Phorbol esters induce synthesis of thromboplastin activity in human monocytes

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12-*O*-Tetradecanoylphorbol 13-acetate (TPA), phorbol 12,13-diacetate and phorbol 12,13-didecanoate were all potent inducers of thromboplastin activity in human monocytes *in vitro*, whereas 4 α -phorbol 12,13-didecanoate and 4 α -phorbol had no such effect. A concomitant increase in titrable apoprotein III antigen was found (apoprotein III is the protein component of thromboplastin). The increase was inhibited by cycloheximide and actinomycin D and partly by α -amanitin. The increase of thromboplastin activity was therefore most likely due to synthesis *de novo* of apoprotein III. The response was approximately halved in the absence of serum or Ca²⁺. Retinol had a weak inhibitory effect, and retinoic acid was inhibitory only at concentrations that also induced signs of cytotoxicity. TPA caused an initial rise in monocyte cyclic AMP concentration of about 90–120 min duration. No increase in ⁴⁵Ca²⁺ influx was induced over 2 h. Good correlation exists between induction of apoprotein III synthesis in monocytes *in vitro* and mouse skin-tumour promotion *in vivo* by the various phorbol derivatives. Substances inactive in tumour promotion do not induce the synthesis of apoprotein III. General activating and cytotoxic effects of TPA were monitored by determining release of lysozyme, β -glucuronidase and lactate dehydrogenase.

Human monocytes develop a potent procoagulant on stimulation with a number of membrane-perturbing agents [endotoxin (Rivers *et al.*, 1975; Prydz & Allison, 1978), lectins (Lyberg & Prydz, 1980), immune complexes (Rothberger *et al.*, 1977; Prydz *et al.*, 1979) and the bivalent ionophore A23187 (Prydz & Lyberg, 1980)]. This procoagulant is functionally identical with thromboplastin (Factor III) of the extrinsic blood-coagulation system and is neutralized by an antiserum against brain thromboplastin (Prydz & Allison, 1978; Prydz *et al.*, 1979).

Thromboplastin is a phospholipid–protein complex. The protein component is apoprotein III (Bjørklid *et al.*, 1973; Bjørklid & Storm, 1977), which is an integral membrane glycoprotein of mol.wt. about 52 000.

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDD, phorbol 12,13-didecanoate, PDA, phorbol 12,13-diacetate; 4 α -PHR, 4 α -phorbol, 4 α -PDD, 4 α -phorbol 12,13-didecanoate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

The phorbol diester series of tumour promoters, of which TPA is the most potent, interact with the plasma membrane of a number of cell types, including fibroblasts, causing changes in cell morphology and growth properties (Driedger & Blumberg, 1977; Diamond *et al.*, 1974), and HeLa cells, altering cell-surface receptors involved in growth regulation (Lee & Weinstein, 1978). Most circulating blood cells respond in some way to TPA. The compound causes platelet aggregation and release of platelet factors (Zucker *et al.*, 1974), stimulates the hexose monophosphate shunt activity (Repine *et al.*, 1974), plasminogen-activator synthesis (Granelli-Piperno *et al.*, 1977) and specific granule release (Estensen *et al.*, 1974) in polymorphonuclear leucocytes, and acts as a mitogen or a co-mitogen in lymphocytes (Touraine *et al.*, 1977; Kensler & Mueller, 1978; Mastro & Mueller, 1974). It further inhibits spontaneous and induced differentiation in murine erythroleukaemia cells in culture (Rovera *et al.*, 1977; Yamasaki *et al.*, 1977) and induces differentiation in a human promyelocytic cell line (HL-60) (Rovera *et al.*, 1979).

We report in the present paper that TPA and some related phorbol esters are powerful stimulators of thromboplastin synthesis in human monocytes, and that there is a positive correlation between the tumour-promoting activity of a given phorbol ester and its ability to induce such thromboplastin synthesis. No activity was induced in granulocytes or lymphocytes under the conditions used.

The fact that these compounds, along with a series of other plasma-membrane-active compounds, induce thromboplastin synthesis in monocytes, suggests that they trigger mechanisms that are at least partly mediated by common signals. We have looked at two potential signal compounds, the cyclic AMP response and the role of Ca^{2+} .

