with either IBMX+taxol (Fig. 1B) or TPA+taxol (Fig. 1C). The initial bright submembranous fluorescence remains constant during the entire course of the experiment and asters are never formed.

An ultrastructural analysis confirmed these observations: in oocytes treated with taxol alone, tight bundles of microtubules are found radiating from the cortex (Fig. 3B). Oocytes treated with IBMX+taxol display loose bundles of microtubules, parallel to the plasma membrane, but without any typical aster organization (Fig. 3C).

Is prevention of aster formation related to the lack of nuclear components or to a cytoplasmic effect of TPA and/or IBMX?

The observed inability of taxol to organize cytasters in IBMX- or TPA-containing media does not result from a side-effect of these drugs since cytasters are formed in treated oocytes undergoing
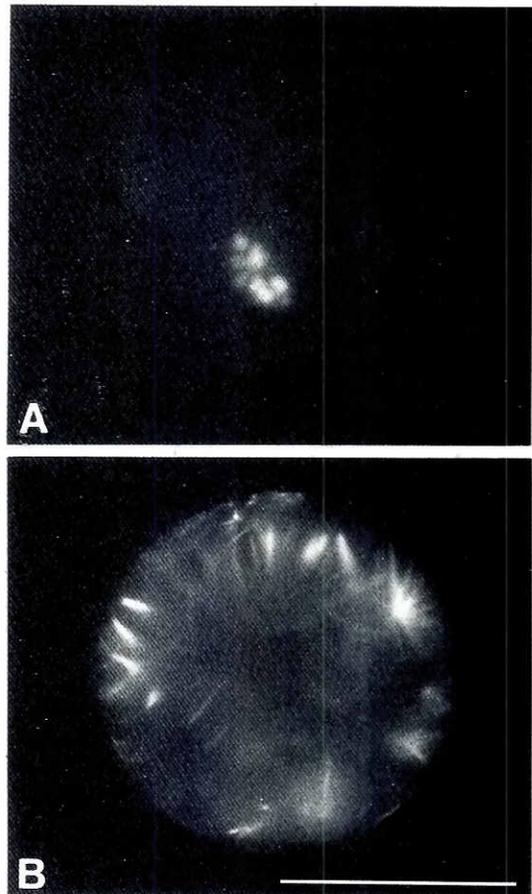

Fig. 2. Double fluorescence staining of a taxol-treated oocyte for (A) chromosomes, and (B) cytasters, with Hoechst dye 33258 and anti alpha tubulin respectively. *Bar, 50 μm.*

GVBD. This was achieved spontaneously in 20 to 40% of the oocytes treated with 10 ng ml^{-1} TPA. In addition, we achieved a similar result by incubating oocytes in the presence of taxol alone for 1 h, then transferring them to an IBMX+taxol-containing medium for a further 15 h. Under these conditions, about 50% of the oocytes undergo GVBD.

In both cases, maturing oocytes display numerous cortical asters, in spite of the presence of the protein kinase activators, whereas oocytes which fail to undergo GVBD display a bright submembranous fluorescence as seen in Fig. 1B,C.

Does microtubule nucleating activity develop in anucleate fragments?

In order to confirm the correlation between GVBD and the ability of PCM foci to nucleate microtubule assembly, we analyzed the

