Characterization of the Growth Inhibition Induced by Tumor-promoting Phorbol Esters and of Their Receptor Binding in A549 Human Lung Carcinoma Cells

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ABSTRACT

Exposure of A549 human lung carcinoma cells to $10^{-8}$ M 12-O-tetradecanoylphorbol-13-acetate (TPA) resulted in a change in cell morphology and caused the arrest of cell growth. After 4–5 days of exposure to TPA the cells started to proliferate again. However, on removal of the cells from the culture flask and reseeding, the cells had regained their sensitivity towards TPA. Cells which were subcultured in the presence of $10^{-6}$ M TPA for 9 weeks were permanently refractory to the growth-inhibitory properties of TPA. Incubation of A549 cells with $[^{3}H]$phorbol-12,13-dibutyrate ($[^{3}H]$PDB) showed that the cells possess specific phorbol ester receptors. Exposure of the cells to $10^{-7}$ M PDB preceding the receptor binding assay led rapidly to a decline in the binding of $6.0 \times 10^{-7}$ M $[^{3}H]$PDB, in case of preincubation for 24 h to 38% of the binding in cells not pre-exposed to PDB. The receptor binding capacity after pretreatment with PDB was only weakly decreased in the cells which were desensitized towards the TPA-induced growth inhibition. Thus the decrease in receptor binding on exposure to phorbol esters does not appear to cause the refractoriness of the cells towards the effect of TPA. It seems more likely that this decrease in binding capacity is part of the events by which phorbol esters cause inhibition of cell growth.

INTRODUCTION

Certain phorbol esters, diterpenes which are potent tumor promotors, exert a large variety of effects on animal and human cells (8). TPA, the phorbol ester with the greatest potency as tumor promotor, has been shown to be mitogenic in rodent cell lines (8). TPA, the phorbol ester with the greatest potency as tumor promotor, has been shown to be mitogenic in rodent cell lines (8). The features of the growth inhibition are characterized. In order to elucidate the mechanism by which TPA causes growth arrest in the A549 cells we investigated the hypothesis that the effect is mediated by specific phorbol ester receptors which are considered to trigger many, if not all, cellular responses to phorbol esters (for reviews, see 3–5). We found that on prolonged exposure to TPA the cells became refractory towards the growth-inhibitory effects of phorbol esters and that the binding of labeled PDB to receptors was markedly decreased. Consequently, we investigated the relationship between the TPA-induced inhibition of cell growth, the desensitization towards this effect, and the loss in receptor binding capacity, by comparing the time course of the desensitization with that of the appearance of the decrease in receptor binding.

MATERIALS AND METHODS

Chemicals. Phorbol esters purchased from Sigma Chemical Co. (St. Louis, MO) were dissolved in DMSO and stored at $-20^\circ$. The stock solutions were diluted with Hanks' buffered salt solution. The final concentration of DMSO in the incubation mixtures was $0.1\%$ or less. $[^{3}H]$PDB (specific activity, $11 \mathrm{Ci}/\mathrm{mmol}$) was purchased from New England Nuclear (Boston, MA). Catalase, retinoic acid, and phospholipase C (from Clostridium perfringens) were also obtained from Sigma Chemical Co.

Cell Growth Studies. A549 cells were obtained from the American Type Culture Collection (Rockville, MD). The cell line, which possesses epithelial-like morphology, was initiated in 1972 through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male (11). The cells were maintained in Nutrient F-12 medium (Coon's modified; KG Biologicals Inc., Lenexa, KA) complemented with 10$\%$ fetal bovine serum, penicillin (100 units/ml), and streptomycin (10 mg/ml) and kept in a humidified incubator with 5$\%$ CO$_2$. The medium was replaced after 3 days. The cells were routinely subcultured once weekly.

Cells were plated in either flasks (25 cm$^2$; G. L. Sciex Inc., Verviers, CA) or dishes (6.0 x 1.5 cm; Primaria, Falcon Labware, Oxnard, CA). Phorbol esters were added 4 h after seeding, when the cells had attached to the surface of the dish. An equivalent volume of DMSO was added to controls and had no effect on growth. After removing the cells from the dishes by trypsinization they were counted using a hemocytometer. Each assay was performed with duplicate samples.