Materials and methods

Preparation of monocyte cultures

Human monocytes were collected and isolated as described in detail previously (Lyberg & Prydz, 1980). In summary, leucocytes were harvested individually from healthy fasting blood donors (with their informed consent) by using a blood processor (Haemonetics, Natick, MA, U.S.A.). After sedimentation with dextran (Dextran T 500; Pharmacia, Uppsala, Sweden), mononuclear cells were isolated on Lymphoprep gradients (Nyegaard, Oslo, Norway) by the method of Bøyum (1968). The cells were washed three times in MEM spinner medium (Gibco-Biocult, Paisley, Renfrewshire, Scotland, U.K.) containing 10 units of heparin/ml and twice in RPMI 1640 (Gibco-Biocult) containing 20% (v/v) foetal-calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.), 2 mM-glutamine and 30 mM-Hepes, pH 7.3. (Foetal-calf serum from some other sources may activate the monocytes.) The cells were then resuspended in this RPMI medium, and left to adhere to plastic culture dishes (Falcon, Oxnard, CA., U.S.A.) in humidified air at 37°C for 2 h. Regularly, 1.5×10^7 – 2.0×10^7 mononuclear cells were plated per 35 mm-diameter dish. When 60 mm-diameter dishes were used, 3×10^7 – 7×10^7 mononuclear cells were plated. The non-adherent cells were removed by five washes with the same medium. By this procedure, regularly more than 95% of the nucleated cells are monocytes, as evidenced by non-specific esterase staining (Yam *et al.*, 1971) and differential counting of May-Grünwald-Giemsa-stained preparations. There are regularly 1–5 platelets per nucleated cell, but this number can be decreased to less than one platelet per cell by careful washing of the cells and, if necessary, a repetition of the Lymphoprep gradient. Platelets do not, however, contain apoprotein III antigen (Bjørklid *et al.*, 1978), and in preliminary experiments the number of platelets present (between less than one and about ten platelets per nucleated cell) did not significantly

influence the relative increase of thromboplastin activity.

The various phorbol esters were dissolved in acetone and added to the cells in fresh growth medium for the incubation times indicated. Retinol and retinoic acid were dissolved in dimethyl sulphoxide. The additions were present throughout the incubation.

After incubation the medium was decanted, and the cells were scraped off with a 'rubber policeman', washed three times in ice-cold 0.15 M-NaCl and finally resuspended in 1.0 ml of barbital-buffered saline (0.588 g of sodium diethylbarbiturate, 8.564 g of NaCl and 21.5 ml of 0.1 M-HCl/litre), pH 7.35. The dishes were monitored microscopically to ensure complete removal of the cells.

Assays

After freezing and thawing and a manual homogenization (ten strokes in a Potter-Elvehjem Teflon-glass homogenizer) the cells were tested for thromboplastin activity (Hvatum & Prydz, 1966), protein (Markwell *et al.*, 1978) and deoxyribose (Burton, 1956) with bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) and deoxyribose (Sigma) as standards. Thromboplastin activity was normalized on the basis of protein or deoxyribose as appropriate.

Culture medium and cell homogenates were assayed for lysozyme (EC 3.2.1.17) (Gordon *et al.*, 1974), β -glucuronidase (EC 3.2.1.31) (Gianetto & de Duve, 1955) and lactate dehydrogenase (EC 1.1.1.27) (Kirkeby & Prydz, 1959). Cyclic AMP was measured by the method of Skomedal *et al.* (1980), and uptake of Ca^{2+} in monocytes was monitored by incubating cells in 35 mm-diameter dishes with TPA in the presence of 0.8 μCi of $^{45}\text{Ca}^{2+}$ and 0.8 μCi of [^3H]inulin/ml of medium. After the desired incubation period the medium was removed, centrifuged to remove any floating cells and a 0.1 ml sample was counted for radioactivity. The cells were scraped off in 0.5 ml of water, homogenized, and a 0.1 ml sample was counted for radioactivity. Duplicate samples were counted in 5 ml of Dilusolve (Packard Instruments, Downers Grove, IL, U.S.A.) in a Packard Tri-Carb scintillation counter. Trapped extracellular $^{45}\text{Ca}^{2+}$ was corrected for by using the [^3H]inulin radioactivity. The antiserum against apoprotein III was prepared in a goat as described by Bjørklid & Storm (1977). The amount of antigen was measured by an antibody-neutralization technique (Prydz & Gladhaug, 1971).