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Fig. 1. Anti alpha-tubulin immunofluorescence staining of competent mouse oocytes at the time of their isolation from follicles (0 h), and after 1, 6, and 16 h of culture. *In oocytes treated with taxol 8.5 μg ml^{-1} (row A), microtubules are organized into numerous asters in the submembranous cortical cytoplasm. In oocytes treated with either IBMX (45 μg ml^{-1})+taxol (row B) or TPA (10 ng ml^{-1})+taxol (row C), germinal vesicle breakdown is prevented. A bright submembranous fluorescence is observed instead of asters. Bar, 50 μm.*
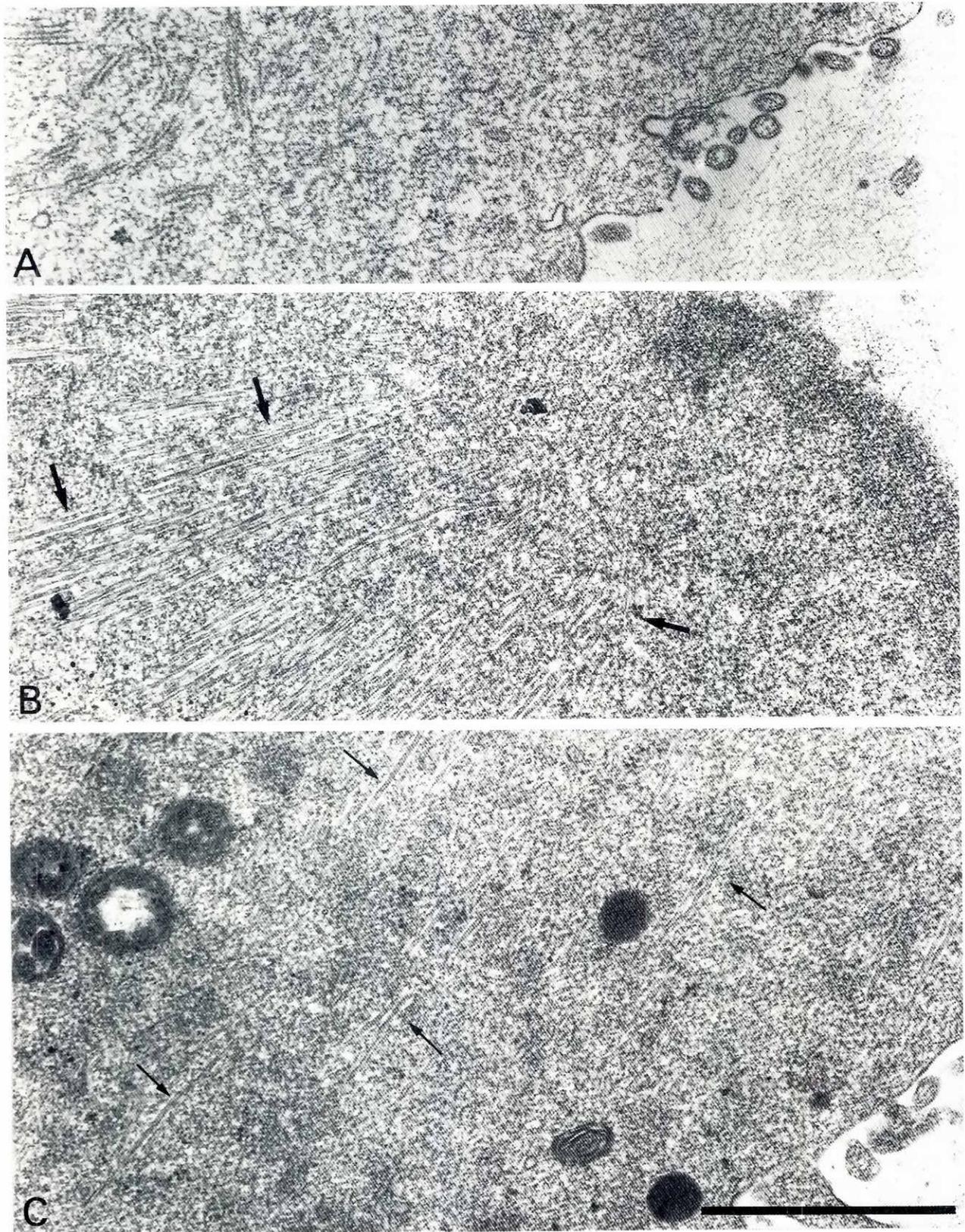
Fig. 3. Ultrastructural analysis: (A) *Control cortex.* **(B)** *Tight bundles of microtubules (arrows) are radiating in the cortex of taxol-treated oocytes, while oocytes treated with IBMX+taxol* **(C)** *show loose bundles of microtubules (arrows), parallel to the plasma membrane, but without aster organization. Bar, 1 μm.*

pattern of taxol-induced microtubule organization in both nucleate and anucleate oocyte fragments cultured for 16 h. Only about one quarter of the collected oocytes gave rise to surviving fragments and, in agreement with previous data, anucleate fragments were more likely to survive (Schultz *et al.*, 1978). In the present study, 86 nucleate and 122 anucleate fragments were obtained from about 400 oocytes. All these fragments had to be processed individually for the immunofluorescence study, which resulted in an additional loss of material. The total numbers of fragments analyzed under each of the three sets of experimental conditions are given in Table 1.

Fig. 4 shows the different patterns of microtubule organization encountered in this study. Nucleate fragments which underwent GVBD in the presence of taxol all display the same pattern as taxol-treated whole oocytes (Fig. 1A, 16 h): several large asters are seen in the cortical area (Fig. 4A,B). The same pattern is found in more than 50% (13/21) of their anucleate counterparts (Fig. 4A'). In the remaining 9 anucleate fragments, tubulin is organized into dense bundles of microtubules with an heterogeneous distribution. However a clear-cut aster-like configuration was never seen (Fig. 4B').

