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Mechanism of the Stimulatory Effect of Phorbol 12-Myristate 13-Acetate on Cellular Production of Plasminogen Activator (41849)

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Abstract. The human Bowes melanoma cell line secretes a plasminogen activator identical to the extrinsic tissue plasminogen activator (EPA) and different from the urokinase-like plasminogen activators mostly found in human tumor lines. In the continuous presence of 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) the 24-hr production of EPA was increased 5.3-fold (average). Preincubation of the cultures for a limited time period (optimally 3 to 6 hr) also resulted in an increase of the subsequent 24-hr production. EPA produced in the presence of PMA was serologically indistinguishable from that produced spontaneously and its molecular weight as defined by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and fibrin–agar zymography was the same as that of spontaneously produced EPA. Treatment of the cells with actinomycin D inhibited PMA-induced EPA production. Also, RNA extracted from PMA-treated cells became enriched in mRNA for EPA. It is concluded that PMA acts by enhancing the transcription of the EPA gene. Cell-associated EPA levels were increased, even when tested as early as 3 hr after initiation of the PMA treatment, thus failing to support the view that increased EPA synthesis occurred as a result of depletion of the cellular pool.

Phorbol esters such as phorbol 12-myristate 13-acetate (PMA) have strong inflammatory and tumor promoting activities (1). The mechanisms underlying these activities are not known. At the cellular level, PMA modulates formation of various proteins. Thus, in rat pituitary cells it stimulates prolactin synthesis and release (2). In certain cell systems it has been described to induce ornithine decarboxylase, an enzyme that is rate limiting in polyamine synthesis (3). In other systems it causes a decrease in production of fibronectin (4). Prostaglandin metabolism has also been reported to be affected (5, 6). Recently, PMA has been shown to stimulate the synthesis of immune interferon (7) and interleukin 2 (8) by lymphocytes.

PMA can also enhance or induce production of plasminogen activator(s) (PA(s)) by normal or transformed cells (9–16). PAs are cellular proteases which can only act by activation of plasminogen into plasmin. Many cell lines produce a PA with molecular weight (≃33,000 and 55,000), enzyme kinetic, and serological properties of urokinases. Other cell lines, in particular those derived from melanomas (e.g., the Bowes melanoma line) produce an activator of a higher molecular weight (~72,000) with properties indistinguishable from those of the so-called tissue or extrinsic plasminogen activator (EPA) that was previously isolated from fresh tissues such as uterus (17, 18).

Observations on the secretion of PA by specialized cells—granulosa cells (19), trophoblastic cells (20), macrophages (21), and neuroblastoma cells (22)—have led to the concept that the plasminogen-dependent proteolytic systems play an important role as space-creating agents in organogenesis, regeneration of damaged tissues, and various other remodeling processes of tissues. In addition, suggestive evidence has been presented for PA secretion to be associated with cellular transformation (23–32), and in particular with metastatic behavior of tumor cells (33).

It is not known whether modulation of PA formation by PMA has any significance in terms of the well-known cocarcinogenic potential of PMA. Neither is the molecular–biological basis of the effects of PMA fully understood. Following exposure of cells to PMA, the cellular content of EPA mRNA rapidly increases (34). Although this indicates that the process of transcription is affected, the possibility remains that PMA also, or even primarily, affects other stages of the protease formation and excretion.

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This kinetic study of the stimulation of PA production by PMA in Bowes melanoma cells gives evidence in favour of an effect on transcription and against an effect on excretion.

Materials and Methods. *Cell culture.* The human melanoma cell line, Bowes, was obtained from Dr. D. B. Rifkin (Rockefeller University, New York, N.Y.). The cells were grown in culture flasks (75-cm^2, Costar, Cambridge, Mass., No. 3075) using modified Eagle's minimum essential medium supplemented with 10% (final concentration) heat-inactivated newborn calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The cells were rendered mycoplasma-free by passage as a solid tumor in nude mice; attempts to subsequently clone the cell line were unsuccessful.

