Phorbol ester-induced actin cytoskeletal reorganization requires a heavy metal ion

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The cell-permeant heavy metal chelator *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) was found to counteract phorbol ester-induced actin reorganization in PTK2 and Swiss 3T3 cells. By using fluorescence and the higher resolution technique of photoelectron microscopy to monitor actin patterns, 15-min pretreatment with 25–50 μM TPEN was found to dramatically reduce actin alterations resulting from subsequent phorbol ester treatment in PTK2 cells. Similar results were obtained with Swiss 3T3 cells using 50 μM TPEN for 1.5 h. Phorbol ester-induced actin alterations are thought to depend on activation of protein kinase C (PKC). In contrast to the phorbol ester effect, the PKC-independent actin cytoskeletal disruption caused by staurosporine and cytochalasin B was unaffected by TPEN pretreatment. TPEN did not block phorbol ester-induced activation of PKC in Swiss 3T3 cells, as observed by the phosphorylation of the 80K PKC substrate protein (MARCKS protein). TPEN also did not inhibit partially purified PKC from Swiss 3T3 cells in an in vitro PKC-specific commercial assay. To establish that the effect of TPEN is the removal of metal ions and not some other nonspecific effect of TPEN, a series of transition metal ions was added at the end of the TPEN pretreatment. The results indicate that the transient but dramatic phorbol ester-induced reorganization of the actin cytoskeleton in cultured cells depends on an interaction of PKC with a heavy metal, probably zinc.

Introduction

Protein kinase C (PKC)[1] is a family of closely related serine/threonine kinases well known for

a key role in many cellular signaling pathways. The general model of PKC activation involves a combination of a calcium-mediated physical relocation of the kinase to the membrane (translocation) and its subsequent activation by phospholipase-generated diacylglycerol (reviewed in Nishizuka, 1984; Niedel and Blackshear, 1986; Huang, 1989). However, it is now clear that the picture is much more complex (Pelech et al., 1990; Bell and Burns, 1991; Gschwendt et al., 1991; Mozier et al., 1991; Trilivas et al., 1991). In addition, a putative zinc-binding site ("zinc finger") has been identified in the structure of PKC (Parker et al., 1986), and recent evidence has been presented to suggest that zinc ion may play an important role in PKC action (Csermely et al., 1988; Forbes et al., 1990a,b). In particular, Forbes et al. (1990a,b) have suggested that zinc may mediate the association of PKC with membrane-associated cytoskeleton, possibly actin.

A rapid and dramatic reorganization of the actin cytoskeleton and its associated structures is one of the prominent physiological events resulting from exposure of cultured cells to the tumor promoter phorbol myristate acetate (PMA); (Schliwa et al., 1984; Kellie et al., 1985; Shiba et al., 1988; Sobue et al., 1988; Turner et al., 1989). Several studies have shown that PKC activation is an essential part of the process (Danowski and Harris, 1988; Hedberg et al., 1990), but there have been no studies designed to ask whether heavy metal ions play a role in this PMA-induced actin reorganization. Ideally, one would have available a series of chelators each one highly specific for one metal ion (e.g., Zn^{2+}) with no significant binding to other metal ions. Because such a series of chelators is not available, we addressed the question of heavy metal ion involvement by pretreating Swiss 3T3 and PTK2 cells with the cell-permeant broad-spectrum heavy metal chelator *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN)

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[1] Abbreviations used: BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, acetoxymethyl ester; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; PEM, photoelectron microscopy; PKC, protein kinase C; PMA,

phorbol 12-myristate 13-acetate; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPEN, *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine.

(Csermely *et al.,* 1988). The subsequent actin alterations resulting from PMA exposure were examined by fluorescence microscopy and the higher resolution technique of photoelectron microscopy. We also looked for modulation of actin reorganization induced by staurosporine and by cytochalasin B, agents that act via different yet PKC-independent pathways, and examined whether TPEN inhibits the activity of partially purified PKC from Swiss 3T3 cells. The results suggest that although TPEN does not affect the PKC-independent staurosporine- and cytochalasin-induced actin reorganization, this chelator does significantly reduce the PKC-dependent phorbol ester-induced actin reorganization, and furthermore, it does so without direct inhibition of PKC. This provides a link between the previously reported biochemical studies on the involvement of a heavy metal (i.e., zinc) in PKC model system studies and the observation of actin reorganization in intact cultured cells.

Results

PTK2 epithelial cell cultures consist of colonies of fairly flat polygonal cells. As with many cell types cultured on a solid substrate, PTK2 cells form a highly organized actin cytoskeleton that can be observed by fluorescence with fluorescent phalloidin derivatives or with an anti-actin antibody. Figure 1A is a fluorescence micrograph of rhodamine-phalloidin visualized actin patterns in a control culture of PTK2 cells. Typical features include an abundance of straight actin microfilament bundles that traverse the cytoplasm and delineate cell–cell boundaries (Figure 1A, arrows) and also form semicircular bands along the unbounded peripheries of cells at the colony edge (Figure 1A, arrowheads). Exposure to the tumor promoter PMA, which is a potent activator of PKC, has a profound effect on this actin organization in PTK2 and similar cells (Schliwa *et al.,* 1984; Hedberg *et al.,* 1990). As shown in Figure 1B, 20-min exposure to 200 nM PMA results in a drastic depletion of microfilament bundles, whereas aggregates and bands of actin staining appear within the cytoplasm and along the edges of cells (Figure 1B, arrows). The process continues over ~1 h until the cells are severely arborized with no evidence of an organized actin cytoskeleton, and then in the course of several hours the culture recovers to a nearly normal appearance (not shown).

To investigate whether the cell-permeant chelator TPEN might modulate the PMA-induced actin alterations, PTK2 cultures were pretreated with 50 μM TPEN for 30 min and then exposed to dimethylsulfoxide (DMSO)-containing control medium (Figure 1C) or to 200 nM PMA (Figure 1D) in the continuing presence of TPEN for an additional 30 min. The actin patterns in TPEN-only control cultures (Figure 1C) were very similar to those of untreated control cultures (Figure 1A), with the exception that the colonies of TPEN-treated cells had somewhat irregular peripheries and the cells at the colony edges tended not to have the usual peripheral band of organized microfilaments (Figure 1A, arrowheads). The cultures that were pretreated with TPEN and then exposed to PMA (Figure 1D) were significantly less sensitive to PMA-induced actin reorganization than cultures that were exposed to PMA only (Figure 1B). Although actin aggregates characteristic of PMA treatment were frequently visible in regions of TPEN-PMA treated cultures, actin microfilament bundles were nearly as abundant as in cultures that were not exposed to the tumor promoter. The extent of the PMA-induced actin disruption was clearly reduced by TPEN even in cultures that were pretreated with only 15 min of 25 μM TPEN (not shown).