Receptor Binding of $[^{3}H]$PDB to Cells. Cells grown in Linbro multiwell plates (2.4 x 1.7 cm; Flow Laboratories, Inc., McLean, VA) were incubated in 0.5 ml Hanks' buffered salt solution containing BSA (4 mg/ml) with $[^{3}H]$PDB, and either with or without unlabeled PDB at a concentration of 1000-fold in excess of the $[^{3}H]$PDB. After a 30-min incubation at 37$^\circ$ the medium was aspirated, and the cultures were rinsed quickly 3 times at 4$^\circ$ with 0.5 ml of Hanks' solution containing BSA (4 mg/ml). Cells were solubilized in 0.1% NaOH, and cell-associated radioactivity was determined by liquid scintillation counting in a Packard Tri-Carb Scintillation Spectrometer at an efficiency of about 45% in Formula 963 aqueous counting cocktail (New England Nuclear). Aliquots of the medium were removed to measure directly the concentration of free $[^{3}H]$-
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PDB. Receptor binding was calculated as the difference in cell-associated radioactivity in the absence and presence of PDB. Throughout this paper, "binding" and "receptor binding" refer to the values obtained in this way. In 4–12 experiments non-specific binding was 25 ± 3% (SD) of the total cell-associated radioactivity at 3 nM [3H]PDB, 27 ± 3% at 6 nM [3H]PDB, 41 ± 7% at 15 nM [3H]PDB, 43 ± 6% at 30 nM [3H]PDB, 46 ± 7% at 45 nM [3H]PDB, 51 ± 5% at 60 nM [3H]PDB, and 54 ± 8% at 180 nM [3H]PDB. Non-specific binding in the A549 cells which were refractory towards the growth-inhibitory effect of TPA was identical with the non-specific binding in naive cells.

It has been shown that plastic culture dishes possess specific PDB binding sites (7). In control experiments we found that the specific binding to sites in the Linbro dishes in the absence of cells was below 3% of the receptor binding to cells at any [3H]PDB concentration investigated. Cell numbers per culture were monitored throughout all experiments.

[3H]PDB Receptor Binding after Exposure of Cells to PDB. Cells were exposed to 10⁻⁷ M PDB for periods of up to 24 h. Thereafter the cells were washed at 37° for 2 min each with F-12 medium (1 ml) once and with Hanks' solution containing BSA (1 ml) 5 times. In control experiments using [3H]PDB this washing procedure completely removed cell-associated radioactivity. After the washing, [3H]PDB binding was determined as described above.

RESULTS

Effect of TPA on Cell Morphology and Growth. On exposure to 10⁻⁸ M TPA, A549 cells gradually changed their appearance (Fig. 1). Cells became less flattened with a prominent part of the cell adopting a protruding round shape. This alteration in cell morphology emerged 2 h after addition of TPA to the medium. Chart 1 shows that the presence of TPA completely arrested growth of the cells for 4 to 5 days, after which cells started to proliferate again at a rate similar to that of control cell growth.

The phorbol ester PDB also inhibited growth (Chart 2) and, at growth-inhibitory concentrations, it altered cell morphology. However, whereas TPA exerted its growth-arresting effect at concentrations at and above 0.5 nM, concentrations of more than 10 nM PDB were required for marked growth inhibition. Phorbol-12,13-diacetate, a phorbol ester with only weak tumor-promoting properties, was only growth-inhibitory at a concentration of 1 µM.

In an attempt to interfere with the TPA-induced growth inhibition, a number of agents, the antipromotor retinoic acid, the thiol compound glutathione, and the enzymes catalase and superoxide dismutase were incubated together with TPA and A549 cells at concentrations which did not influence cell growth in the absence of TPA. Except catalase, these agents did not change the TPA-induced growth inhibition. When added daily for 3 days at a concentration of 25 µg/ml, catalase abolished the change in cell morphology caused by 10⁻⁸ M TPA. Furthermore, in the presence of catalase, the inhibition of cell growth caused by TPA was significantly, if only to a small extent, decreased. However, the influence of catalase on the stability of TPA was not investigated. Therefore it is possible that the catalase used in this study contained an esterase which could have degraded TPA and thus interfered with the effect of TPA on A549 cells.