For experiments the cells were transferred by trypsinization, into 1.6-cm-diameter wells (24-Multiwell plate, Costar, No. 3524) at 1.5 to 4 $\times$ 10^5 cells per well. After culturing for 1 to 3 days, the medium was removed, the cells were washed twice with serum-free medium, and used for the experiments described in the Results section.

Stimulation of EPA production by PMA. PMA was added at indicated time intervals in serum-free medium, and the cultures were incubated at 37°C. At indicated times the culture fluids were removed, supplemented with 0.01% Tween 80 (final concentration) and frozen at −20°C for subsequent assay.

For determination of cell-associated EPA, the medium was removed, the cells were washed twice with phosphate-buffered saline (PBS) and 0.2 to 0.5 ml of PBS containing 0.2% Triton X-100 was added to each well. Lysates were stored at −20°C and were assayed within 1 week.

EPA determination. Assays for EPA in samples of serum-free culture medium or cell lysates were performed using microtiter plates (Falcon Plastics, Oxnard, Calif., cat. No. 3040F). The plates were coated with ^{125}I-labeled fibrin as described by Hoylaerts *et al.* (35). Purified human plasminogen (9 μg) was added to all wells. Subsequently dilutions of the samples to be assayed or of an urokinase standard preparation were added. The volume of the total reaction mixture was 200 μl. The plate was rotated at 37°C and at fixed time intervals aliquots were taken to measure released ^{125}I in a gamma scintillation counter (Packard, PGD, Auto-Gamma). Units of activity were expressed as international units per milliliter using urokinase as the standard.

Protein determination. Protein was determined fluorimetrically using bovine serum albumin as a standard (36).

Immunological characterization. Serum-free culture medium in which PA content had previously been determined was diluted to 10 to 20 units/ml with serum-free medium containing 0.01% Tween 80.

Equal volumes of increasing concentrations of antibody against the human EPA were added and the mixtures were incubated at 37°C for 1 hr. The reaction mixture was then diluted and the activity was determined.

Measurement of rapidly labeled cellular RNA. Cells were labeled with [^{3}H]uridine (New England Nuclear Corp, Boston, Mass; 27.4 Ci/mmole) at concentrations indicated in the text. After 1 hr incubation at 37°C, the cells were washed with ice-cooled PBS and lysed by adding an aliquot of a 1% solution of sodium dodecyl sulfate. An equal volume of 20% trichloroacetic acid was added to the lysates. The precipitates were caught on membrane filters which were counted in a liquid scintillation counter.

mRNA extraction and purification. Cells were harvested in PBS containing 0.01% EDTA and extracted as described by Opdenakker *et al.* (37). Briefly, the cells were washed twice with cold PBS and resuspended in 15 ml/10^9 cells of a hypotonic buffer containing 10 m*M* NaCl, 3 m*M* magnesium acetate, and 20 m*M* Tris–HCl, pH 7.4. Ribonucleosyl vanadyl complexes (10 m*M*) were added as a RNase inhibitor. After swelling for 10 min at 4°C, cells were lysed with 1/3 vol of hypotonic buffer supplemented with 5% sucrose and 1.2% Triton N-101. After homogenization nuclei were removed by centrifugation and RNA was extracted with the phenol method in the presence of 1% SDS. The total RNA was precipitated overnight at −20°C with 2 vol of ice-cold ethanol and 1/10 vol of 1 *M* NaCl.

The poly(A)-rich RNA fraction was isolated by affinity chromatography on oligo(dT)-cellulose (type 7) essentially as described by Aviv and Leder (38) except for the use of sodium instead of potassium salt. After chromatography RNA was precipitated with ethanol/

NaCl overnight. Before oocyte injection this precipitated RNA was washed twice with cold 75% ethanol, dried under vacuum, and dissolved in bidistilled water for 1 hr at 4°C.

Translation in oocytes and PA assay. Mature oocytes, stage V (39) were injected with 50 nl of RNA solutions and incubated in groups of 10 at 19°C for 48 hr, according to the procedures originally described by Gurdon *et al.* (40). The oocyte culture medium was harvested, supplemented with Tween 80 to a final concentration of 0.01%, and assayed for the presence of PA.