The effect of TPEN on phorbol ester-induced actin alterations was also documented at higher resolution by photoelectron microscopy (PEM) of samples prepared in parallel to those shown in Figure 1, A–D. PEM is an emission-based, surface-sensitive form of electron microscopy that does not require that the samples be stained, shadowed, or coated with metal (Griffith and Birrell, 1985). The technique allows direct examination of cytoskeletal structures in thin regions of cultured cells provided that the surface membrane is first removed by neutral detergent treatment. The immunogold technique can be used in PEM for selective labeling of specific structures but usually the individual cytoskeletal systems are recognizable by virtue of their characteristic appearance (Hedberg and Griffith, 1986). Because of the ease of sample preparation, the available resolution (~10 nm), and the unique information content of PEM images, this technique has proven useful for documentation of alterations in cytoskeletal organization (Birrell *et al.,* 1989; Hedberg *et al.,* 1990).

In Figure 2, A–D, photoelectron images of control, PMA-treated, and TPEN-treated cells corresponding to comparable regions of the fluorescence samples of Figure 1, A–D are shown. Although unlabeled, the actin cytoskeleton in the PEM images is easily recognized by comparison with the rhodamine-phalloidin flu-
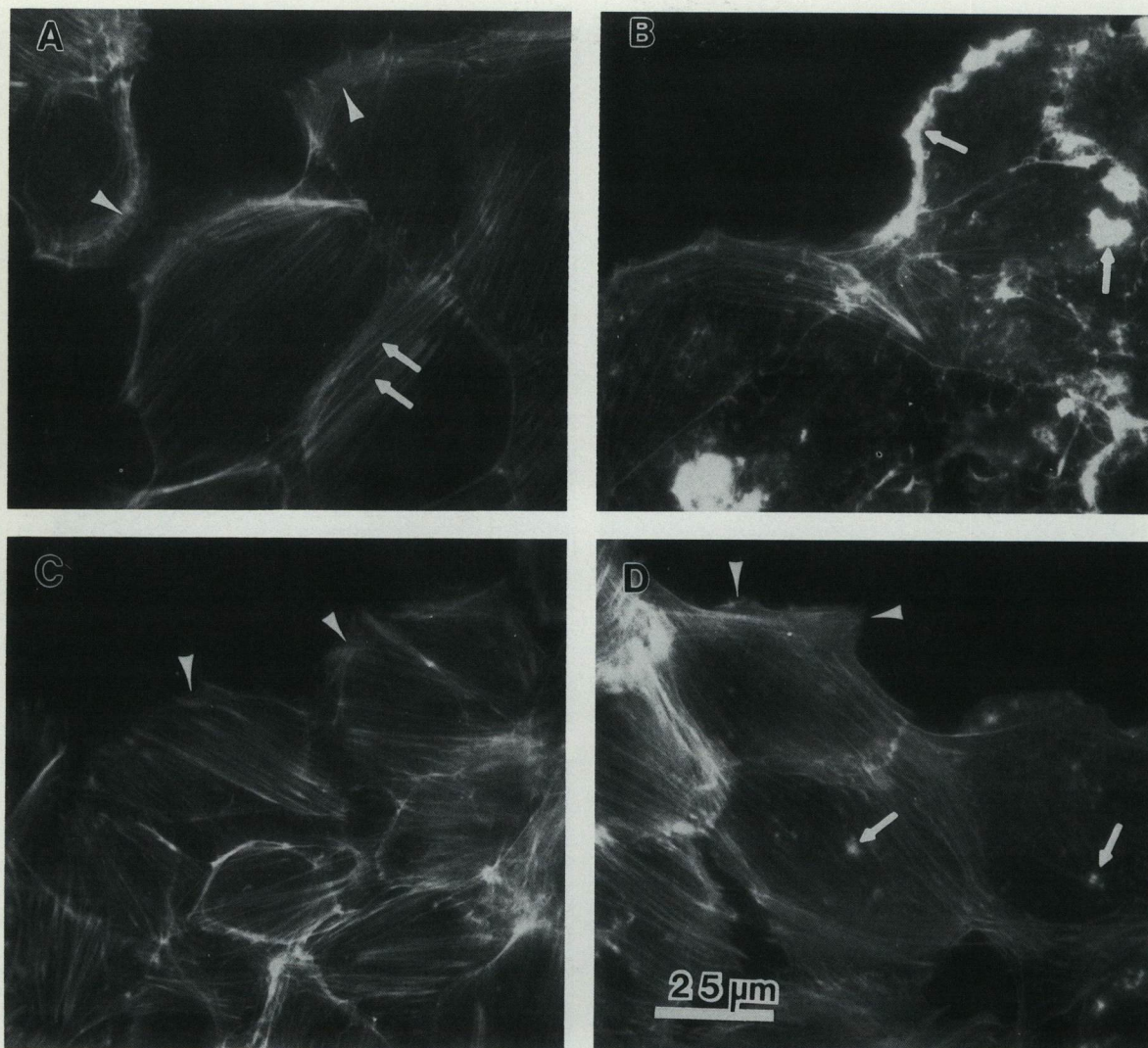
Figure 1. **Pretreatment with TPEN counters phorbol ester induced-loss of actin microfilaments in PTK2 cells.** Cell cultures were pretreated with (A, B) control medium; or (C, D) 50 μM TPEN in culture medium for 0.5 h, followed by addition of (A, C) DMSO vehicle in medium or (B, D) 200 nM PMA for 20 min. Arrows in (A) indicate actin microfilament bundles; arrows in (B, D) indicate actin filament aggregates. Arrowheads in (C, D) point out irregular cell peripheries frequently seen in TPEN-treated cultures.

orescence images of Figure 1. The micrographs of control and TPEN-treated cytoskeletal preparations (Figure 2, A, C, and D) are characterized by the actin microfilament bundles that dominate the images (arrows). These traverse the cytoplasmic space and can be frequently seen to terminate in fine brush-like regions of actin filament-membrane contact (arrowheads), as well as forming a dense band of cytoplasmic filaments 5–10 μm from the edge of the cell in control cultures (Figure 2A). This is in stark contrast to the appearance of cytoskeletal preparations from cells that were exposed to PMA without TPEN pretreatment. In the cell shown

in Figure 2B, recognizable microfilament bundles are absent and a band of aggregated actin appears in a long mass near the edge of the cell (arrows). In the absence of an intact actin cytoskeleton, the thick curving keratin bundles of the PTK2 intermediate filament system are exposed and are easily recognized (black arrowheads).

