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Tumor promoter induces reorganization of actin filaments and calspectin (fodrin or nonerythroid spectrin) in 3T3 cells

(phorbol 12-myristate 13-acetate/vinculin/trifluoperazine/adhesion plaque/focal contact)

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ABSTRACT **We have used immunofluorescence, differential-interference-contrast, and interference-reflection microscopy to examine the translocation of actin filaments and calspectin (fodrin or nonerythroid spectrin) in 3T3 cells induced by phorbol 12-myristate 13-acetate (PMA). The two cytoskeletal proteins were observed to localize in dot structures that corresponded to the cell–substratum contact sites (focal contact) of the cytoplasmic surface of the plasma membrane. The induction of these cytoskeletal changes was specific for tumor promoters. High-resolution microscopy revealed that calspectin was intensely concentrated in ring-like structures surrounding actin dots. It was also located within the areas of actin dots, but to a lesser extent. Trifluoperazine and other phenothiazine derivatives inhibited the formation of those dot structures that appeared after the addition of PMA. Some serine protease inhibitors were also demonstrated to influence cytoskeletal changes by PMA. Our results provide evidence that calspectin is closely associated with actin filaments in dot structures induced by PMA. Possible mechanisms for these cytoskeletal changes produced by PMA are discussed.**

Tumor promoters are known to have various effects on cellular functions. Phorbol 12-myristate 13-acetate (PMA), a potent tumor promoter, induces change in shape, redistribution of cytoskeletal proteins, increase in plasminogen activator production, reduction of cell surface-associated fibronectin, stimulation of DNA synthesis, of cell growth, and of hormone secretion, and enhancement of membrane fluidity (see ref. 1 for a review). Among these alterations, the most prominent cytoskeletal change is a rapid dissolution of stress fibers with an increasing formation of large actin aggregates, i.e., actin dots, at the cell–substratum contact sites (2–4). It has been reported (3, 4) that vinculin and α-actinin, which might be involved in the actin filament–membrane interaction, also redistribute in association with reorganization of actin filaments during PMA treatment. These dramatic changes of cytoskeletal proteins resemble those induced by oncogenic viruses (1). Therefore, tumor promoters are considered to be a good tool to study cytoskeletal changes that are associated with cellular transformation.

In analogy with the erythrocyte cytoskeleton, a member of the spectrin family in nonerythroid cells (so-called calspectin or fodrin) has been suggested as another candidate for an actin filament attachment site in the plasma membrane. In fact, biochemical and immunocytochemical studies support the idea that it mainly locates at the inner surface of the plasma membrane (5–9). In cultured cells, it remains on the cytoskeletal meshwork even after cells are permeabilized with detergent (10, 11). Like erythrocyte spectrin, it also binds to globular (G) and filamentous (F) forms of actin, resulting in an increased viscosity of actin filaments *in vitro* (12, 13). In spite of these observations, no one has been able to demonstrate the direct interaction between calspectin and actin filaments that accompanies the modulation of cellular functions.

In this report, by using techniques of immunofluorescence, differential-interference-contrast, and interference-reflection microscopy, we compared the location of actin filaments with that of calspectin during PMA treatment. Consequently, we found that a part of calspectin redistributed into the cell–substratum contact sites corresponding to the areas of actin dots.

MATERIALS AND METHODS

Materials. BALB/c 3T3 cells were purchased from American Type Culture Collection. Phenothiazine derivatives, including trifluoperazine, chlorpromazine, and fluphenazine, were generous gifts from Yoshitomi Pharmaceutical (Osaka, Japan). The sources of materials used in this work were as follows: PMA, phorbol 12,13-dibenzoate, phorbol 12,13-diacetate, and 4α-phorbol 12,13-didecanoate from LC Services (Woburn, MA); aprotinin, rhodamine-labeled phalloidin, and N^6,$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate (Bt_2cAMP) from Sigma; leupeptin and pepstatin from Peptide Institute (Osaka, Japan); benzamidine and 1,10-phenanthroline from Nakarai Chemicals (Kyoto, Japan); rabbit anti-vinculin antiserum from Transformation Research (Framingham, MA); fluorescein isothiocyanate-conjugated anti-rabbit IgG from Miles–Yeda (Rehovot, Israel).