Electrophoresis and zymography of PA. A vertical slab-gel electrophoresis apparatus was used. The separation gel contained 9% acrylamide and 0.23% bisacrylamide in 0.375 *M* Tris–HCl buffer, pH 8.8, with 0.1% sodium dodecyl sulfate (SDS). Stacking gels were made of 4% acrylamide and 0.23% bisacrylamide in 0.138 *M* Tris–HCl buffer, pH 6.8, containing 0.1% SDS (41). Activator samples, containing 0.1 to 1 units, were applied in a volume of 20 μl. The samples contained 2% SDS. Zymography was carried out by the fibrin–agar overlay method (42). The gels were washed in 2.5% Triton X-100 to remove the SDS. After a brief rinse in distilled water, the gels were layered on indicator gel slabs containing 1.25% agar, purified human plasminogen (5 μg/ml), thrombin (0.06 units/ml), and fibrinogen (2 mg/ml). The polyacrylamide gel indicator slab assemblies were incubated at 37°C in a humid environment. After the reaction, the electrophoretic gel was removed and the fibrin layer was stained.

Reagents. Bovine fibrinogen (Poviet, Organon-Teknika, Oss, The Netherlands), thrombin (Leo Pharmaceuticals, Ballerup, Denmark), human urokinase, human plasminogen (NH_2-terminal glutamic acid-plasminogen), ^{125}I-labeled fibrinogen, antiserum against EPA, and purified EPA from Bowes melanoma cell cultures were kindly provided by Dr. D. Collen. The anti-EPA antiserum was purified by affinity chromatography on sepharose-bound EPA and had no neutralizing activity against urokinase, as previously shown (18). Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma Chemical Company, St. Louis, Missouri. Oligo(dT)-cellulose (type 7) was obtained from PL Biochemicals Inc., Milwaukee, Wisconsin.

Results. *Effect of PMA on cell viability.* Since determination of EPA in culture medium could only be done in the absence of serum, it was necessary to define conditions under which the combined cytotoxic effect of PMA and nutrient depletion were minimized. Figure 1 shows the results of an experiment in which the effect of replenishment of growing cultures with serum-free medium containing PMA was tested on further outgrowth of the cells. It can be seen that for a period of 48 hr after removing the serum the cells continued to divide. The effect of PMA on this further outgrowth of the cultures was dose- and time-dependent. With small doses (1 and 10 ng/ml) the cells continued to divide, although to a lesser degree than in the controls, for the whole 48-hr observation period. With higher doses (50 and 100 ng/ml) there was still some increase in cell number for 24 hr but then the cell number decreased indicating that, with time, PMA became cytotoxic. PMA at 500 ng/ml was cytotoxic from the beginning.

Kinetics of the enhancement of EPA release by PMA treatment. Incubation of melanoma cells in the presence of PMA resulted in enhanced production of extracellular EPA. The time and dose response relationships of this effect were investigated.

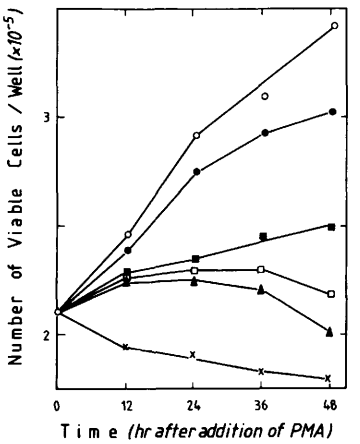

FIG. 1. Effect of PMA on multiplication of Bowes melanoma cells. Cultures were seeded at 1.6×10^5 cells/1.6-cm-diameter well in medium containing 10% serum. After 48 hr incubation the cultures were washed and refed with serum-free medium containing PMA in final concentrations of 0 (○), 1 (●), 10 (■), 50 (□), 100 (▲), or 500 (×) ng/ml.

In a first type of experiment confluent melanoma cell cultures were incubated for 48 hr in the presence of various concentrations of PMA; samples of the supernatant fluid were taken at several time intervals and their content of EPA was determined. The results were plotted in different ways. In Fig. 2a the yields of PA are given as units per milliliter of culture fluid; in Fig. 2b the same data are plotted as units per 10^5 viable cells. The dose–response curves in these different plots were very similar: increments in production with the very high doses of PMA (100 and 500 ng/ml) were somewhat less pronounced when the effects of PMA on cell viability were not taken into account. Expression of PA production per milligram of cellular protein also yielded a similar dose–response curve. Indeed, cellular protein content of the cultures varied very little with dose of PMA: only with 500 ng/ml was there a decrease of about 10%.