In contrast, and as expected from Fig. 1B,C, taxol induces the appearance of a bright homogeneous submembrane fluorescence in both nucleate and anucleate fragments of IBMX- as well as TPA-treated oocytes (Fig. 4C,C' and D,D' respectively).

Discussion and conclusions

The initial purpose of the present work was to analyze the capacity of mouse oocytes to nucleate microtubule assembly in the absence of mixing between the nucleoplasm and cytoplasm. Taxol was used since it reveals the nucleating activity of PCM in both mouse and amphibian oocytes (Maro *et al.*, 1985; Rime *et al.*, 1987; Jessus *et al.*, 1988). It has been shown that this nucleating activity is modified during meiotic maturation as nucleating stimuli such as heavy water (Heidemann and Kirschner, 1975), heat-shock (Hanocq-Quertier *et al.*, 1987), microinjected centrosomes (Karsenti *et al.*, 1984) or taxol (Jessus *et al.*, 1988) induce cytaster formation in maturing, but not in full-grown amphibian oocytes.

As in *Xenopus* oocytes (Jessus *et al.*, 1988), external taxol rapidly induces tubulin assembly in the submembranous cortical cytoplasm of mouse oocytes. After GBVD, cortical microtubules also reorganize into numerous cortical asters. This confirms previous observations made on both mouse (Maro *et al.*, 1985) and rat oocytes (Albertini, 1987), with short taxol treatments. However, in these two species, meiotic spindles are never observed. In contrast, in taxol-treated oocytes of the surf clam *(Spisula solidissima),* the first meiotic spindle forms, but does not migrate toward the periphery, and persists while the second spindle polymerizes onto the first (Kuriyama, 1986). This discrepancy could be explained by the presence of centrioles associated with the spindle in the surf clam oocyte, while mammalian oocytes lack centrioles. Their meiotic spindles are indeed fitted with an electron-dense amorphous region made of pericentriolar material (PCM) (Szöllösi *et al.*, 1972) known to nucleate microtubule formation (Gould and Borisy, 1977). Maro *et al.* (1985) have shown that each spindle pole of a maturing mouse oocyte is composed of about three PCM foci that are dispersed on nocodazole-induced spindle dissolution. It may thus be hypothesized that the absence of centrioles allows the dispersal of PCM in taxol-treated mouse oocytes, while in other

species such as *Spisula*, centrioles keep PCM in two clusters from which a spindle is organized.

A clear-cut correlation between GVBD and the occurrence of microtubule nucleating activity is demonstrated by the retention of an homogeneous cortical layer of microtubules for as long as 16 h in continuously taxol-treated oocytes prevented from undergoing GVBD by the activation of either PK-A or PK-C (Fig. 1B,C). However, this does not allow us to conclude that a GV factor is required for the acquisition of the aster formation response to taxol. Indeed, under both sets of experimental conditions, prevention of GVBD results from prevention of MPF activation (Bornslaeger *et al.*, 1986a,b). The absence of MPF should therefore be an alternative explanation for the lack of any cortical microtubule nucleating activity in metabolically GV-arrested oocytes. From this point of view, the obvious aster formation in the cortical area of taxol-treated anucleate oocyte fragments is quite interesting. It has indeed been shown that such anucleate fragments, isolated before GVBD, undergo certain of the changes in protein synthesis associated with meiotic maturation in both mice (Schultz *et al.*, 1978) and sheep (Sun and Moor, 1991). They also display a MPF activity after several hours of culture (Balakier and Czolowska, 1977; Balakier and Masui, 1986). Gautier (1987) also showed that nucleoplasm is not an absolute requirement for MPF production in the Axolotl oocyte.

The microtubule nucleating activity of PCM foci seems thus to be closely related to the acquisition of MPF by the ooplasm. This relationship is strengthened by the recent demonstration that activation of MPF coincides with the M-phase specific phosphorylation of a group of proteins (Kuang *et al.*, 1989), and that this M-phase specific phosphorylation is required for the microtubule nucleating capacity of centrosomes (Centonze and Borisy, 1990). However, it is not known whether MPF itself is involved in activation of PCM, either directly or indirectly. The molecular target of this phosphorylation has not been identified since PCM has not yet been defined biochemically. A very interesting candidate might however be the newly discovered γ-tubulin (Oakley and Oakley, 1989) shown very recently to be a highly conserved component of the centrosome-associated PCM (Zheng *et al.*, 1991; Stearns *et al.*, 1991).

