1 Amano, T., Hamprecht, B., and Kemper, W., *Expl Cell Res.*, **85**, 399–408 (1974).
2 Klee, W. A., and Nirenberg, M., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 3474–3477 (1974).
3 Glick, M. C., Kimhi, Y., and Littauer, U. Z., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 1682–1687 (1973).
4 Minna, J., Glazer, D., and Nirenberg, M., *Nature new Biol.*, **235**, 225–231 (1972).
5 Hamprecht, B., and Schultz, J., *Hoppe-Seyler's Z. Physiol. Chem.*, **354**, 1633–1641 (1973).
6 Daniels, M. P., and Hamprecht, B., *J. Cell Biol.*, **63**, 691–699 (1974).
7 Glick, M.C., in *Miami Winter Symposia*, 7 (edit. by Lee, E. Y. C., and Smith, E. E.), 213–240 (Academic, New York, 1974).
8 Blume, A., *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **67**, 786–792 (1970).
9 Hamprecht, B., and Amano, T., *Analyt. Biochem.*, **57**, 162–172 (1974).
10 McMorris, F. A., and Ruddle, F. H., *Devl Biol.*, **39**, 226–246 (1974).

Tumour promotor induces plasminogen activator

INFECTION of chick embryo fibroblasts with Rous sarcoma virus (RSV) induces a cell-specified plasminogen activator[1]. Induction occurs with transforming viruses but not with lytic viruses or with oncornaviruses which are not themselves transforming[2]. Similarly, many mammalian cell lines and embryo cultures transformed with either viruses or chemical carcinogens may be high producers of plasminogen activator in contrast to their untransformed counterparts[3–6]. A correlation has been demonstrated between production of plasminogen activator and various features of the transformed phenotype, such as cell locomotion, morphology and loss of anchorage-dependent growth[4,7]. Several established cell lines which are not highly tumorigenic or transformed by the usual criteria are, however, active producers of plasminogen activator; there are examples also of transformed or tumorigenic cell lines which do not produce significant levels of plasminogen activator[3,8–10].

We have been interested in the regulation of the expression of plasminogen activator in the expectation that an understanding of the mechanism of its control and an ability to alter its levels with exogenous agents would provide insights into its physiological significance in the normal and malignant state. We reported that glucocorticoids inhibit expression of plasminogen activator in certain cell lines[10]. Troll *et al.* found that a phorbol ester, phorbol-12-myristate-13-acetate (PMA), a component of croton oil which causes epidermal inflammation, hyperplasia and tumour promotion in treated skin[11], also induces increased trypsin-like proteolytic activity in treated mouse skin[12]. In view of these findings, we tested the effects of PMA on plasminogen activator levels in cultured cells, and report here that PMA is a potent inducer of plasminogen activator in chick embryo fibroblasts and HeLa cells.