Over a total of 15 experiments a dose of 100 ng/ml of PMA caused an average 5.3-fold increase in 24 hr production of EPA (SE = 0.91). Between 24 and 48 hr, EPA production had a tendency to level off, especially in cultures treated with >50 ng/ml of PMA.

In the previous experiment PMA was left in the culture medium for the whole duration of the experiment. Under these conditions the drug may be cumulatively absorbed and disturb cell metabolism more than necessary for the study of the initial phases of induction. It therefore seemed advisable to design an experimental protocol in which short pulses of PMA treatment were given. Under these circumstances a fixed amount of PMA may be expected to adsorb to the cells; removal of excess PMA at a suitably chosen time may then avoid variability due to additional uptake of PMA from the medium.

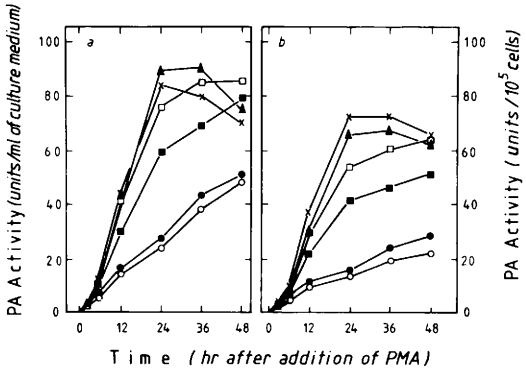

FIG. 2. Effect of increasing doses of PMA on production of EPA by Bowes melanoma cells. Confluent cultures were washed, refed with serum-free medium containing PMA in final concentrations of 0 (○), 1 (●), 10 (■), 50 (□), 100 (▲), or 500 (×) ng/ml, and incubated for various time intervals. Culture fluids were harvested for PA assay. The activity was expressed as units/ml of culture medium (a) and as units/10^5 viable cells (b).

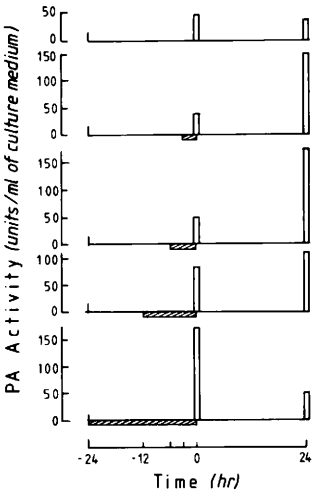

FIG. 3. Twenty-four-hour yields of EPA in Bowes melanoma cell cultures pretreated for various time intervals with 100 ng/ml of PMA. Confluent cultures were washed, refed with serum-free medium, and incubated for 24 hr. PMA was added to these cultures for time intervals of 3, 6, 12, and 24 hr (horizontal shaded bars) preceding the end of this incubation period. Supernatant fluids were harvested, and the cultures were washed, refed with fresh serum-free medium, reincubated for another 24-hr period, and harvested again.

To determine the minimum amount of time necessary for PMA to trigger maximal induction of EPA, cultures were refed with serum-free medium and incubated for 24 hr. PMA (100 ng/ml) was added to these cultures for time intervals of 3, 6, 12, and 24 hr preceding the end of this incubation period. Supernatant fluids were collected, and the cultures were washed, refed with fresh serum-free medium, incubated for another 24-hr period, and harvested again. The results of this experiment are shown in Fig. 3. Enhancement of total EPA production (i.e., production in the two harvests taken together) was as pronounced in cultures preexposed to PMA for 3 or 6 hr

as in cultures incubated for 12 or 24 hr. With short pulses the enhancement was seen in the second harvest, while with long treatment courses it was more apparent in the first harvest. This is what could be predicted from the results of Fig. 2 if the time course of EPA production is the same in the pulsed and in the continually treated cultures. Thus, a short pulse of PMA treatment of 3 to 6 hr appeared sufficient to obtain maximal stimulation of EPA production. Consequently, this experimental design was preferred for further kinetic studies.