Desensitization of Cells to TPA-induced Growth Arrest. The inhibition of the growth of A549 cells by TPA at either 10⁻⁸, 10⁻⁷, or 10⁻⁶ M was between 92 and 89%, as assessed by cell number after 5 days' exposure to the phorbol ester (Chart 3,4). Cells were incubated with 10⁻⁸ M TPA for 6 days. Subsequently TPA...
was removed from the culture flasks, and in some of these flasks TPA was newly added at either $10^{-8}$, $10^{-7}$ or $10^{-6}$ M. At this stage the added change in cell morphology caused initially by TPA had disappeared. When the growth of the cells in the continued presence of TPA was compared with that of the cells from which TPA had been removed, the extent of growth inhibition observed was only between 20 and 29% (Chart 3B). Similarly continual treatment of the cells with $10^{-7}$ M PDB instead of TPA for 6 days also led to a loss of sensitivity of the cells towards the growth-inhibitory effect of PDB. However when the refractory cells were removed from the culture flasks after exposure to TPA for 6 days and freshly seeded, the subsequent addition of TPA was again able to inhibit cell growth, now by 75–78% (Chart 3C). Furthermore, when the cells were subcultured in the continued presence of $10^{-8}$ M TPA for several weeks, they gradually became completely refractory to the growth-inhibitory properties of TPA (Chart 4), so that after 9 weeks' continued exposure to TPA the phorbol ester was hardly able to slow cell growth at all (Chart 3D). This cell variant with acquired insensitivity towards the growth-inhibitory properties of phorbol esters is being referred to as A549-TPA hereafter.

Characterization of Phorbol Ester Receptors in A549 Cells. For the investigation of phorbol ester receptors in A549 cells we used $[^3H]$PDB. This phorbol ester has been frequently used as ligand in phorbol ester receptor binding studies, because PDB is less lipophilic than TPA (9). Chart 5A shows the time course of receptor binding of $[^3H]$PDB to confluent A549 cells at $37^\circ$. Maximal receptor binding was achieved after 30 min. The binding reaction was reversible. When a 1000-fold excess of unlabeled PDB was added to cultures that had been exposed previously to $[^3H]$PDB for 30 min, a very fast dissociation of the ligand-receptor complex was observed (Chart 5B). It is well documented that the time course of phorbol ester receptor binding and receptor modulation are temperature-dependent (7, 14). We restricted our binding studies to an environment of $37^\circ$. However, in order to ensure that the washing procedure used to eliminate loosely bound ligand did not remove receptor-bound $[^3H]$PDB, we determined that appreciable dissociation of bound $[^3H]$PDB from the receptor at $4^\circ$ did not occur within 5 min incubation.
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In addition to TPA, both phorbol and phorbol-12,13-diacetate inhibited binding of 6 nm [³H]PDB to receptors in A549 cells (results not shown). TPA was the most potent inhibitor, followed in potency by PDB.

Chart 6 shows [³H]PDB receptor binding to A549 cells as a function of [³H]PDB concentration. Phorbol ester receptor binding to normal A549 cells was compared with binding to receptors in A549-TPA cells, which have been permanently refractory to the growth-inhibitory effect of TPA by exposure to 10⁻⁸ M TPA for 9 weeks. The graph shows that both cell populations possess at least two kinds of receptor binding sites, only one of which is saturable at [³H]PDB concentrations of up to 180 nM. The shape of the binding curve at [³H]PDB concentrations of above 60 nM indicates that non-saturable receptor sites of low affinity may be involved. The reasons for the peculiar shape of the binding curve have not been investigated in this study, but one might speculate that it may be a consequence of the “mobile” nature of the phorbol ester receptor which becomes membrane-bound in the presence of ligand (22). Scatchard analysis was attempted, but the unusual shape of the binding curve renders the analysis impossible to interpret.

In several binding experiments, A549 cells were incubated with catalase for 12 h before and during the binding studies at the concentration at which it decreased the growth-inhibitory properties of TPA. At three different [³H]PDB concentrations the amount of labeled ligand which was bound to the receptors ranged between 93 and 102% of control binding. Thus catalase had no effect on phorbol ester receptor binding.