The presence of microtubule nucleating centers in anucleate fragments (Fig. 4A') strongly indicates that PCM foci were already dispersed in the cytoplasm of the oocyte at the time of pseudocleavage. This agrees with the previous demonstration that,
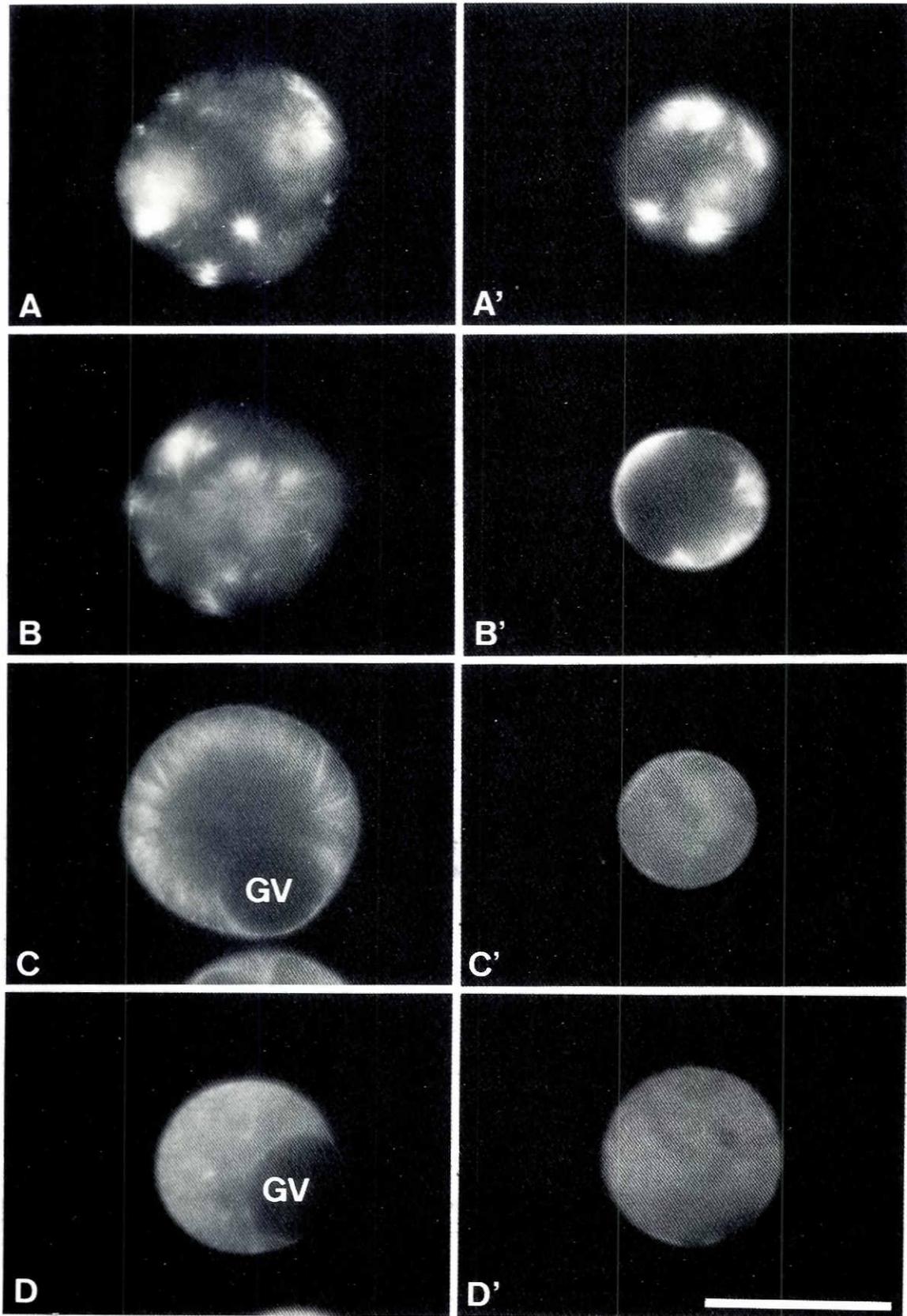
Fig. 4. **Anti alpha-tubulin immunofluorescence staining of mouse oocyte** *nucleate* (**A,B,C,D**) *and anucleate* (**A′,B′,C′,D′**) *fragments cultured for 16 h in taxol-containing medium* (**A,A′,B,B′**) *and in taxol+IBMX* (**C,C′**) *or taxol+TPA-* (**D,D′**) *containing media. GV, germinal vesicle. Bar, 50 μm.*