Photoelectron images of cells pretreated with TPEN (Figure 2, C and D) reveal numerous microfilament bundles in the cytoplasmic space. With subsequent PMA exposure, these bundles persist (Figure 2D), consistent with the fluorescence observation (Figure 1D) that TPEN acts
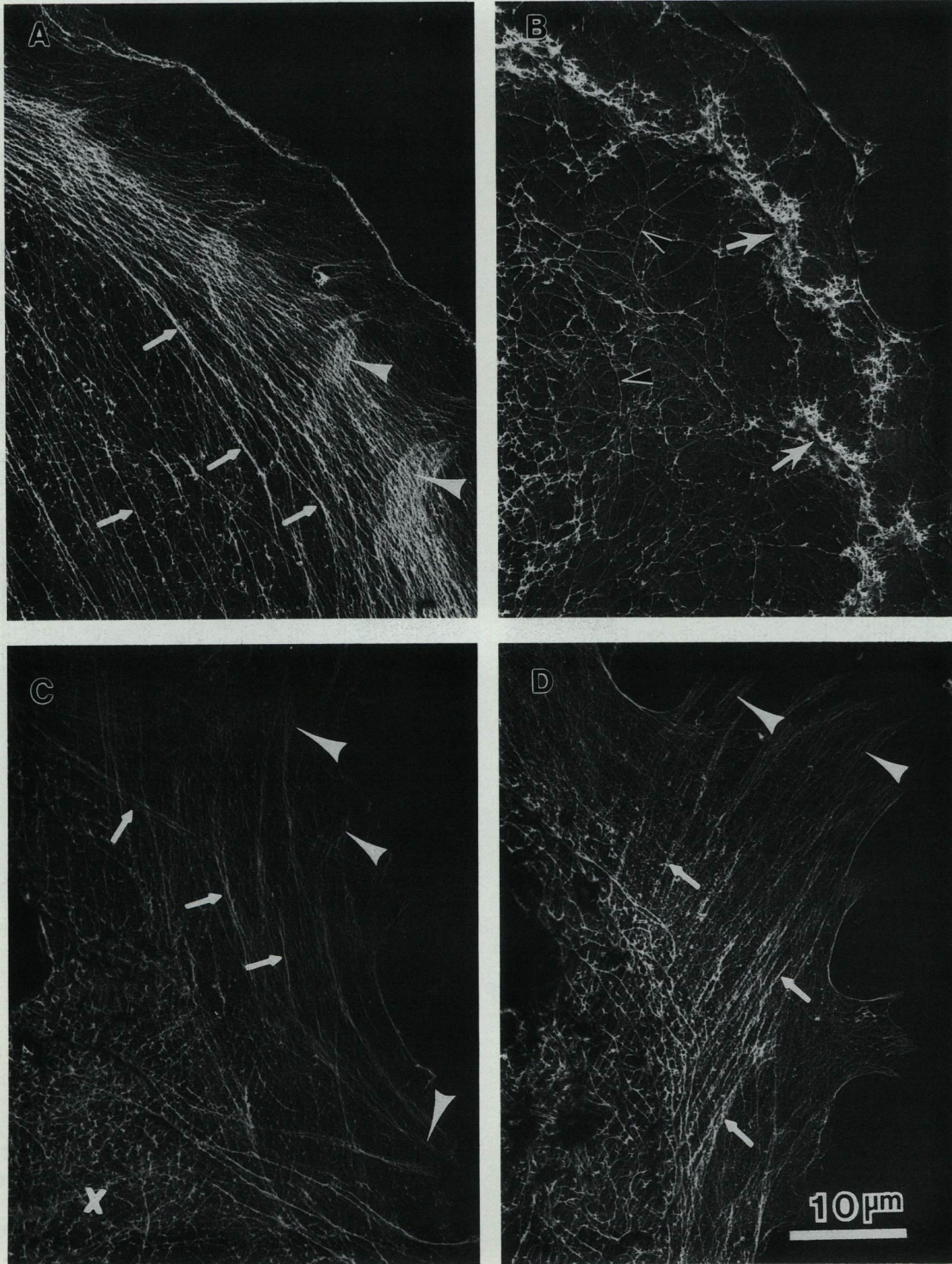
Figure 2. Photoelectron micrographs of samples prepared in parallel to those of the experiment shown in Figure 1. PTK2 cells were grown on PEM sample mounts, exposed to (A, B) control medium or to (C, D) 50 μM TPEN for 0.5 h, then treated with addition of either (A, C) DMSO vehicle in medium or (B, D) 200 nM PMA for 20 min. Cytoskeletal whole-mount specimens were prepared as described in Materials and methods. Arrows indicate abundance of microfilament bundles in control (A) and TPEN-treated (C, D) samples. Arrows in (B) are actin aggregates; arrowheads in (A, C, D) are adhesion plaque regions where microfilament bundles terminate on the lower cell surface.
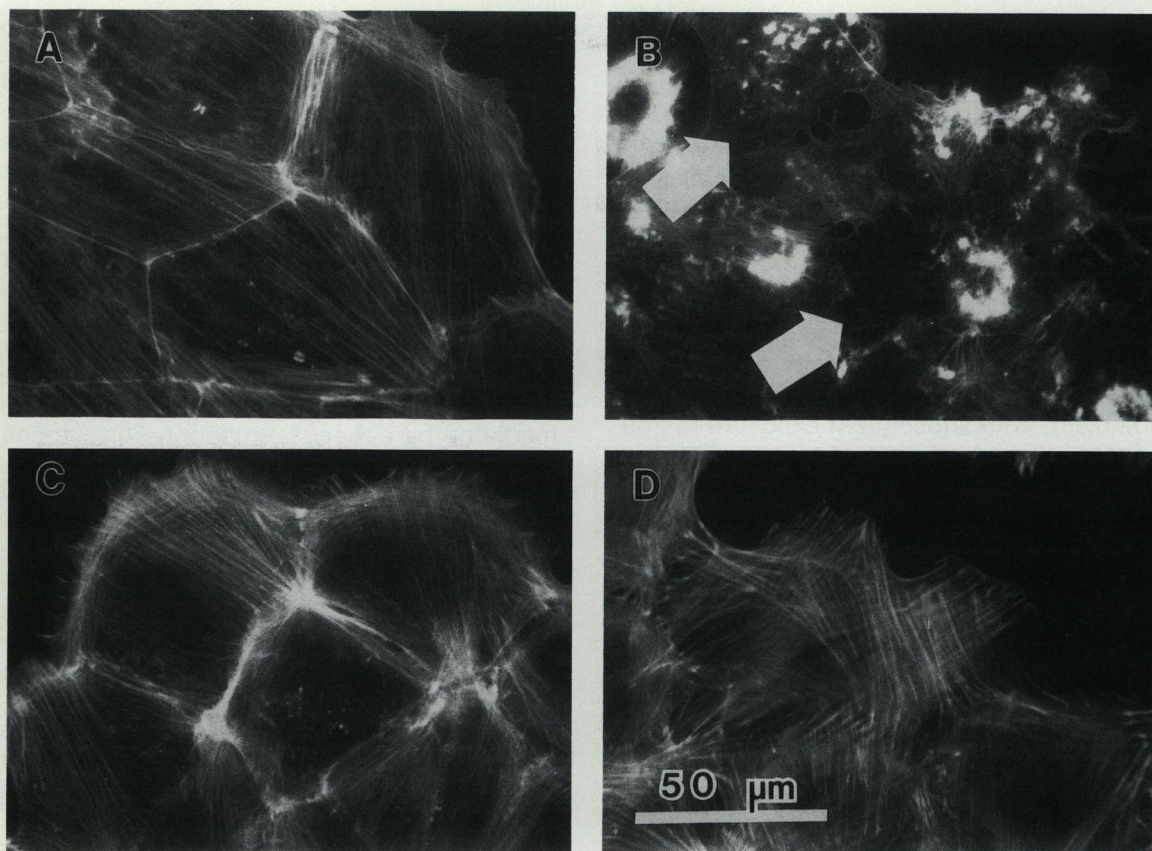
Figure 3. Rhodamine-phalloidin fluorescence of actin patterns in PTK2 cells, demonstrating that ZnCl$_2$ but not FeSO$_4$ can reverse the ability of TPEN to counter the phorbol ester-induced actin disruption. (A–D) Pretreated with 50 μM TPEN in culture medium for 30 min; followed by (A, B) 100 μM ZnCl$_2$; (C, D) 100 μM FeSO$_4$. After 10 min, cultures were then challenged with (A, C) addition of DMSO vehicle only or (B, D) 200 nM PMA for an additional 20 min. Arrows indicate cells depleted of actin microfilament bundles, which dominate in cultures treated with TPEN plus ZnCl$_2$ followed by PMA (B).