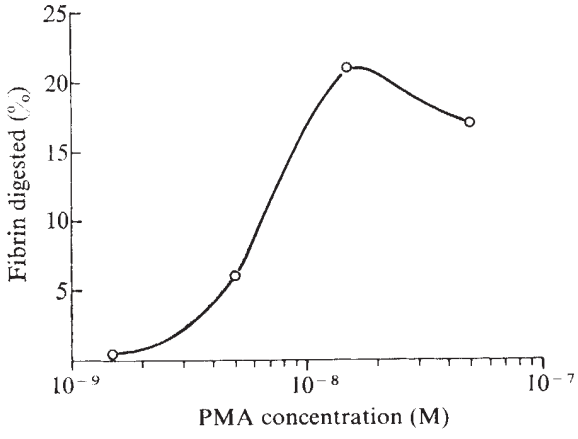

Fig. 1 A dose study of PMA in CEF cells. Subconfluent plates of CEF (approximately 5×10^6 cells per 9-cm dish) were re-fed growth medium containing PMA at the indicated concentrations. After 24 h cells were washed twice with phosphate-buffered saline and collected by scraping into 2 ml of swelling buffer (10 mM Tris, 10 mM NaCl, *p*H 8.0) without detergents. Lysates were diluted 1:10 into assay buffer (100 mM Tris, 10 mM NaCl, 3 mM NaN_3, *p*H 8.0) and assayed in triplicate as described by Unkeless *et al.*[1] on fibrin-coated plates containing 40,000–70,000 c.p.m. of ^{125}I-fibrin (10 μg cm^{-2}) in the presence of 4 μg per ml human plasminogen previously purified by lysine–Sepharose affinity chromatography[13]. Aliquots were taken after 3 h and the solubilised fibrin determined by scintillation counting (Nuclear Chicago) in Aquasol (New England Nuclear Corp.) using the ^{3}H channel. Percentage fibrin digested was determined from the ratio of the counts released in the presence of sample (adjusted for fibrin released in the presence of plasminogen but in the absence of sample) to the total counts released by trypsin.

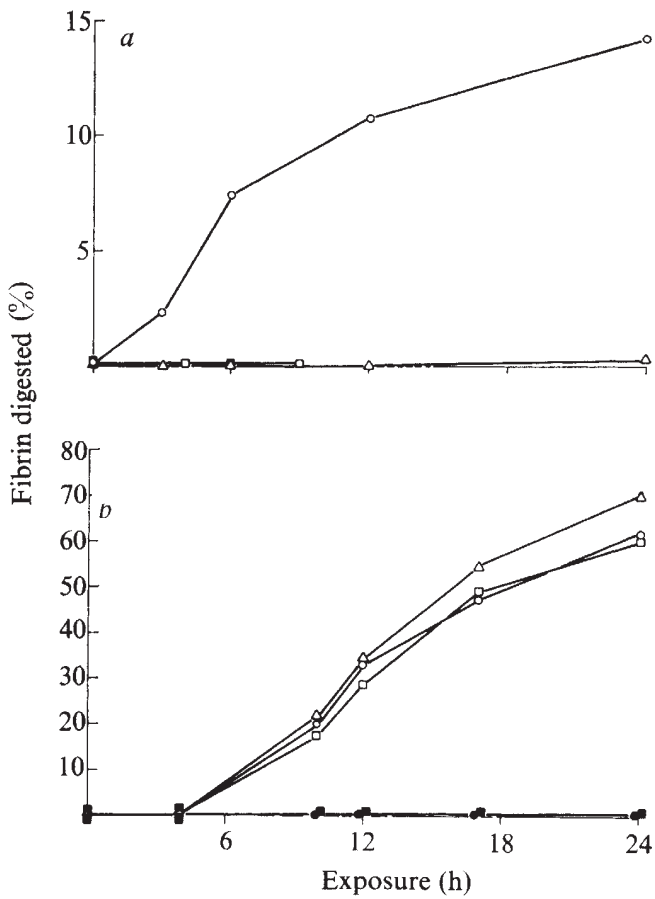

Fig. 2 Time course of induction. *a*, Cell lysate assay. Agents were added to replicate subconfluent plates of CEF at zero time and at the subsequent times indicated in the figure cells were collected and lysates assayed as described in Fig. 1. △, Untreated CEF; ○, CEF treated with 5×10^{-8}M PMA; □, CEF treated with 5×10^{-8} M PMA and 1 μg ml^{-1} actinomycin D. *b*, Intact cell assay. Assays were carried out according to Unkeless *et al.*[2]. CEF (2×10^5) were plated in triplicate on to 6-cm plates coated with 50,000 c.p.m. ^{125}I-fibrin (10 μg cm^{-2}). The medium was Dulbecco's with 10% foetal calf serum. Six hours after plating (zero time), various agents were added to the plates. Aliquots were taken at the times indicated in the figure and percentage fibrin digested determined as described in the cell lysate experiments. ●, Untreated CEF; □, CEF treated with 1.5×10^{-8}M PMA; ○, CEF treated with 1.5×10^{-8} M PMA and 10^{-6} M phorbol; ■, CEF treated with 10^{-6} M phorbol; △, CEF treated with 5×10^{-8} M PMA.

Plasminogen activator was assayed by published methods[1,2] (see legends to Figs and Table for details). The assay is based on quantification of the solubilisation of ^{125}I-labelled fibrin due to proteolytic digestion in the presence of plasminogen and sample. The sample may be either intact cells, plated directly on to a fibrin-coated dish, or a lysate of cells. In our studies, omission of plasminogen from the assay of PMA-induced cells or cell lysates reduced fibrinolysis by more than 95%, indicating that the fibrinolysis was plasminogen dependent. This justifies our referring throughout to the induction of a plasminogen activator.