Increased transcription of EPA-mRNA in PMA-treated melanoma cells. Two types of experiments were done to determine whether the increase in production of EPA might be due to increased transcription. In a first type of experiment the effect of actinomycin D on production of EPA was studied; in the second type of experiments the amount of EPA-specific mRNA was directly determined.

Table I shows the effects of PMA and of actinomycin D on overall RNA synthesis in the Bowes melanoma cells. Actinomycin D at 100 ng/ml inhibited the synthesis of rapidly labeled RNA by 86%, and this dose was used to evaluate the effect of such inhibition on EPA synthesis. PMA at 10 and 100 ng/ml caused a slight increase in [^{3}H]uridine incorporation when measured at 6 hr after labeling, and a slight decrease when measured after 22 hr.

In order to study the effect of inhibition of RNA synthesis on EPA production by control and PMA-treated cells, sets of cultures were refed with 0 or 100 ng/ml of actinomycin D. After 30 min, each set was divided in three subsets and 0, 50, or 100 ng/ml of PMA was added to the media of these subsets. The cultures were then incubated for 3 or 6 hr, washed, refed with serum-free medium, and reincubated. EPA was assayed on samples of the medium harvested after 24 hr. The results are represented in Fig. 4. It can be seen that actinomycin D inhibited spontaneous as well as PMA-induced EPA production. This inhibition occurred despite the fact that cell viability after 48 hr incubation was 90% in cultures pretreated with actinomycin D for 3 hr and 80% in cultures pretreated for 6 hr.

In a second type of experiment the amount of poly(A) RNA with specific EPA-mRNA activity was directly determined. Confluent cultures of melanoma cells (stationary 175-cm^2 flasks) were washed and refed with serum-free

TABLE I. EFFECT OF ACTINOMYCIN D AND PMA ON INCORPORATION OF [^{3}H]URIDINE INTO RNA OF BOWES MELANOMA CELLS

Dose of drugs[a] (ng/ml)		Acid-insoluble cellular radioactivity at:			
		6 hr		22 hr	
Actinomycin D	PMA	cpm/10^5 cells	% of control	cpm/10^5 cells	% of control
—	—	25,750	100	26,160	100
—	—	28,300			
20	—	11,170	41	—	
100	—	4,450	16	7,320	28
500	—	2,750	10	—	
—	10	29,420	109	24,010	91
—	10	29,660			
—	100	29,100	113	18,725	75
—	100	32,105			
100	10	4,840	19	7,020	27
100	10	5,600			
100	100	5,320	17	5,350	20
100	100	4,130			

[a] At time 0 hr cell monolayers were decanted, washed, and refed with medium containing the appropriate concentration of actinomycin D. PMA, at the appropriate concentration was added at time 30 min. At 6 and 22 hr sets of cultures were decanted, washed, and refed with 1 ml of medium containing 1 μCi of [^{3}H]uridine. After 1 hr absorption of the label the cells were washed with ice-cooled PBS and processed for measuring acid-insoluble radioactivity.

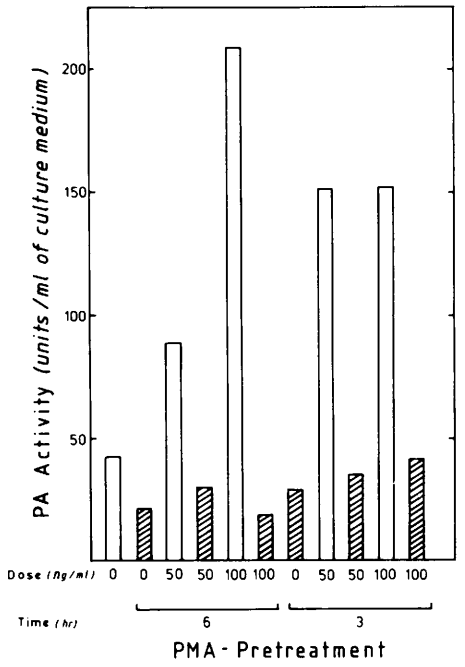

FIG. 4. Inhibitory effect of actinomycin D on production of PA by Bowes melanoma cells pretreated with PMA. Confluent cultures were preexposed to actinomycin D (0.1 μg/ml) for 30 min, then treated for 3 or 6 hr with PMA, washed, refed with serum-free medium, and incubated. After 24 hr culture fluids were harvested for PA assay. Empty bars: not treated with actinomycin D; filled bars: treated with actinomycin D.