Cell Culture. BALB/c 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum in a humidified atmosphere of 5% CO_2/95% air. For immunofluorescence, differential interference contrast, and interference reflection microscopy the cells were grown on glass coverslips. Phorbol diesters were dissolved in dimethyl sulfoxide (2 mg/ml) and added to the culture medium at the indicated concentrations. Control cells were treated with 0.01% dimethyl sulfoxide.

Preparation of Antibodies. Calspectin was purified from bovine brains with the method of Sobue *et al.* (12). Antiserum to calspectin was raised in New Zealand rabbits (11). The IgG fraction from the anti-calspectin antiserum was prepared by the $(NH_4)_2SO_4$ fractionation, followed by DE-AE-cellulose column chromatography as described (11). Highly purified calspectin (5 mg) was coupled to 2 ml of cyanogen-activated Sepharose 4B for use in the affinity column. The IgG fraction was passed through the affinity column, after which the column was extensively washed with isotonic phosphate-buffered saline (PBS). Calspectin antibodies bound to the affinity column were eluted with 0.1 M glycine hydrochloride, pH 2.3. The eluted material was

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Abbreviation: PMA, phorbol 12-myristate 13-acetate.

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immediately neutralized with 1 M Tris base and dialyzed against PBS containing 1 mM NaN_3.

Immunofluorescence Microscopy. The cells were washed twice with buffer A (60 mM Pipes/25 mM Hepes/10 mM EGTA/2 mM $MgCl_2$, pH 6.9) and fixed with 3.5% (wt/vol) paraformaldehyde in buffer A for 20 min at room temperature. The fixed cells were permeabilized with 0.2% (vol/vol) Triton X-100 in buffer A for 5 min. In some experiments, before fixation the cells were incubated with 0.1% (vol/vol) Triton X-100 in buffer A containing protease inhibitors (leupeptin at 1 μg/ml and 0.25 mM phenylmethylsulfonyl fluoride) for 1 min at room temperature and then fixed with 3.5% (vol/vol) paraformaldehyde in buffer A. After fixation, the cells were washed four times with PBS and incubated for 60 min with rhodamine-labeled phalloidin or calspectin antibodies. The cells were then washed three times with PBS and stained with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG for 30 min with the exception of rhodamine-labeled phalloidin staining. The stained cells were extensively washed with PBS and were mounted in 50% (vol/vol) glycerol in PBS. The cells were viewed with an Olympus fluorescence, differential-interference-contrast, and interference-reflection optic microscope. Photographs were recorded on Kodak Tri-X film for fluorescence and differential-interference-contrast microscopy and on Kodak Panatomic-X film for interference-reflection microscopy. In double-fluorescence experiments, the fixed cells were stained with a mixture of rhodamine-labeled phalloidin and calspectin or vinculin antibodies.

RESULTS

BALB/c 3T3 cells were elongated in appearance. When exposed to PMA, the cells became rounded, and a majority of cells displayed active ruffles at their periphery. In some cells, prominent protrusions were observed. The distribution of actin filaments was determined by fluorescence microscopy using rhodamine-labeled phalloidin, which specifically binds to the filamentous actin. The location of calspectin was examined by using the specific antibodies against calspectin with fluorescein isothiocyanate-conjugated second antibodies. The specificity of calspectin antibodies was examined by immunoblot analysis (14). The antibodies specifically recognized the 240/235-kDa doublet polypeptides in the blot containing the total protein from 3T3 cells. These doublet polypeptides were identical to the brain calspectin used for the immunogen.

The effect of PMA on cytoskeletal changes including actin filaments and calspectin was investigated over a wide range of PMA concentrations (0.5–200 ng/ml). PMA in the range of 5–50 ng/ml induced a dramatic change. At >50 ng/ml, PMA had a cytotoxic effect on 3T3 cells as determined by trypan blue dye exclusion. Accordingly, a PMA concentration of 25 ng/ml was chosen for further experiments.