Table 1 Maximum-fold increase in cell-associated plasminogen activator over endogenous levels after exposure to PMA

Cell	Origin	Time in culture	Maximum-fold increase in plasminogen activator
CEF	Chick embryo fibroblast	1–3 weeks	10
HeLa[20]	Human cervical carcinoma	20 yr	10
HEC	Hamster embryo cells	1–3 weeks	3
REC	Rat embryo cells	1–3 weeks	2
HTC[21]	Rat hepatoma	10 yr	2
L[20]	Mouse fibroblast	20 yr	1

Exposure was for 24 h and ranged from 10 to 1,000 ng ml^{-1}. Cells were grown as monolayer cultures in either Dulbecco's modified Eagle's minimal essential medium (DMEM) with 5% foetal calf serum (FCS) (L cells), DMEM+10% FCS (CEF, HEC, REC, HeLa) or Ham's F12 Medium + 10% FCS (HTC). Embryo cultures were prepared by standard procedures[22,23] from 9–12-d chick embryos, midterm pregnant rats or late term pregnant hamsters. Cell lysates were prepared and assayed as described in the legend to Fig. 1.

The dose–response curve of chick embryo fibroblasts (CEF) to PMA is shown in Fig. 1. Induction occurred at a concentration as low as 5×10^{-9} M PMA (3 ng ml^{-1}). Maximal induction was observed at 1.5×10^{-8} M. The time course of induction of plasminogen activator in CEF as assayed in cell lysates and intact cells is shown in Fig. 2*a* and *b*. Increased fibrinolytic activity in lysates (cell-associated plasminogen activator) was observed as early as 3 h after exposure to PMA (Fig. 2*a*). The longer delay in the appearance of extracellular fibrinolytic activity assayed with intact cells (Fig. 2*b*) reflects the lower sensitivity of this assay, since in Fig. 2*b* foetal calf serum was used as a source of plasminogen. When intact cells were assayed using foetal calf plasminogen, purified by lysine–Sepharose affinity chromatography[13], rather than serum, induction was detected after 3 h (data not shown). Phorbol itself, which is inactive as a tumour-promoting agent[11], neither induced nor blocked induction (Fig. 2*b*). Actinomycin D completely blocked the response to PMA (Fig. 2*a*), suggesting that PMA induction requires RNA synthesis.

The induced levels of plasminogen activator gradually returned to control values within 12 h of removal of PMA. Curiously, the deinduction was also blocked by actinomycin D. In this regard, deinduction resembles the glucocorticoid-induced repression of plasminogen activator expression in rat hepatoma cells, which is also blocked by actinomycin D (ref. 10).

PMA is not toxic to early passage CEF at 5×10^{-8} M as judged by growth rate during a 4-d exposure; however, it did inhibit growth of late (eighth) passage CEF. A growth inhibitory effect of PMA on late passage CEF has also been reported by Diamond *et al.*[14]. In spite of the different effects on growth at early and late passage, the kinetics of induction of plasminogen activator were found to be the same at both passage times.

Several cell types were screened for response to PMA (Table 1). PMA produced approximately a tenfold elevation of plasminogen activator in HeLa and CEF. A two- to threefold increase was seen with hamster embryo cells, rat embryo cells and a rat hepatoma cell line, and no induction was found with the mouse L cell line. Unresponsive cell types may prove useful in an analysis of the mechanism of the PMA response.

Phorbol esters, in addition to causing epidermal hyperplasia, inflammation and tumour promotion, have various other biological effects. These include alterations in cell morphology, growth properties and increased ^{3}H-choline incorporation in cultured cells[14–16]; mitogenesis in mouse 3T3 cells and lymphocytes[17]; a rapid but short lived increase in cyclic GMP in lymphocytes and mouse 3T3 cells[17]; decreased cyclic AMP levels in mouse skin[18]; and enhancement of cell transformation by chemical carcinogens in cell culture[19]. Our discovery that extremely low concentrations of PMA induce a marked increase in plasminogen activator in such diverse systems as CEF and HeLa cells, together with the previously described *in vitro* effects of this compound, suggest that PMA induces a constellation of changes reminiscent of the phenotype of cells transformed by oncogenic viruses or chemical carcinogens, a major difference being that the effects of PMA are readily reversed when the agent is removed. An analysis of the mechanism of action of PMA in cell culture may, therefore, shed light on the general problem of malignant transformation.