to counter the effect of the phorbol ester. It is also clear from the photoelectron micrographs that the irregular cell peripheries observable by fluorescence of TPEN-treated cells (Figures 1, C and D) are due to the presence of adhesion plaque-like regions near or at the very edge of the cell (Figure 2, C and D, arrowheads). These structures, like the microfilament bundles themselves, also appear to be less sensitive to PMA-induced disruption in cells pretreated with TPEN.

To test whether the ability of 50 μM TPEN to reduce the actin disruption induced by PMA was related to its role as a chelator, we also asked whether the effect of TPEN could be reversed by the addition of various transition metal ions. PTK2 cells were first pretreated with 50 μM TPEN alone for 30 min, which is sufficient to accomplish significant protection against PMA-induced actin disruption, as was shown in Figures 1 and 2. Subsequently, metal ions (Mn^{2+}, Fe^{2+}, Ni^{2+}, Co^{+2}, Cu^{2+}, and Ni^{2+}) were added to

give 100 μM final concentration for an additional 10 min followed by challenge with addition of either control medium or PMA to 200 nM final concentration for 20 min. The metal ions alone did not alter actin patterns significantly in either control or PMA-treated cells. The addition of 100 μM ZnCl$_2$ to the TPEN-treated culture before the addition of PMA is sufficient to cancel the protective effect of TPEN pretreatment, as seen in Figure 3, A and B. Microfilament-depleted cells and actin aggregates dominate in the image of the sample, which now resembles a culture exposed to PMA without benefit of prior TPEN treatment (compare Figure 3B with 1B). The ability of Zn^{+2} to reverse the TPEN effect was not duplicated by 100 μM FeSO$_4$ (Figure 3, C and D) nor by 100 μM MnCl$_2$ or 100 μM NiCl$_2$ (not shown). However, 100 μM CuCl$_2$ and 100 μM CoCl$_2$, like ZnCl$_2$, did reverse the TPEN effect.

Because Ca^{2+} is the intracellular ion with a documented role as a second messenger, the

effect of TPEN was also compared with that of the cell-permeant calcium chelator 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, acetoxymethyl ester (BAPTA-AM) (Tsien, 1980). PTK2 cultures were pretreated with 25 μM BAPTA-AM for 30 min, which is sufficient to hold intracellular Ca^{2+} close to a baseline level of 100 nM (Rodland *et al.*, 1990). In contrast to the somewhat irregular, more angular cellular peripheries typical of TPEN pretreated cells, BAPTA-AM pretreated cells had a flatter, more pancake-like cell morphology with a somewhat less distinct actin staining pattern than controls (not shown). With subsequent exposure to 200 nM PMA for 20 min, the BAPTA-AM pretreated cells did experience a major reorganization of actin patterns and depletion of straight microfilament bundles, although the characteristics of the disruption were not exactly that of PMA alone (not shown). We conclude from the BAPTA-AM experiments that lowering the intracellular Ca^{2+} levels may change the details of the appearance of the actin alterations but does not otherwise counteract the effect of PMA on actin filaments. These results suggest that although TPEN reduces phorbol ester-induced actin disruption by acting as a chelator, the ion involved is not Ca^{2+}.

We also examined whether TPEN might have a general stabilizing effect on the actin cytoskeleton that would be effective regardless of the particular pathway of actin reorganization involved. For this experiment we used two agents that disrupt cytoskeletal actin patterns by two different means, staurosporine and cytochalasin B. In cultured cells exposed to nanomolar amounts of staurosporine, microfilament bundles quickly dissociate into an abundance of fine curving filaments with eventual depletion of recognizable actin filament structures. Although staurosporine is a kinase inhibitor frequently employed in cell biology for its potent inhibition of PKC, its effect on the actin cytoskeleton is as readily observed in PKC-depleted as in control cells, indicating that this pathway of actin reorganization is PKC-independent (Hedberg *et al.*, 1990). In contrast, the treatment of cells with the cytochalasins rapidly results in a progressive shortening of actin filaments to yield a cytoplasm filled with punctate actin aggregates. The mechanism of cytochalasin action is thought to involve direct binding to filamentous actin, resulting in the severing of existing filaments and inhibition of the elongation of nascent filaments (Schliwa, 1986).

For these experiments, PTK2 cell cultures were left untreated (Figure 4, A, C, and E) or pretreated (Figure 4, B, D, and F) with 50–80 μM TPEN for 1 h (higher concentrations induce significant cell rounding and detachment). They were then exposed for an additional 20 min to control medium (Figure 4, A and B), 40 nM staurosporine (Figure 4, C and D), or 10 μM cytochalasin B (Figure 4, E and F). A comparison of the actin patterns in the TPEN pretreated cells to those of cells not pretreated with TPEN suggests that TPEN doses that are more than adequate to reduce the PMA-induced actin alterations do not modulate the actin reorganization mediated by either staurosporine or cytochalasin B.

The ability of TPEN to reduce PMA-induced actin reorganization is not limited to PTK2 cells. Similar results were obtained with Rat-1 (not shown) and with Swiss 3T3 cells (Figure 5, A–D). It was noted, however, that the required amount of pretreatment time varied with cell type. For PTK2 cells, it was sufficient to pretreat with 25 μM TPEN for 15 min to demonstrate a reduced sensitivity to phorbol ester, whereas Swiss 3T3 cultures had to be pretreated with 25–50 μM TPEN for 1–3 h to achieve similar results. As with PTK2 cells, control Swiss 3T3 cells have an abundance of cytoplasmic microfilament bundles (Figure 5A). Exposure to 200 nM PMA for 20 min results in a depletion of actin microfilaments, the appearance of actin aggregates, and morphological changes (Figure 5B). Pretreatment with 25 μM TPEN for 3 h did not alter actin organization (Figure 5C) but was sufficient to reduce actin alterations caused by subsequent addition of PMA (Figure 5D). As with PTK2 cells, the addition of $ZnCl_2$ with TPEN to Swiss 3T3 cells served to negate the effect of TPEN (not shown).