Cytoskeletal changes during exposure to PMA are shown in Fig. 1. Untreated cells showed many extended stress fibers (Fig. 1*A*), whereas the distribution of actin filaments was rapidly altered by PMA. Within 5 min, there was a substantial loss of stress fibers. Between 5 and 30 min, a majority of stress fibers had disappeared, and some actin filaments had redistributed in protrusions and peripheral ruffles. In addition, <30% of the total cells showed dot-like structures of actin in their cytoplasm. These actin dots progressively increased over the next 60 min (>80% of PMA-treated cells, Fig. 1*B*).

Calspectin was diffusely distributed over the entire cell in addition to higher concentrations in the perinuclear region of the cytoplasm (Fig. 1*C*). Although PMA treatment did not induce a significant change in the intracellular location of calspectin for the first 5 min, a portion of the calspectin was redistributed into dot-like structures during the following 5–30 min. After 60 min, a predominance of dot-like structures containing calspectin was observed (Fig. 1*D*). The effects of PMA were reversible. A complete reversal of morphological and cytoskeletal changes was attained within

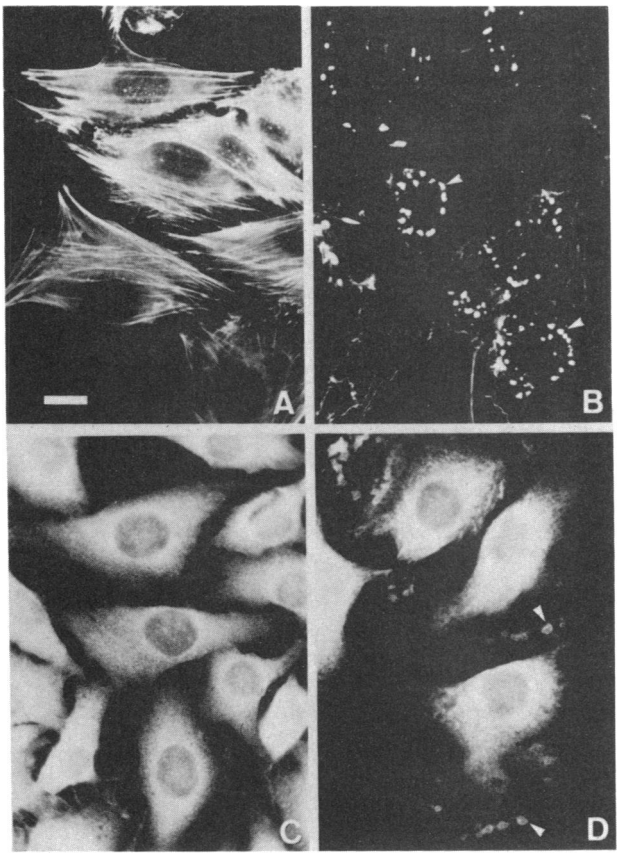

FIG. 1. Effect of PMA on reorganization of actin filaments (*A* and *B*) and calspectin (*C* and *D*) in 3T3 cells. Control 3T3 cells were treated with 0.01% dimethyl sulfoxide (*A* and *C*); 3T3 cells were treated with PMA at 25 ng/ml for 60 min (*B* and *D*). Actin and calspectin dots were detected in the cytoplasm (*B* and *D*, arrowheads). (Bar = 10 μm.)

Table 1. Effect of phorbol diesters on a redistribution of actin filaments and calspectin

Phorbol diester	Stress fibers, % of cells	Actin dots, % of cells	Calspectin dots, % of cells
PMA			
50 ng/ml	11	89	88
25 ng/ml	15	82	84
5 ng/ml	48	60	61
PBz_2			
125 ng/ml	26	69	68
25 ng/ml	49	33	34
5 ng/ml	81	8	8
PAc_2			
125 ng/ml	95	9	8
25 ng/ml	100	3	4
5 ng/ml	100	1	1
$4\alpha PDco_2$			
125 ng/ml	100	1	1
25 ng/ml	100	0	0
5 ng/ml	100	0	0

After phorbol diester treatment for 60 min, 200 cells on each coverslip were scored. Numbers represent the percentage of phorbol diester-treated cells containing stress fibers, actin dots, or calspectin dots. PBz_2, phorbol 12,13-dibenzoate; PAc_2; phorbol 12,13-diacetate; $4\alpha PDco_2$, 4α-phorbol 12,13-didecanoate.

6 hr after the removal of PMA from the culture medium (Fig. 1*D*).