Chemicals, biochemicals and radiochemicals

Ionophore A23187 was kindly provided by Dr. R. L. Hamill, Lilly Research Corporation, Indianapolis, IN, U.S.A. Endotoxin was lipopolysaccharide B from *Escherichia coli* 0111:B4 obtained from

Table 1. *Stimulation of thromboplastin activity of human monocytes in vitro*

Abbreviation used: PPD, purified protein derivative of Bacille Calmette-Guérin.

Stimulant	Optimum concn. ($\mu\text{g/ml}$)	Thromboplastin activity (% of unstimulated control activity)	Requirement for serum	Time of maximum activity (h after beginning of stimulation)
Albumin/anti-albumin	25	1315 ± 237	High partial	14–18
Phytohaemagglutinin	25	1123 ± 248	None	
Concanavalin A	25	1400 ± 339	None	4–6
Wheat-germ agglutinin	10	532 ± 100	None	
Endotoxin	5–50	754 ± 175	None	4–10
Ionophore A23187	0.25	494 ± 54	Absolute	8–12
PPD	25	324 ± 30	None	2–6
TPA	1	2557 ± 210	Low partial	16–20

Difco Laboratories, Detroit, MI, U.S.A. Immune complexes were formed by incubating human serum albumin (Sigma) with the corresponding antiserum (Dakopatts, Copenhagen, Denmark) at equivalence for 90 min at room temperature. Phytohaemagglutinin HA 16 was obtained from Wellcome Research Laboratories, Beckenham, Kent, U.K. Purified protein derivative of Bacille Calmette-Guérin (tuberculosis bacterium) was obtained from the National Veterinary Institute, Oslo, Norway, and anthralin from Norsk Medisinaldepot, Oslo, Norway.

4 α -PHR, TPA, PDD, 4 α -PDD and PDA were obtained from P-L Biochemicals, Milwaukee, WI, U.S.A.

Retinol and retinoic acid, concanavalin A, wheat-germ agglutinin, *N*⁶,2-*O'*-dibutyryl cyclic AMP, 3-butyl-1-methylxanthine, cycloheximide, dexamethasone, sodium pyruvate, β -NADH, phenolphthalein glucuronic acid and dried *Micrococcus lysodeikticus* cells were obtained from Sigma. Actinomycin D and α -amanitin were from Serva, Heidelberg, Germany.

[2,8-³H]Adenosine 3':5'-cyclic phosphate, [³H]-inulin and ⁴⁵Ca²⁺ (as ⁴⁵CaCl₂) were from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Effect of TPA on thromboplastin activity of human monocytes

Results for the stimulatory effect on thromboplastin synthesis in human monocytes of several membrane-perturbing agents are given in Table 1. Preliminary experiments showed that TPA was one of the most powerful of these stimulatory agents.

The dose response for TPA was maximal at 1 $\mu\text{g/ml}$ (Fig. 1), but at this concentration evidence of cytotoxicity was seen after incubation for more than 8 h as judged by increased uptake of Trypan Blue and increased release of lactate dehydrogenase. The stimulatory activity was observed,

Fig. 1. *Effect of various concentrations of TPA on thromboplastin activity of human monocytes*

The incubation time was 14 h. Each value is the mean for duplicate cultures; 1.5×10^7 – 2.0×10^7 mononuclear cells were plated per 35 mm-diameter dish. For further details, see the Materials and methods section.

however, at much lower doses (1–10 ng/ml), not accompanied by signs of toxicity.

The response to TPA showed a time-dependent steady increase over about 18 h, after which a decline was observed (Fig. 2). The kinetics of this response differed from those of most other known inducing agents (Table 1) by being slower.

The increase in thromboplastin activity was inhibited in a dose-dependent way by cycloheximide and actinomycin D and also partially by α -amanitin (Table 2). Dexamethasone at a final concentration of 10 $\mu\text{g/ml}$ did not influence the TPA-induced response (95% of stimulated control). In the absence of foetal-calf serum from the culture medium the increase was about half (54–65%) of that in cultures with TPA and serum present. Removal of Ca²⁺ from the medium also decreased the response to about half

(40–65%) of that in TPA-stimulated cultures with Ca^{2+} and induced signs of cytotoxicity. TPA did not cause increased uptake of $^{45}\text{Ca}^{2+}$ into monocytes, and exogenous Ca^{2+} is therefore probably not necessary for the thromboplastin response to occur.