Because PMA-induced actin reorganization depends on activation of PKC (Danowski and Harris, 1988; Hedberg *et al.*, 1990), it was possible that TPEN might modulate the effect of PMA by serving as a PKC inhibitor. To address this issue, we chose to examine Swiss 3T3 mouse fibroblasts. Mouse fibroblasts have been found to express only one of the three major known PKC isozymes (type III or PKC-α) (McCaffrey *et al.*, 1987) and therefore are a somewhat simpler system than the cell lines that express multiple isozymes. We tested whether an endogenous PKC substrate, the MARCKS protein, would be phosphorylated after PMA treatment of TPEN-pretreated cultures (Figure 5E). The phosphorylation of the MARCKS protein has been shown to be a reli-

able marker of PKC activation in Swiss 3T3 and other cells (Rodriguez-Pena and Rozengurt, 1985; Blackshear et al., 1986). In the case of Swiss 3T3, the MARCKS protein can be observed as a band of about 80K molecular weight on autoradiograms of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of ^{32}P-labeled cellular proteins. In Figure 5E, lane 1 represents a control culture, lane 2 a culture exposed to 200 nM PMA for 10 min, lane 3 a control culture treated with 50 μM TPEN for 1.5 h, and lane 4 a culture pretreated with 50 μM TPEN and then exposed to 200 nM PMA for 10 min. The position of the MARCKS protein is indicated by arrowheads. PMA exposure resulted in a phosphorylated band at this position in both untreated and TPEN treated cultures (lanes 2 and 4, respectively), whereas the cultures that were not stimulated by PMA did not have this band (lanes 1 and 3, respectively). Thus, TPEN pretreatment of Swiss 3T3 cells did not prevent PKC-mediated phosphorylation of this endogenous substrate.

In another test of whether TPEN might act as an inhibitor of PKC and to obtain more quantitative data, PKC was partially purified from cultures of Swiss 3T3 cells and assayed using a commercially available assay kit with a PKC-specific synthetic peptide substrate. In this in vitro assay, 10–50 nM staurosporine reduced kinase activity by ~75%, whereas 25–75 μM TPEN had no inhibitory effect on kinase activity. Thus, although TPEN appears to selectively reduce actin cytoskeletal disruption resulting from the potent activation of PKC by phorbol ester, the mechanism probably does not involve kinase inhibition. Rather, it appears that TPEN modulates the phorbol ester-induced actin alterations through its ability to chelate metal ions.

Discussion

PKC exists as a family of closely related isoenzymes that exhibit similar physical properties and regulation by phospholipid (in particular, phosphatidylserine), Ca^{2+}, and diacylglycerol or phorbol ester. The complete amino acid sequence of bovine PKC indicates that in addition to at least one probable Ca^{2+} binding site in the regulatory domain of the enzyme, the neighboring putative phorbol ester binding site in this domain contains a sequence of repeating cysteines (Parker et al., 1986). It has been proposed that this cysteine-rich region of PKC is required for significant activation of PKC by nanomolar concentrations of phorbol ester (Gschwendt et al., 1991). In thyroid hormone receptors similar

domains of repeating cysteines have been shown to constitute Zn^{2+} binding sites (Hard et al., 1990), and such cysteine-rich regions have come to be called zinc fingers (Evans and Hollenberg, 1988). Although it has not yet been firmly established that the cysteine-rich sites in PKC do in fact bind Zn^{2+}, a number of recent studies suggest that zinc (which is the most abundant intracellular heavy metal) (Wong and Fritze, 1969) may play a role in the distribution of PKC between the cytosol and other cellular compartments. For example, increasing the intracellular zinc ion concentration through the use of an ionophore can lead to the association of PKC with the cytoskeletal fraction of lymphocytes (Zalewski et al., 1990). The zinc-PKC-cytoskeletal association is particularly intriguing because this may help to explain the role of PKC in the temporary but dramatic reorganization of the actin cytoskeleton caused by exposure of cultured cells to the potent tumor promoter, PMA.

The disruption of the actin cytoskeleton by the phorbol ester tumor promoters has been observed in a wide variety of cells, both as changes in actin patterns (e.g., Schliwa et al., 1984) and as resultant alterations in cell morphology as the actin alterations progress (Sastrodihardjo et al., 1987; Danowski and Harris, 1988; Bershadsky et al., 1989; Harrison and Mobley, 1990). The actin alterations occur within minutes of PMA treatment and reverse over the period of several hours, consistent with the immediate activation of PKC followed by its subsequent down-regulation in the continuing presence of phorbol ester (Rodriguez-Pena and Rozengurt, 1984). The process clearly requires activation of PKC as it does not occur in PKC-depleted cells (Danowski and Harris, 1988; Harrison and Mobley, 1990; Hedberg et al., 1990). Nevertheless, it appears that PKC activation alone is not sufficient for actin cytoskeletal alterations to occur (Delescluse et al., 1988). The previous reports of a Zn^{2+}-mediated association of PKC with the cytoskeletal fractions of hemopoietic model systems suggest that intracellular Zn^{2+} concentrations might modulate the association of activated kinase with specific substrates, for example those that regulate actin organization. If this were also true in the case of the PMA-induced actin reorganization in cultured cell systems, then it should be possible to use a cell-permeant chelator to intervene in this process and to reverse the effect of the chelator by adding back excess Zn^{2+}.