Table 1 shows the effect of other phorbol diesters on reorganization of actin filaments and calspectin. Phorbol 12,13-dibenzoate, another potent tumor promoter, also induced reorganization of actin filaments and calspectin. Phorbol 12,13-diacetate and 4α-phorbol 12,13-didecanoate, which are inactive as tumor promoters, failed to induce such cytoskeletal changes.

We then analyzed dot structures in detail with double-fluorescence, differential-interference-contrast, and interference-reflection microscopy. The location of the cytoskeletal proteins was examined with double-fluorescence microscopy using rhodamine-labeled phalloidin for actin filaments and

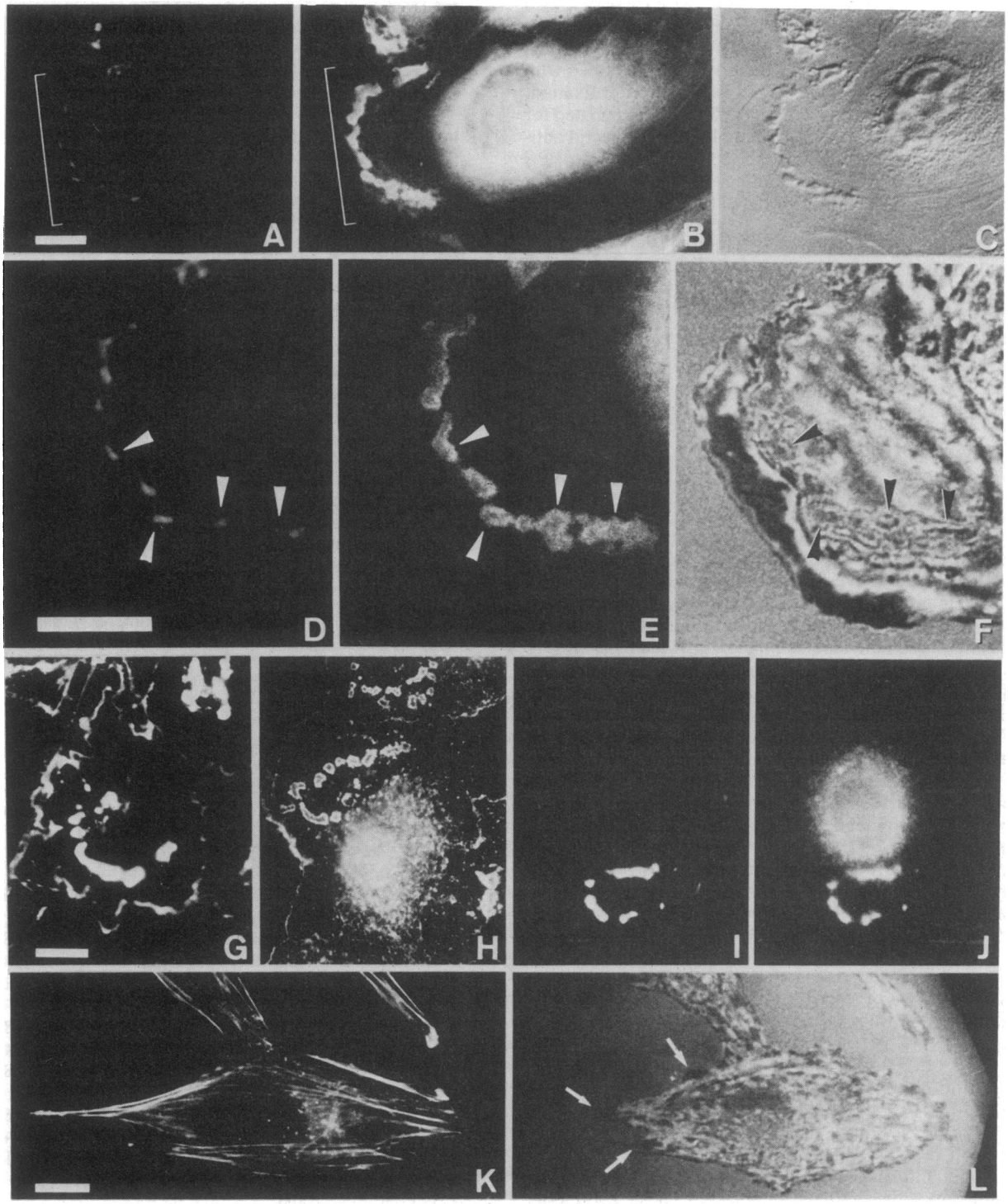

FIG. 2. Double-labeled fluorescence staining of PMA-treated 3T3 cells with rhodamine-labeled phalloidin (*A*) and calspectin antibodies using fluorescein isothiocyanate-conjugated second antibodies (*B*). (*C*) Differential-interference-contrast micrograph of the cell in *A* and *B*. Marked areas in *A* and *B* are enlarged by high-resolution-fluorescence (*D* and *E*) and interference-reflection (*F*) microscopy. Arrowheads, typical examples of the ring-like structures. PMA-treated cells were permeabilized with Triton X-100 before fixation and then stained with rhodamine-labeled phalloidin (*G*) or calspectin antibodies (*H*). Double-fluorescence micrographs of PMA-treated cells with rhodamine-labeled phalloidin (*I*) and vinculin antibodies (*J*). Fluorescence micrograph of untreated cells stained with rhodamine-labeled phalloidin (*K*) and interference-reflection micrograph of the corresponding cells (*L*). Arrows, adhesion plaques. (Bars = 10 μm.)

rabbit anti-calspectin antibodies stained with fluorescein isothiocyanate-conjugated anti-rabbit IgG for calspectin. There was a strong topological correspondence between actin and calspectin dots (Fig. 2 *A* and *B*). Differential-interference-contrast microscopy revealed the protuberances from the ventral surface of the plasma membrane (Fig. 2*C*). It was apparent that the protuberances precisely coincided with dot structures of actin and calspectin. Calspectin dots, however, were much larger than actin dots. Double-labeled dot structures were further observed with high-resolution microscopy. Calspectin was intensely concentrated