at the time of GVBD, while most of the PCM foci are located near the chromosomes, some are dispersed into the cytoplasm (Maro *et al.*, 1990). If this were not the case in all oocytes, anucleate fragments without PCM foci could be generated. This might explain why some anucleate fragments cultured for 16 h in control medium do not display any asters. Differences in the stage of acquisition of meiotic competence (Wickramasinghe *et al.*, 1991) at the time of pseudocleavage could be an alternative explanation for this duality in the response of anucleate fragments to taxol. This however seems unlikely since all the oocytes used in this study were selected according to their optimal size (75-80 μm in diameter). Those oocytes failing to undergo completion of meiosis (extrusion of the first polar body) always display a well developed first metaphase spindle (Wickramasinghe *et al.*, 1991; Van Cauwenberge and Alexandre, unpublished observations).

Materials and Methods

Collection and culture of oocytes

Germinal vesicle stage oocytes were obtained from the ovaries of 10-week old NMRI mice, removed and placed in a modified Krebs-Ringer bicarbonate culture medium (KRB-4) (Mulnard and Puissant, 1984), supplemented with crystallized and lyophilized bovine serum albumin (BSA, Sigma). Oocytes were freed of their surrounding granulosa cells by repeated passages through a micropipette. In this way, 20 to 40 oocytes were obtained from each mouse. The oocytes were cultured in KRB4-BSA medium under paraffin oil at 37°C in a humidified atmosphere of 5% CO_2 in air.

Oocyte treatments

Pseudocleavage of GV-intact oocytes was achieved according to the method of Wassarman *et al.* (1976): oocytes were collected and cultured for 3 h in the presence of IBMX 45 μg ml⁻¹ and cytochalasine B (CCB) 5 μg ml⁻¹. They were then transferred to a KRB-4 medium containing IBMX, CCB and pronase 0.5 mg ml⁻¹ in order to remove the *zona pellucida* (15 min at room temperature). The depellucidated oocytes were subsequently transferred to the former medium containing IBMX and CCB. During this treatment, oocytes divide incompletely into nucleate and anucleate compartments. Total separation of nucleate and anucleate fragments was then achieved by gentle pipetting using a mouth-controlled micropipette with a bore size smaller than the diameter of an oocyte. They were cultured separately in individual drops of experimental medium in a 60-well Greiner dish.

Whole oocytes, as well as nucleate and anucleate fragments, were cultured for up to 16 h in the continuous presence of the following drugs: Taxol: 8.5 μg ml⁻¹ (10 μM). Taxol was a gift from Dr. M. Suffness of the NIH, Bethesda, USA.

3-isobutyl-1-methylxanthine, IBMX (Aldrich): 45 μg ml⁻¹.

12-O-tetra-decanoyl phorbol-13-acetate, TPA (Sigma): 10 ng ml⁻¹.

Immunofluorescent staining of microtubules

Mouse oocytes were freed from their *zona pellucida* by brief exposure to acid Tyrode solution (Nicolson *et al.*, 1975) and rinsed in phosphate buffer saline (PBS) containing EDTA (1 mg ml⁻¹), and then in Hanks balanced salt solution minus glucose (BSS). They were simultaneously fixed and permeabilized for 20 min at room temperature in a mixture containing equal amounts of 3.5% paraformaldehyde in 0.1 M phosphate buffer and 0.1% saponin in BSS (Reima and Lehtonen, 1985), then exposed to an anti alpha tubulin monoclonal antibody (Amersham) diluted 1:4000 in BSS containing 0.05% saponin and 0,5% bovine serum albumin (BSA) for 40 min at room temperature. They were then rinsed twice for 20 min in BSS containing 0.05% saponin, once in the same solution supplemented with 0.1% BSA and subsequently exposed to a fluorescein or Texas Red-labeled sheep anti-mouse Ig G antibody (Amersham) diluted 1:40 in BSS containing 0.05% saponin and 0.9% BSA for 40 min, in the dark at room temperature. Oocytes were then rinsed. Some were incubated in Hoechst dye 33258 (Calbiochem)

5μg ml⁻¹ in PBS for 10 min at 20°C in order to stain the chromosomes. They were mounted in 50% glycerol in PBS buffer and examined with a Leitz microscope equipped with an epi-illuminator and appropriate filters.

Transmission electron microscopy

Oocytes were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer with 5% sucrose for 30 min., washed with the same buffer, postfixed with 1% osmium tetroxide in cacodylate, dehydrated in ethanol and embedded in epoxy resin (EPON 812). Ultra-thin sections were stained with uranyl acetate followed by lead citrate and were observed with an AEI EM6B electron microscope.

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