TPEN is a relatively new chelator (Anderegg et al., 1977) and has a high affinity for transition
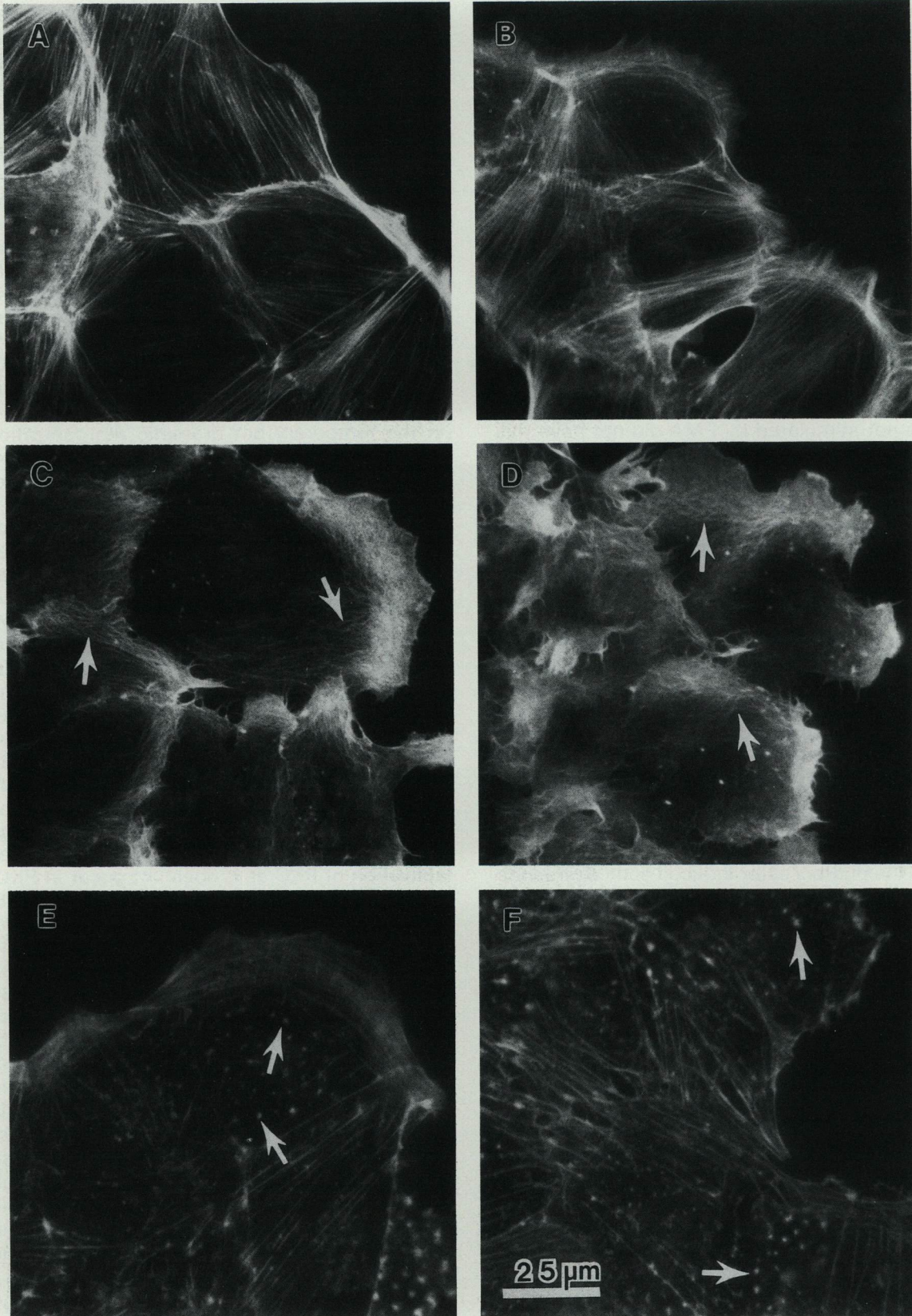
metal ions and a low affinity for calcium and magnesium (Arslan *et al.,* 1985). TPEN resembles the more familiar chelating agent EDTA in that it has six coordinating ligands that surround the metal ion to form an octahedral complex. The detailed geometry has been established in crystal structure studies of TPEN metal ion complexes (Gagne *et al.,* 1982; Chang *et al.,* 1990). All six ligands of TPEN are nitrogens (4 from pyridine and 2 derived from ethylene diamine), whereas EDTA has two nitrogen and four oxygen donors. Calcium binding generally involves oxygen ligands (e.g., carboxylate or carbonyl interactions), as demonstrated from crystal structure studies of calcium complexes (Einspahr and Bugg, 1984). By replacing all oxygens with nitrogens in the chelator, the affinity for calcium and magnesium is greatly reduced without reducing the affinity for transition metal ions. Anderegg *et al.* (1977) report remarkably high affinities of a range of transition metal ions for TPEN. For example, the association constants for TPEN with the transition ions Ni^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, and Mn^{2+} are 3×10^{21}, 3×10^{20}, 4×10^{16}, 4×10^{15}, 4×10^{14}, and 2×10^{10} M^{-1}, respectively (Anderegg *et al.,* 1977). In contrast, Arslan *et al.* (1985) report TPEN affinity constants for Ca^{2+} and Mg^{2+} of 2×10^4 M^{-1} and 50 M^{-1}, respectively. Thus, the affinity of TPEN for calcium ions is ~11 orders of magnitude lower than for Zn^{2+} ions, and it is even less for magnesium ions. At typical intracellular concentrations of 10^{-6} M for Ca^{2+} and 10^{-3} M for Mg^{2+}, the sequestering of these ions by TPEN is negligible. Another important feature of TPEN is that it is soluble in organic solvents, moderately soluble in water, and largely uncharged at physiological pH. The solubility and neutrality of TPEN makes it possible for this chelator to freely cross the plasma membranes into cells (Arslan *et al.,* 1985).

Using TPEN as a chelator, we have examined whether metal ions might be required for the actin disruption induced by phorbol esters. Both fluorescence and photoelectron microscopy were used to observe and document the resultant actin alterations in an epithelial (PTK2) and a fibroblast (Swiss 3T3) cultured cell line. In both cell types, treatment of the cells with 200 nM PMA resulted in rapid depletion of normally abundant cytoplasmic microfilament bundles and appearance of dots and (especially in PTK2) arcs and rings of actin filament aggregates (Figures 1, A and B; 2, A and B; and 5, A and B). The loss of microfilaments in particular, and to a somewhat lesser extent the development of the actin aggregates, was counteracted by pretreatment of the cells with TPEN (Figures 1, C and D; 2, C and D; and 5, C and D). The effect of TPEN was reversible; the addition of $ZnCl_2$, $CoCl_2$, and $CuCl_2$ before challenge with the phorbol ester completely eliminated the ability of TPEN to offset the PMA-induced actin reorganization (Figure 3).

The observation that a given ion is able to negate the effect of TPEN on the phorbol ester-induced actin reorganization does not in itself implicate that ion in this PKC-mediated event. In fact, any transition metal ion with an association constant for TPEN that is greater than that of the involved ion should be able to reverse the effect of TPEN by displacing the involved ion, assuming equal access of these ions to the interior of the cell. Thus, one would predict that in a test of a series of transition metal ions, those with association constants for TPEN that are less than the relevant ion would not reverse the TPEN effect, whereas those ions with equal or greater association constants would reverse the TPEN effect. In these experiments, we find that Fe^{2+} and Mn^{2+} (which have lesser association constants for TPEN than does Zn^{2+}) do not reverse the TPEN effect, whereas Zn^{2+}, as well as Co^{2+} and Cu^{2+} (with higher association constants for TPEN than Zn^{2+}), do reverse the TPEN effect. Because Zn^{2+} is the first ion in the series to reverse the TPEN effect, these data suggest that Zn^{2+} may in fact be the ion involved. The one odd point in the series is the fact that Ni^{2+}, with the highest association constant for TPEN of any of the ions tested (3×10^{21} M^{-1}), did not reverse the effect of TPEN. This may be due to a lesser cellular permeability of Ni^{2+} than of the other ions. Although calcium chelation is not likely to be involved in the effect of TPEN (because of the very low association constant of Ca^{2+} for TPEN), the cell-permeant calcium chelator BAPTA-AM was also tried in these experiments with the expected result that it did not mimic the effect of TPEN (not shown).