in ring-like structures surrounding actin dots (Fig. 2 *D* and *E*). It was also located within the areas of actin dots, but to a lesser extent. Interference-reflection microscopy developed by Curtis (15) was used to evaluate the distance between the ventral surface of the plasma membrane and the substratum. With this technique it was found that dot structures containing actin and calspectin exhibited central gray dots surrounded by white rings (Fig. 2*F*). This result suggests that dot structures loosely bind to the substratum. Conversely, adhesion plaques, the location of the stress fiber termini, had an intensely dark image with an arrowhead-like shape indicating a tight apposition to the substratum (Fig. 2 *K* and *L*). Even if cells were extracted with detergent prior to fixation, both actin and calspectin remained on dot structures (Fig. 2 *G* and *H*). These observations imply that calspectin dots, in addition to actin dots, link to the detergent-resistant cytoskeletal meshwork. Unlike calspectin dots, the size of vinculin dots was the same as that of actin dots (Fig. 2 *I* and *J*).

We examined whether other agents could mimic or modulate the above cytoskeletal changes caused by PMA. When trifluoperazine was added to the culture medium prior to PMA treatment, the PMA-induced actin- and calspectin-dot formation was completely inhibited. Although the PMA-induced disappearance of stress fibers was not affected by trifluoperazine, actin filaments were concentrated at the cell periphery (Fig. 3 *B* and *F*). Other phenothiazine derivatives, including chlorpromazine and fluphenazine, also inhibited PMA-induced actin- and calspectin-dot formation, but to a lesser extent. Some serine protease inhibitors, including benzamidine and 1,10-phenanthroline, partially inhibited the PMA-induced disappearance of stress fibers. Additionally, the size of PMA-induced actin and calspectin dots tended to be unequal with these protease inhibitors (Fig. 3 *C*, *D*, *G*, and *H*). Other protease inhibitors, leupeptin, pepstatin, and aprotinin, did not show any significant effect on PMA-induced cytoskeletal changes. As a control, neither phenothiazine derivatives nor protease inhibitors used in this experiment could produce cytoskeletal changes without PMA (data not shown).