medium containing 0, 1, 10, or 100 ng/ml of PMA. After 6 hr incubation at 37°C, the medium was decanted and the cells were detached by adding PBS with 0.01% EDTA. Cytoplasmic RNA was extracted with the phenol method, and the poly(A)-rich fraction was isolated by chromatography on oligo-dT cellulose. For each dose of PMA about 10^8 cells were available for extraction. The yields of poly(A) RNA were 27.3, 25.4, 40.6, and 81.3 μg per 10^8 cells in the case of cells treated with 0, 1, 10, and 100 ng of PMA, respectively. Thus, there was a clear-cut increase in amount of total cytoplasmic mRNA. In order to determine whether PMA caused a specific increase in the amount of EPA-specific RNA, xenopus oocytes were injected with 10 and 20 ng/oocyte of the poly(A)-RNA preparations, and PA was assayed on the medium harvested after 48 hr incubation. The results of this experiment are represented in Fig. 5. It can be seen that with increasing doses of PMA added to the melanoma cells, the poly-A RNA became indeed enriched in mRNA specific for EPA. From the lateral shift in the dose–response curves shown in the figure the increase in specificity induced by 100 ng/ml of PMA can be estimated to be about 6-fold. Taking into account the 3-fold increase in total poly(A) RNA per cell, one can estimate at 18-fold the increase in total EPA-specific mRNA content in cells treated for 6 hr with 100 ng/ml of PMA.

Serological characterization of activator produced in the presence of PMA. In order to verify that the PA produced after stimulation with PMA was of the same type as that produced by unstimulated cells (and not, e.g., of the urokinase type), aliquots of culture supernatants of both PMA-stimulated and unstimulated melanoma cells were incubated with increasing concentrations (0.1 to 1000 μg IgG/ml) of a highly specific, neutralizing antibody against EPA, and devoid of activity against urokinase. After incubation for 60 min at 37°C the mixture was assayed for enzymatic activity. For both supernatants the kinetics of neutralization were identical as shown in Fig. 6: 2.5 μg IgG/ml of the antibody reduced the enzymatic activity of 10 to 20 units/ml by 50% and 10 μg IgG/ml neutralized it completely.

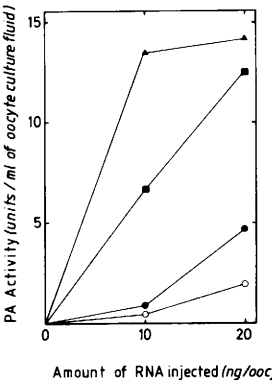

FIG. 5. Increased production of EPA-mRNA by Bowes melanoma cells after exposure to PMA. Confluent cultures were washed, refed with PMA at 0 (○), 1 (●), 10 (■), or 100 (▲) ng/ml in serum-free medium, incubated for 6 hr, and processed for mRNA extraction. Translation was tested by injection in oocytes. The oocyte culture fluid was harvested and assayed for EPA.

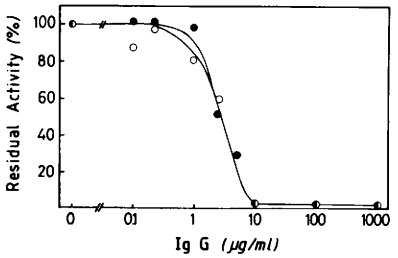

FIG. 6. Serological neutralization of EPA produced in Bowes melanoma cells. Diluted or undiluted aliquots of culture supernatants (10 to 200 units/ml) of both PMA-treated (●) and nontreated (○) cells were incubated with equal volumes of increasing concentrations (0.1 to 1000 μg IgG/ml) of a highly specific antibody against EPA. After incubation for 60 min at 37°C the mixture was assayed for enzymatic activity.