Figure 4. **Rhodamine-phalloidin fluorescence of actin patterns in PTK2 cells exposed to TPEN and then staurosporine or cytochalasin B.** (A, C, E) Controls not exposed to TPEN; (B, D) pretreated with 80 μM TPEN for 1 h; (F) pretreated with 50 μM TPEN for 1 h. Cultures were subsequently treated for 20 min with (A, B) no addition; (C, D) 40 nM staurosporine; or (E, F) 10 μM cytochalasin B. Arrows in (C, D) indicate fine wavy actin filaments typical of staurosporine treatment; arrows in (E, F) indicate a few of the punctate actin aggregates typical of cytochalasin treatment.
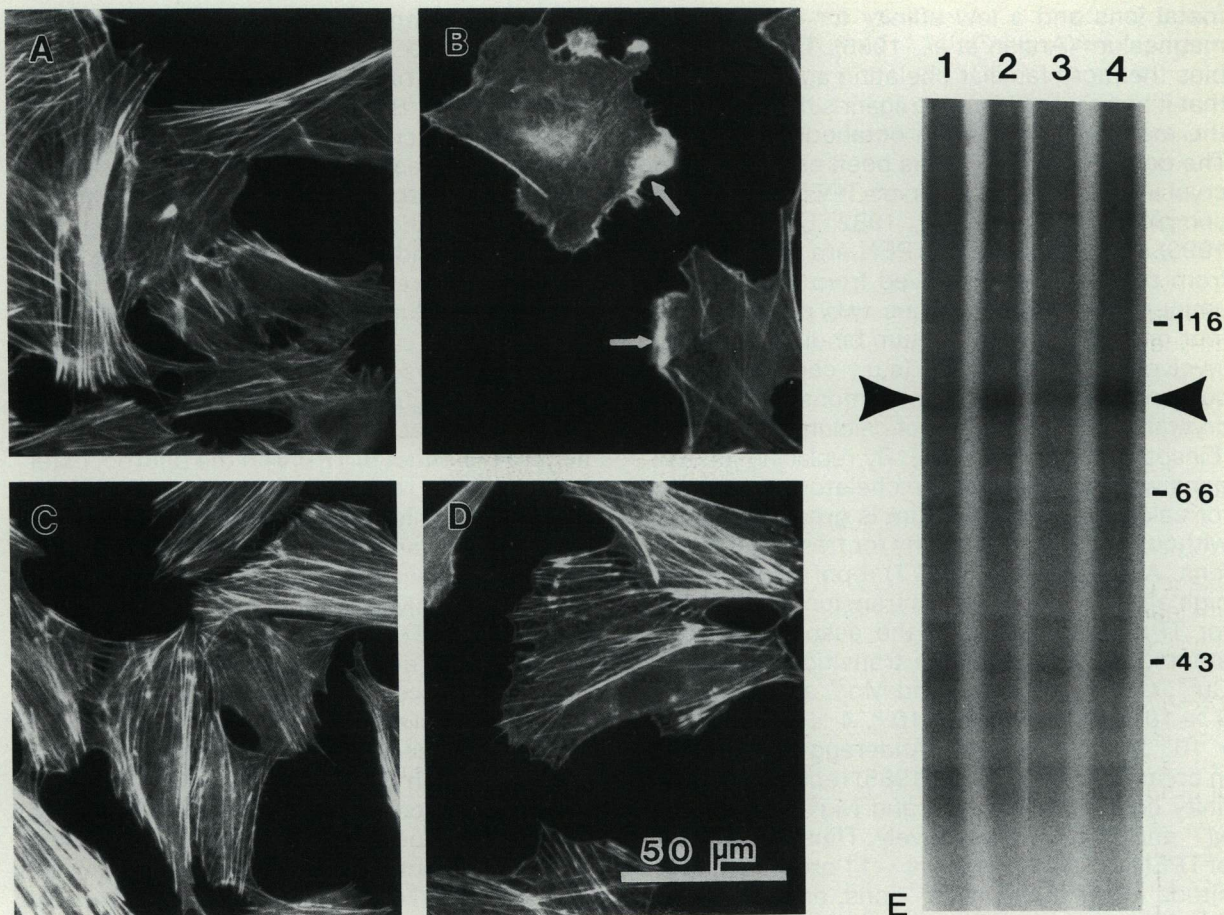
Figure 5. **Counteracting effect of TPEN on PMA-induced actin reorganization in Swiss 3T3 fibroblasts, shown by fluorescence with rhodamine-phalloidin.** (A, B) Not exposed to TPEN; (C, D) pretreated with 25 μM TPEN for 3 h. (A, C) Cultures subsequently received DMSO vehicle only in the same medium; (B, D) cultures subsequently received 200 nM PMA for 20 min in the same medium. Arrows in (B) indicate PMA-induced actin redistribution from microfilament bundles to the cell periphery. (E) Autoradiogram of a 10% polyacrylamide gel of ^{32}P-labeled phosphoproteins showing that TPEN does not block phorbol ester induced phosphorylation of the 80K PKC substrate protein (MARCKS protein) in Swiss 3T3 cells. Lanes 1 and 2, no pretreatment of cultures; lanes 3 and 4, cultures pretreated with 50 μM TPEN for 2 h. Cultures were challenged with control medium containing DMSO vehicle only (lanes 1,3) or 100 nM PMA (lanes 2,4) for 10 min before the gel samples were prepared as in Materials and methods. Arrowhead, position of 80K PKC substrate phosphoprotein, visible in lanes 2 and 4.

Thus, the ability of TPEN to counteract actin microfilament disruption is most likely dependent on its function as a heavy metal chelator. Although these experiments cannot prove absolutely that Zn^{2+} is the relevant ion, these data taken together with the literature evidence for zinc involvement in PKC function are highly suggestive of a role for Zn^{2+}.

An alternative explanation for the effect of TPEN is that TPEN binds to and stabilizes actin filaments in a Zn^{2+}-reversible manner. However, TPEN pretreatment did not detectably reduce actin cytoskeletal disruption caused by the drug cytochalasin B or by staurosporine (Figure 4). These agents induce characteristic actin patterns that are distinct from each other and from

those induced by PMA and do so by different mechanisms that are not thought to involve PKC. Thus, of these three different mechanisms of actin filament disruption, TPEN appears to be specific for the one that is PKC-dependent.

TPEN might also exert its effects through inhibition of PKC. We examined whether TPEN pretreatment might inhibit activation of PKC in intact Swiss 3T3 cells by observing the PMA-stimulated phosphorylation of the MARCKS, or 80K PKC substrate, protein (Figure 5E). The MARCKS protein is a widely used indicator of PKC activation in intact cells (Niedel and Blackshear, 1986), and in this assay TPEN did not prevent activation of PKC. Up to 75 μM TPEN also did not inhibit partially purified PKC from

Swiss 3T3 cells when assayed using a commercial "PKC-specific" kit. Thus, it is not likely that TPEN (in the 25–75 μM concentration range) acts as a PKC inhibitor in intact cells.