Decreased [³H]PDB Receptor Binding after Exposure to PDB. When receptor binding was studied in the cells which were exposed to 6 nm [³H]PDB for periods of above 2 h a marked reduction in binding was observed (Chart 7). More dramatically, exposure of the cells to 10⁻⁷ M PDB preceding the receptor binding determination led to a rapid decline in binding (Chart 7). After exposure to 10⁻⁷ M PDB for 90 min, receptor binding was only 42% of control binding. Similarly, after incubation with PDB for 24 h, the cells displayed receptor binding studied with 6 nm [³H]PDB which was 38% of the receptor binding in cells not previously exposed to PDB. In these experiments the PDB, which was bound to the cells during the 24-h incubation period, was completely removed by repeated rinsing of the cells before the binding assay was performed. The efficiency of the washing procedure in removing PDB was tested using labeled PDB. The decreased ability to bind [³H]PDB after initial treatment with unlabeled PDB has been demonstrated for a number of other cell types and in several cases was shown to be due to the down-regulation of the receptors (12, 15). Receptor down-regulation means a decrease in binding sites with unaltered affinity of the receptor for the ligand. A549 cells which had been treated with 10⁻⁷ M PDB for 24 h were incubated with [³H]PDB at several different concentrations, and the amount of receptor-bound label was determined. The shape of the binding curve obtained was similar to the curve obtained with naïve cells (data not shown). As the shape of the PDB binding curves obtained did not allow the mathematical evaluation by Scatchard analysis, it is impossible to judge whether the decrease in binding was due to a change in the number of binding sites available or to a decrease in the affinity of the receptors for PDB. In order to see to what extent the change in binding on exposure to PDB was reversible, cells were incubated with medium for 3 h after they had been exposed to PDB for 24 h and subsequently washed thoroughly. Following this recovery period, receptor binding in the cells was restored to 70% of control binding (Chart 8). The decrease in binding of [³H]PDB to its receptors was only weak in cells which had been maintained in culture dishes with 10⁻⁷ M PDB for 6 days before the binding assay, a treatment which made them refractory to the growth-inhibitory potential of phorbol esters (Chart 8B). In these cells the binding of 6 nm [³H]PDB to receptors

![Chart 6](image-url)

**Chart 6.** Binding of [³H]PDB to receptors in A549 cells (O) and A549-TPA cells, i.e., A549 cells which were subcultured in the presence of 10⁻⁸ M TPA for 9 weeks (X). Non-specific binding was between 25% (at 3 nM [³H]PDB) and 54% (at 180 nM [³H]PDB) of total cell-associated radioactivity. Values are the mean of 5-10 experiments; points without error bars are the mean of 2 experiments. Bars, SD.

![Chart 7](image-url)

**Chart 7.** Time course of the decrease in [³H]PDB receptor binding. Cells were exposed to 10⁻⁷ M PDB for the indicated time periods and then rinsed thoroughly, and receptor binding was determined with 6 nm [³H]PDB. Values are mean of 2 experiments (point without error bars) or the mean of 4-6 experiments. Bars, SD.
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Chart 8. Loss of ligand binding capacity of \( ^{3}H \)PDB receptors in A549 cells (A) and A549-TPA cells, A549 cells which were cultured in the presence of \( 10^{-7} \) TPA for 9 weeks (B). Binding was estimated with \( 6 \) nm \( ^{3}H \)PDB, and columns indicate control receptor binding (D); receptor binding to cells after 24 h exposure to \( 10^{-7} \) m PDB, without (B) or with a subsequent incubation period of 3 h in medium only (C); and receptor binding to cells maintained in \( 10^{-7} \) m PDB for 6 days without (D) or with subsequent incubation for 3 h in medium only (E). Number of experiments is shown in brackets below the bars: * \( P < 0.01 \); ** \( P < 0.005 \); *** \( P < 0.001 \) compared to control binding. Bars, SD.

...was 75% of the binding to receptors in untreated cells (Chart 8). Also, in the A549-TPA cells which had been desensitized permanently to the growth-inhibitory effect of TPA, the ability of \( ^{3}H \)PDB to bind to receptors was only little decreased by pre-treatment with \( 10^{-7} \) m PDB for 24 h, to 77% of binding in untreated cells (Chart 8).

The results shown in Chart 8 were obtained in incubations with \( ^{3}H \)PDB at a concentration of \( 6 \) nm. In further experiments similar changes in binding on pretreatment of the cells with \( 10^{-7} \) m PDB were also seen with \( 30 \) nm \( ^{3}H \)PDB, even though the decrease in receptor binding at this concentration after PDB treatment was only 31% as compared to 62% in the case of \( 6 \) nm \( ^{3}H \)PDB.