Additional evidence that no second type of protease, e.g., urokinase-like, was involved in the PMA-induced enhancement of plasminogen activator, came from analysis by electrophoresis and zymography of the product. As shown in Fig. 7 only a single band of enzyme activity was detectable in the culture supernatants and cell lysates from both control and PMA-treated cells. The activity migrated at a position that coincided with that of purified EPA and that differed from the position of urokinase.

Another control experiment consisted in adding PMA to the assay of EPA. This did not influence the assay results, indicating that the increases in EPA concentration noted in PMA-treated cultures was not due to a direct effect of PMA on the assay system.

Effect of PMA on cell-associated EPA. Increased EPA-mRNA levels and EPA release, as seen in the experiments described above, might be secondary to PMA-induced depletion of the preexisting cellular pool of EPA. Thus, if PMA would primarily act by enhancing externalization of cell-associated EPA, the cell could react with increased transcription and translation of EPA mRNA so as to replenish the EPA pool. In this case one would expect to see a decrease in the level of cell-associated EPA early after exposure of the cells to PMA. Figure 8 shows the results of an experiment done to test this possibility. Cell-associated EPA was measured before PMA treatment and after 3, 6, 15, 24, 36, and 48 hr of PMA treatment. At all time points the level of cell-associated EPA showed a dose-dependent increase.

Discussion. Many human tumor cell lines produce proteases which activate plasminogen. Most of these are of the urokinase type and various studies have shown that their production is increased by treatment with phorbol

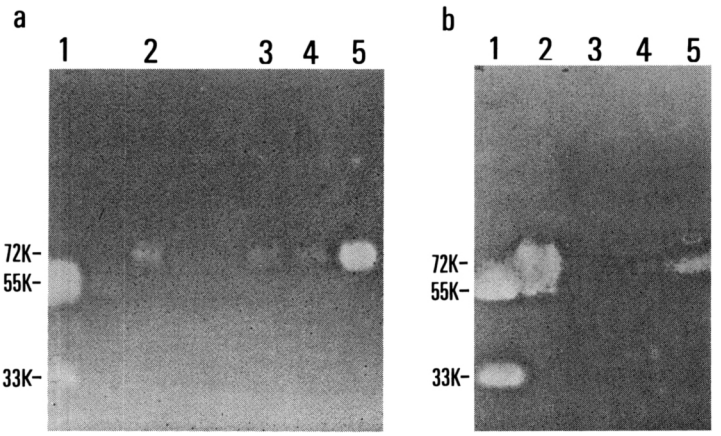

FIG. 7. Fibrin–plasminogen–agar zymogram of PA electrophoresed by SDS–polyacrylamide gel. After the appearance of the zones of fibrinolysis the indicator gel was stained and photographed. (a) Culture fluids: Lanes 1 and 2, human urokinase and purified EPA from Bowes melanoma cells as standards, respectively; Lanes 3, 4, and 5, nontreated, PMA-treated (10 ng/ml), and PMA-treated (100 ng/ml) for 24 hr, respectively. (b) Cell lysates: Lanes 1 and 2, human urokinase and purified EPA from Bowes melanoma cells as standards, respectively; Lanes 3, 4, and 5, nontreated, PMA-treated (10 ng/ml), and PMA-treated (100 ng/ml) for 24 hr, respectively.

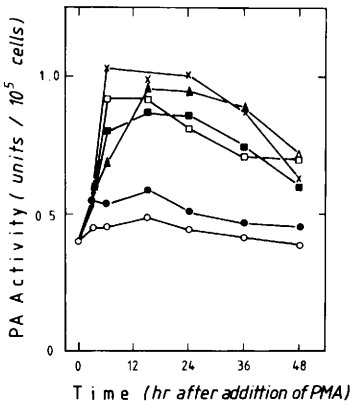

FIG. 8. Effect of increasing doses of PMA on the intracellular level of PA in Bowes melanoma cell cultures. Confluent cultures were washed, refed with serum-free medium containing PMA in final concentrations of 0 (○), 1 (●), 10 (■), 50 (□), 100 (▲), or 500 (×) ng/ml and incubated for various time intervals. After culture fluid was removed, cells were washed with PBS, and lysed by 0.2% solution of Triton X-100.

esters. The Bowes melanoma cell line studied here produces a different plasminogen activator called tissue type or extrinsic plasminogen activator (EPA) (18). We found that treatment with the phorbol ester PMA caused a distinct increase in the production of this EPA by Bowes melanoma cells. Under optimal conditions of dosage and time of exposure to PMA the production could be increased up to 13-fold.