DISCUSSION

Rifkin *et al.* (2) first reported PMA-induced reversible changes in actin filament distribution in chicken embryo fibroblasts. Subsequently, PMA-induced cytoskeletal changes have been noted in many studies (2–4, 16–18). Independent lines of evidence have led to the conclusion that cytoskeletal changes induced by tumor promoters seem to

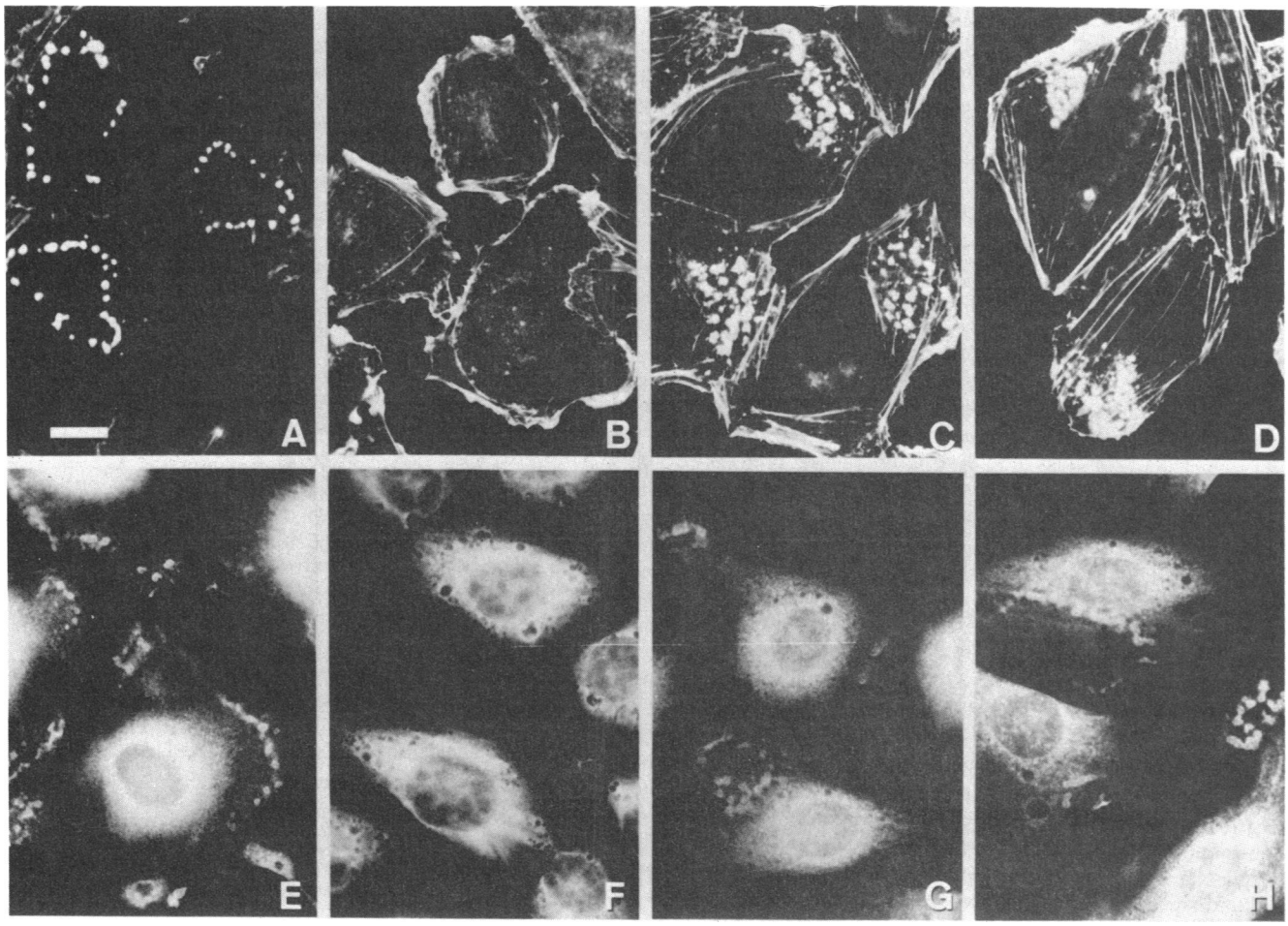

FIG. 3. Effects of various agents on PMA-induced cytoskeletal changes. The cells were stained with rhodamine-labeled phalloidin (*A–D*) and calspectin antibodies (*E–H*). The cells were preincubated with 25 μM trifluoperazine for 2 hr followed by the addition of PMA at 25 ng/ml for 60 min (*B* and *F*). The cells were incubated for 60 min with PMA at 25 ng/ml (*A* and *E*), with PMA at 25 ng/ml plus 2 mM benzamidine (*C* and *G*), and with PMA at 25 ng/ml plus 0.3 mM 1,10-phenanthroline (*D* and *H*). (Bar = 10 μm.)