Influence of Treatment with Phospholipase C on Cell Growth. It has recently been suggested that the cellular effects of phorbol esters are mediated through the activation of the phospholipid and Ca\(^{2+}\)-dependent enzyme protein kinase C, which is probably identical to the phorbol ester receptor (for reviews, see 5 and 22). The endogenous ligands of this enzyme are diacylglycerols, products of the breakdown of phospholipids, which is catalyzed by phospholipase C (22). In analogy to work reported by Jeng et al. (16) we tested the hypothesis that diacylglycerols enzymatically produced by phospholipase C are the physiological ligands of phorbol ester receptors in A549 cells and exert effects in these cells similar to those elicited by the phorbol esters. Addition of phospholipase C (0.5 unit/ml) daily for 4 days to cultures of A549 cells slowed cell growth to \( 65 \pm 5\% \) \((n = 7)\) of control growth when cell numbers were assessed after 5 days. Cell morphology was not changed by the treatment with phospholipase C, nor was \( ^{3}H \)PDB receptor binding altered by the exposure of the cells to phospholipase C (0.5 unit/ml) 3 h before and during the determination of the binding.

DISCUSSION

Our results show that A549 human lung carcinoma cells are a cell type which responds to exposure to phorbol esters by growth arrest (Charts 1 and 2). This inhibition of proliferation is concurrent with a marked change in cell morphology (Fig. 1). On comparison of the different human cell systems in which phorbol esters cause growth arrest, the behavior of the A549 cells appears to resemble that of A431 human epidermoid carcinoma cells (29) and SVK 14 cells, which were derived from human epidermal keratinocytes by transformation with simian virus 40 (19). TPA was not toxic in either of these cell lines or in the A549 cells, since the number of cells after exposure to TPA was never less than the number initially seeded. In MCF-7 breast cancer cells (23), in Epstein-Barr virus-negative human lymphoma cells BJAB and Ramos (2), and in the leukemia cell lines in which phorbol esters induce differentiation (13, 26), exposure to TPA leads to growth arrest followed by cell death. In contrast, after a period of 4–5 days' exposure to TPA, A549 cells started to grow again (Chart 1). A similar recovery from the growth-inhibitory effect of TPA was observed in SVK 14 cells (19). A detailed investigation of the time course of the growth inhibition observed in A549 cells exposed to TPA shows that the loss of responsiveness occurred in two different situations. Firstly, cells attached to the culture flask recovered from the growth inhibition caused by TPA after about 4–5 days as described above and grew only slightly more slowly in the presence of TPA than the cells from which TPA had been removed (Chart 3B). However, these cells regained most of their responsiveness to the TPA-induced growth arrest on detachment from the plastic surface and fresh seeding (Chart 3C). Secondly, culturing the cells in the presence of TPA for 9 weeks made these cells, when freshly seeded, essentially resistant to the growth inhibition induced by TPA (Chart 3D and Chart 4). We concluded that these cells were desensitized permanently. There are a number of biological effects elicited by phorbol esters in cultured cells towards which the cells become refractory after a period of continued exposure to the phorbol ester. For example, mouse preadipose cells (27) and B-16 melanoma cells (20) were desensitized towards phorbol ester-induced inhibition of differentiation, and mouse pituitary cells developed a decreased responsiveness towards the phorbol ester-stimulated secretion of corticotropin (24). Likewise prolonged treatment of Swiss 3T3 cells with PDB rendered the cells refractory to subsequent mitogenic stimulation by PDB (7).

There are several explanations for the phenomenon of loss in responsiveness of cells to physiologically potent substances such as hormones. One explanation is that on prolonged exposure to a stimulant, cells acquire an increased ability to metabolize the stimulant and may thus ameliorate its effect. This explanation is unlikely to be applicable to the TPA-induced growth inhibition in A549 cells, as the cells lost their responsiveness towards TPA even at a concentration as high as \( 10^{8} \) m (Chart 3). It is more likely that the cells became desensitized towards the TPA-induced growth inhibition.