The enhancing effect of PMA on production of extracellular EPA became apparent within 3 to 6 hr after its addition. Also, short incubation times (e.g., 3 hr) were sufficient to obtain full stimulation of EPA production similar to those seen in cultures exposed for 24 hr. This raises the possibility that continuous presence of PMA in or at the cells may not be required for full stimulation. However, PMA is known to firmly attach to the cells, and therefore extensive washing may still leave considerable quantities associated with the cells (43).

Spontaneous as well as PMA-enhanced production were blocked by pretreatment of the cells with a dose of 100 ng/ml of actinomycin D. The same dose also inhibited the synthesis of rapidly labeled RNA by 84%, suggesting that new mRNA synthesis is required for PMA to be able to enhance EPA synthesis. Additional evidence supporting an effect of PMA on transcription is that incubation of the cells for 6 hr with PMA at 100 ng/ml caused a 3-fold increase in total cellular poly(A^+) RNA and an 18-fold increase in EPA-specific mRNA. While this increase would amply explain the usual 6-fold increase in EPA production by the cells, it is not certain whether it is brought about by increase in synthesis of the mRNA or a decrease in breakdown. However, taken together with the results from the experiments with actinomycin D, our data are highly suggestive for an effect of PMA on the rate of transcription of EPA-specific mRNA. Direct measurements on EPA-mRNA synthesis will require the availability of specific DNA-probes (Opdenakker *et al.*, in preparation).

The question whether PMA acts primarily by enhancing externalization of preexisting intracellular enzyme was addressed by determining cell-associated enzyme at different times after exposure to PMA. At all times, cell-associated EPA was increased rather than decreased in PMA-treated cultures. Cell-associated EPA levels were increased, even when tested as early as 3 hr after initiation of the PMA-treatment, thus failing to support the view that increased EPA synthesis occurred as a result of depletion of the cellular pool. It remains possible, however, that a very early depletion, not detected by current techniques, preceded the increase in cell-associated EPA levels and served as the primary trigger for transcription enhancement.

In certain cell systems PMA has been shown to induce a plasminogen activator with a molecular weight (~50,000 Da) different from that of the plasminogen activator spontaneously produced (~75,000 Da) (9, 44). The two activators may be products of different genes or variants of the same gene product. In the latter case one would expect them to be serologically related. Christman *et al.* (9) have shown by immunoinhibition studies that in several hamster cell lines the two activators are serologically different, and that only one type was induced by PMA. EPA spontaneously produced by our Bowes melanoma cells has been purified to homogeneity and its molecular weight was estimated at 72,000 (16). Analysis by gel electrophoresis indicated that PMA did not induce plasminogen activator(s)

with a different molecular weight. Also, the product released by PMA-treated cells was completely neutralizable by antibody specific for spontaneously produced EPA. Similarly the oocyte translation product of mRNA from PMA-treated melanoma cells was serologically characterizable as EPA (data not shown). Therefore, the possibility that enhanced production of EPA was due to induction of a second type of activator (e.g., an activator of the urokinase type) can be eliminated.

Various hypotheses have been formulated to explain the tumor-promoting effect of PMA. In particular, certain models for two-stage carcinogenesis are based on the assumption that the promotor modifies gene expression resulting in altered management of carcinogen (initiator)-induced lesions. Our studies provide supportive evidence that PMA can indeed affect transcription of certain genes. In the present study we have only studied expression of one gene, that of EPA. Work in progress in which mRNA from PMA-induced melanoma cells is being investigated by translation in cell-free systems, has already revealed that PMA induces production of several other mRNAs (34).

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