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1 Unkeless, J. C., Dano, K., Kellerman, G. M., and Reich, E., *J. biol. Chem.*, **249**, 4295–4305 (1974).
2 Unkeless, J. C., *et al.*, *J. exp. Med.*, **137**, 85–111 (1973).
3 Weinstein, I. B., Wigler, M., and Stadler, U., *WHO–IARC Workshop Rapid Screening Tests to Predict Late Toxic Effects of Environmental Chemicals*, (International Agency for Research on Cancer, Lyon, in the press).
4 Pollack, R., Risser, R., Conlon, S., and Rifkin, D., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4792–4796 (1974).
5 Jones, P., Benedict, W., Strickland, S. and Reich, E., *Cell*, **5**, 323–329 (1975).
6 Ossowski, L., Unkeless, J. C., Tobia, A., Quigley, J. P., and Reich, E., *J. exp. Med.*, **137**, 112–126 (1973).
7 Ossowski, L., Quigley, J. P., Kellerman, G. M., and Reich, E., *J. exp. Med.*, **138**, 1056–1064 (1973).
8 Mott, D. M., Fabisch, P. H., Sani, B. P., and Sorof, S., *Biochem. biophys. Res. Commun.*, **61**, 621–627 (1974).
9 Lang, W. E., Jones, P. A., and Benedict, W. F., *J. natn. Cancer Inst.*, **54**, 173–179 (1974).
10 Wigler, M. H., Ford, J. P., and Weinstein, I. B., in *Proteases and Biological Control* (edit. by Reich, E., Rifkin, D. B., and Shaw, E.), 849–856 (Cold Spring Harbor Laboratory, New York, 1975).
11 Hecker, E., *Cancer Res.*, **28**, 2338–2349 (1968).
12 Troll, W., Rossman, T., Katz, J., Levitz, M., and Sugimura, T., in *Proteases and Biological Control* (edit. by Reich, E., Rifkin, D. B., and Shaw, E.), 977–987 (Cold Spring Harbor Laboratory, New York, 1975).
13 Deutsch, D. G., and Mertz, E. T., *Science*, **170**, 1095–1096 (1970).
14 Diamond, L., O'Brien, S., Donaldson, C., and Shimizu, Y., *Int. J. Cancer*, **13**, 721–730 (1974).
15 Sivak, A., and Van Duuren, B. L., *Science*, **157**, 1443–1444 (1967).
16 Suss, R., Kreibich, G., and Kinzel, V., *Eur. J. Cancer*, **8**, 299–304 (1972).
17 Estensen, R. D., *et al.*, in *Control of Proliferation in Animal Cells* (edit. by Clarkson, B., and Baserga, R.), 627–634 (Cold Spring Harbor Laboratory, New York, 1974).
18 Belman, S., and Troll, W., *Cancer Res.*, **34**, 3446–3455 (1974).
19 Mondal, S., Peterson, A. R., and Brankow, D. W., *Proc. Am. Ass. Cancer Res.*, Abstr., **16**, 74 (1975).
20 Eagle, H., *Science*, **122**, 43–46 (1955).
21 Thompson, E. B., Tomkins, G. M., and Curran, J. F., *Proc. natn. Acad. Sci. U.S.A.*, **56**, 296–303 (1966).
22 Di Paolo, J. A., Donovan, P., and Nelson, R., *J. natn. Cancer Inst.*, **42**, 867–874 (1969).
23 Rubin, H., in *Tissue Culture Methods and Applications* (edit. by Kruse, P. F., and Patterson, M. K.), 119–122 (Academic, New York, 1973).

Evidence for inhibition of platelet activation in blood by a drug effect on erythrocytes

ADP released from erythrocytes causes adhesion and aggregation of platelets[1]. This can presumably happen *in vivo*