The continued presence of TPA for several weeks rather than days appeared to lead to the isolation of a cell variant, A549-TPA, with acquired resistance to the growth-inhibitory potential of TPA. In this behavior the A549 cells are similar to HL60 human promyelocytic leukemia cells, which became resistant to phorbol ester-induced differentiation by being subcultured for 35 cultures in the presence of TPA (30). The decrease of some cellular responses to phorbol esters has been associated with the down-regulation of the phorbol ester receptors (7, 12, 15). A549 cells possess specific phorbol ester receptors (Chart 8), and on ex-
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posure to PDB for 90 min or more they exhibited a markedly decreased capacity to bind \([\text{[^3H]}\text{PDB}](\text{Chart 7})\). Reversal of the decrease in PDB receptor binding was also fast; 3 h after removal of PDB, 70% of the initial receptor sites had their ability for ligand binding restored (Chart 8). If the desensitization of the cells towards the growth-inhibitory effect of phorbol esters was indeed due to the decreased receptor ligand binding capacity, one would expect both phenomena, desensitization and decreased binding, to occur during similar time intervals. However, whereas the desensitization took 4–5 days to develop (Chart 1), the loss of receptor binding occurred within 90 min of exposure to PDB (Chart 7). Furthermore, the binding capacity of the receptors in the desensitized cells was decreased by only 25% (Chart 8), compared with a decrease of 62% in the normal cells. Likewise, the receptors in the A549-TPA cell variant, which had lost permanently its sensitivity towards the growth-inhibitory potential of TPA (Chart 4), demonstrated only a weak decrease in binding (Chart 8). Consequently it may well be that the decrease in the binding capacity of the phorbol ester receptors for \([\text{[^3H]}\text{PDB}](\text{Chart 8})\) is part of the biochemical events by which phorbol esters cause growth inhibition to occur. The decrease in receptor binding does not seem to be associated with the mechanism by which the A549 cells became refractory to the phorbol ester-induced growth inhibition.

There is now good evidence for the contention that the enzyme protein kinase C is a target for phorbol esters (1, 6, 17, 18, 21), probably the most important one, as the tumor promoters directly activate this enzyme (1, 8). It remains to be established whether the TPA-induced inhibition of the growth of A549 cells is mediated by the activation of protein kinase C. An attempt to obtain evidence as to the involvement of the protein kinase C system with this effect yielded an ambiguous result. Incubation of A549 cells with phospholipase C, the enzyme which catalyzes the degradation of endogenous phospholipids to diacylglycerols, the endogenous ligands of the phorbol ester receptor, inhibited the growth of the cells by 35%. If this effect was mediated by diacylglycerols one would expect that the affinity of the receptor for \([\text{[^3H]}\text{PDB}](\text{Chart 6})\) in A549 cells in the presence of phospholipase C should be decreased with an unchanged number of ligand binding sites available. However \([\text{[^3H]}\text{PDB}](\text{Chart 6})\) receptor binding was not significantly altered by phospholipase C. This result is in contrast with the finding that treatment of mouse primary epidermal cells with phospholipase C led to responses similar to those seen when cells were exposed to phorbol esters (16). In these mouse cells binding affinity for \([\text{[^3H]}\text{PDB}](\text{Chart 6})\) decreased by 50% without change in total number of binding sites on incubation with phospholipase C. The possibility cannot be discounted that in our study a phospholipase C-induced change of \([\text{[^3H]}\text{PDB}](\text{Chart 6})\) binding to the population of those receptors which mediate the growth inhibition was so slight that it was not detected by the binding assay. As we did not attempt to measure diacylglycerols produced by phospholipase C in A549 cells it is possible that the amount released by this enzyme was too small to elicit dramatic effects.

Potent tumor-promoting phorbol esters are capable of inhibiting cell multiplication in certain cells such as the A549 cells and of inducing terminal differentiation in some leukemia cells. Over a number of years some authors have pointed out that it might be worth investigating the growth-inhibitory potential of phorbol esters (13, 23, 26, 32). Recently a link has been discovered between the phorbol ester receptor, protein kinase C, and the transduction of physiological signals for intracellular events and for cellular proliferation via the turnove of membranal inositol phospholipids (22). In view of these developments it might be worthwhile to embark on a search for substances which possess biological properties of both diacylglycerols and phorbol ester tumor promoters. These compounds might point in the direction of the development of novel non-cytotoxic inhibitors of malignant growth.

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Fig. 1. Effect of TPA on A549 cell morphology. a shows control cells; b shows cells which have been treated with TPA (10^-6 M) for 24 h, under a microscope. x 200.
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