resemble those of transformed cells. Following virus-infected transformation, actin, vinculin, talin, and α-actinin have been shown to redistribute into dot structures that correspond to close contacts with the substratum (19–24). Based on the morphological observations, Tarone *et al.* (22) pointed out that these dot structures and adhesion plaques were different from each other (22).

In this report, we investigated the effect of PMA on reorganization of actin filaments and calspectin. These two cytoskeletal proteins were redistributed into dot structures that loosely bound to the substratum. A similar translocation of actin filaments and of calspectin into dot structures was also observed in the cells transformed by some avian sarcoma viruses (unpublished results). The dot-shaped concentration of the two proteins by tumor promoter, therefore, might be related to that by viral infection. High-resolution microscopic analyses of dot structures revealed that calspectin had a precise topological distribution in ring-like structures surrounding actin dots, in addition to the areas corresponding to actin dots. These results suggest that at least a part of calspectin might be bound to actin filaments in the areas where the two proteins colocalized. In addition, it was apparent that both cytoskeletal dots were linked to the detergent-resistant cytoskeletal meshwork. Marchisio *et al.* (24) have reported that vinculin forms a doughnut-like ring surrounding actin dots in the cells transformed by Rous sarcoma virus. In contrast to their observations, our results showed that the size of vinculin dots coincided with that of actin dots. This indicates that in a case of PMA-induced transformation the location of calspectin in dot structures is different from that of vinculin.

The common mechanism of cytoskeletal changes induced by tumor promoters and by viral infection is not yet understood. In our preliminary experiments, PMA treatment did not affect the turnover rates of calspectin and actin in 3T3 cells determined by the pulse–chase experiment using [^{35}S]methionine, suggesting cytoskeletal changes might be dependent upon a rearrangement of cytoskeletal proteins (data not shown). One of the intracellular binding sites of tumor promoters has been identified as protein kinase C (ref. 25 for a review). As determined by immunoprecipitation, we found that phosphorylation of calspectin and its interacting proteins, 4.1-related protein (26) and calpactin (27), in ^{32}P-labeled 3T3 cells was not significantly enhanced by PMA. In addition, Bt_2cAMP had also no effect on the cytoskeletal rearrangement and phosphorylation of the above proteins regardless of the presence or absence of PMA (data not shown). Consequently, the direct phosphorylation of calspectin and its interacting proteins resulting in a cascade of cytoskeletal changes is unlikely. Conversely, independent lines of evidence suggest that the oncogene products having tyrosine kinase activity are involved in cytoskeletal changes (24, 28, 29). In association with these, phorbol diesters are known to stimulate a rapid phosphorylation on tyrosine in normal cells (30–32). Stimulation of tyrosine phosphorylation by phorbol diesters suggests that initial stimulation of protein kinase C activates a tyrosine kinase in normal cells. Further studies are required to evaluate the direct or indirect involvement of tyrosine kinase in the PMA-induced cytoskeletal changes.

Other different mechanisms are postulated from the effects of agents on cytoskeletal changes. Phenothiazine derivatives inhibited PMA-induced dot formation. This may be due to either the direct stabilizing effect of their derivatives on plasma membrane (33) or the inhibitory effect on intracellular signal transduction to induce the cytoskeletal rearrangement. Actually, phenothiazine derivatives are known to be calmodulin inhibitors (34) and to inhibit the ligand-induced redistribution of the submembranous cytoskeleton (35). In addition, some serine protease inhibitors were observed to modulate PMA-induced cytoskeletal changes, which implies that serine protease(s) may be involved in these cytoskeletal changes (2). In support of this, degraded fibronectin was found at the focal contacts between dot structures of virus-infected cells and the substratum (23, 36).

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