Our data establish that TPEN can counteract phorbol ester-induced cytoskeletal alterations in cultured cells. Furthermore, this effect is reversible. Two mechanisms that could account for this effect are 1) chelation of a heavy metal ion by TPEN and 2) a direct interaction of TPEN with some cellular component. We have distinguished between these possibilities by examining the effect of addition of a series of heavy metal ions. The results indicate that it is the ability of TPEN to chelate a heavy metal ion, probably Zn^{2+}, that is responsible for the protective effect of TPEN.

There is strong evidence that these cytoskeletal changes are mediated by PKC, but the exact mechanisms and the role of Zn^{2+} remain unclear. In at least two cell lines (rat embryo fibroblasts [Jaken et al., 1989] and Y-1 rat adrenal tumor cells [Papadopoulos and Hall, 1988]) a significant amount (>10%) of cellular PKC is associated with the cytoskeleton, even in the absence of activation by phorbol esters or other agents. Presumably, exposure of these cells to phorbol ester could activate this cytoskeleton-associated PKC, resulting in the phosphorylation of closely associated actin-regulatory proteins and subsequent cytoskeletal alterations. Our observation that chelation by TPEN counters the phorbol ester-induced actin alterations would argue that Zn^{2+} (or some other heavy metal ion) is required for the activation of PKC (a subject of some controversy; see Speizer et al., 1989). However, we do not find detectable inhibition of PKC activity by TPEN at concentrations that are effective in countering the actin alterations. Rather, our data is consistent with the involvement of Zn^{2+} in a phorbol ester-stimulated association of PKC with specific substrates. This may occur as a measurable physical redistribution of PKC to membrane-associated cytoskeleton, such as has been observed in two cultured cell systems (Kiley and Jaken, 1990; Mochly-Rosen et al., 1990). On the other hand, it may involve a more subtle change in kinase-substrate interactions on the molecular level that will become clearer as work in this field progresses. For the present, this study demonstrates an essential role for metal ions in a PKC-mediated alteration in cellular physiology and complements current model system studies and debate on the role of zinc in PKC function.

Materials and methods

Chemicals

TPEN and BAPTA-AM were from Molecular Probes (Eugene, OR), PMA was from LC Services (Woburn, MA), cytochalasin B was from Sigma (St. Louis, MO), and staurosporine was from Calbiochem (LaJolla, CA). All agents were prepared as stocks in DMSO, stored as aliquots at −20°C, and diluted in culture medium just before use. These secondary stocks were bath sonicated ≥2 min before the final dilution into the culture dish. All treatments were in culture medium containing 10% serum and <0.2% DMSO.

Cells

Cell line Swiss 3T3 was from the American Type Culture Collection (Rockville, MD); PTK2 and Rat-1 were gifts from Dr. Lan Bo Chen (Dana-Farber Cancer Institute, Boston, MA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Grand Island, NY) with 10% supplemented bovine calf serum (HyClone, Logan, UT).

Fluorescence microscopy

Cells grown on round glass coverslips were exposed to desired treatments in 6- or 12-well multiwell plates, washed quickly with phosphate-buffered saline (PBS; GIBCO), and fixed for 5 min at room temperature (RT) with 5% paraformaldehyde in PBS. They were then permeabilized with 0.5% Triton X-100 in cold PHEM buffer (60 mM piperazine-N,N'-bis(2-ethanesulfonic acid), 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 2 mM $MgCl_2$, pH 6.9) (Schliwa et al., 1984) for 5 min, followed by three washes in PHEM buffer without Triton, and a final wash with PBS. Labeling for filamentous actin was accomplished by a 10 min RT incubation with a 1:20 dilution in PBS of rhodamine-conjugated phalloidin (Molecular Probes), followed by two 10-min washes with excess PBS. Labeled coverslip cultures were then placed cells-up on a glass slide, overlaid first with a drop of glycerol containing 0.1 M n-propyl gallate (Sigma) to reduce photobleaching (Giloh and Sedat, 1982), and then covered with a second, larger glass coverslip. Samples were observed and photographed with a Zeiss fluorescence microscope equipped with epifluorescence optics (Zeiss, Thornwood, NY).

PEM

Cell cultures intended for examination by PEM were grown on chromium-coated glass 5-mm-diameter round coverslips. After treatments in parallel to those used for the fluorescence samples, the PEM samples were quickly rinsed in RT PBS and then prefixed in 0.15 mg/ml dithiobis (succinimidyl propionate) (Bell, 1981) in PBS for 3 min at RT. After a rinse in PBS, the samples were permeabilized in 0.5% Triton X-100 in PHEM buffer for 5 min at 4°C, rinsed 3 times in PHEM buffer without detergent, then fixed in 2% glutaraldehyde in 0.15 M Na cacodylate, pH 7.2. Samples were stored in fixative at 4°C, then washed in distilled water, dehydrated through increasing concentrations of ethanol solutions, and finally critical point dried from CO_2. Dehydrated specimens were examined immediately or stored under vacuum until examination.

The photoelectron microscope is an oil-free high-vacuum instrument that has been described in detail (Habliston et al., 1991; Rempfer et al., 1991). The images are formed by accelerating, focusing, and recording on electron image film the electrons emitted from the sample surface during UV

light excitation. Typical film exposure times were <1 min for images at ×1000 to ×2000 magnification; no sample damage was seen during specimen observation.

PKC assay

Partially purified PKC from one confluent 100-mm dish of quiescent Swiss 3T3 cells was prepared by DEAE cellulose column chromatography and assayed using procedures and materials provided in a commercial kit (Protein Kinase C Assay System; GIBCO BRL Life Technologies, Inc., Gaithersburg, MD). The procedure used differed slightly from the kit procedure in that the samples were incubated with ^{32}P and substrate for 7 min instead of 5 min and the amount of reaction mixture spotted onto the phosphocellulose disks was 20 μl instead of 25 μl with the calculations adjusted accordingly.

SDS-PAGE analysis of cellular phosphoproteins

Confluent quiescent cultures of Swiss 3T3 cells in 6-well plates were washed with phosphate free DMEM (Flow Laboratories, McLean, VA) and then incubated in 0.75 ml/well DMEM containing 175 μCi ^{32}P orthophosphate and either 50 μM TPEN or no addition of TPEN for 1.5 h at 37°C on a rocking platform. Subsequently, the cultures were challenged with 100 nM PMA or control medium for an additional 10 min. The remainder of the procedure was essentially that of Rozengurt *et al.* (1983), in which the trichloroacetic acid precipitated cellular proteins are resolved by PAGE (on a 10% gel on Gel-Bond [FMC Bioproducts, Rockland, ME]) and visualized by fluorography.

Acknowledgments

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