The effect of retinoids (retinol and retinoic acid) on the thromboplastin response to TPA was also

investigated. Retinol had a very moderate inhibitory effect. Retinoic acid had a strong inhibitory effect, but only at concentrations that also induced signs of cytotoxicity (Table 2).

TPA, in common with other compounds that cause an increase in thromboplastin activity in monocytes, induced no procoagulant activity in populations of granulocytes and non-adherent mononuclear cells (results not shown).

Comparison of thromboplastin-inducing activity of various phorbol esters

Other phorbol esters, structurally related to TPA, vary in their potency as tumour promoters (Boutwell, 1974; Diamond *et al.*, 1978). We have tested their ability to induce thromboplastin synthesis in monocytes. Both PDD and PDA are potent inducers (Fig. 3), whereas 4 α -PDD and 4 α -PHR are inactive in this respect. Another weak tumour promoter, anthralin (Bock & Burns, 1963), is also a weak thromboplastin inducer. Thus there is a very strong correlation between tumour-promoting and thromboplastin-inducing activity.

Effect of TPA on cyclic AMP concentration and effect of cyclic AMP elevation on the thromboplastin response

Concentrations of cyclic AMP in monocytes stimulated with TPA showed an initial 3–4-fold increase, which lasted about 90–120 min and then returned to the basal value, which remained stable

Fig. 2. Time-course for the effect of TPA (1 µg/ml) on thromboplastin activity of human monocytes. Each value is the mean for duplicate cultures. Cell numbers were as in Fig. 1. For further details, see the Materials and methods section.

Table 2. Effect of various compounds on TPA-induced apoprotein III synthesis *in vitro*. The final concentration of TPA was 1 µg/ml, and the incubation time 16–17 h. The stimulation with TPA was 17–34-fold above incubated-control values. Each value is the mean for two to four cultures.

Addition	Final concn. (µg/ml)	Thromboplastin activity (% of stimulated-control value)
Cycloheximide	1	33
	5	9
	10	4
	20	3
Actinomycin D	10	22
	25	8
α-Amanitin	10	89
	25	64
Retinol	1	94
	5	83
	10	54
Retinoic acid	1	101
	5	42
	10	18
Dibutyryl cyclic AMP	491	90
3-Butyl-1-methylxanthine	111	70
Dibutyryl cyclic AMP + 3-butyl-1-methylxanthine	491 + 111	76

Fig. 3. Effect of various phorbol esters ($1\mu\text{g/ml}$) and anthralin ($10\mu\text{g/ml}$) on the thromboplastin activity of human monocytes

The incubation time was 16 h. Each column represents the mean result for duplicate cultures. Cell numbers were as in Fig. 1. For further details, see the Materials and methods section.

Fig. 4. Effect of TPA on the intracellular cyclic AMP concentration

The TPA concentration was $1\mu\text{g/ml}$. Each value is the mean value for duplicate cyclic AMP determinations on two separate cultures; 5×10^7 – 7×10^7 mononuclear cells were plated per 60 mm-diameter dish. ■—■, TPA-stimulated; ●---●, control.

for at least 8 h (Fig. 4). The phosphodiesterase inhibitor 3-butyl-1-methylxanthine (final concn. 0.5 mM) had a slight to moderate inhibitory effect on TPA-induced thromboplastin increase, whereas addition of exogenous dibutyryl cyclic AMP (final concn. 1 mM) had no significant effect (Table 2). Dibutyryl-cyclic AMP and 3-butyl-1-methylxan-

Table 3. Release of lysozyme and β -glucuronidase from monocytes stimulated with phorbol esters and anthralin

The final concentration of the stimulants was $1\mu\text{g/ml}$, except for anthralin ($10\mu\text{g/ml}$), and the incubation time was 14–16 h. Each value is the mean for two to ten cultures. Unstimulated controls released $0.72 \pm 0.09\mu\text{g}$ of lysozyme/h per mg of cell protein and $0.08 \pm 0.01\mu\text{g}$ of β -glucuronidase/h per mg of cell protein.

Stimulant	Activity (% of unstimulated-control value)	
	Lysozyme	β -Glucuronidase
TPA	$666 \pm 114^*$	$432 \pm 69^*$
PDD	650	447
PDA	495	372
4 α -PDD	94	75
4 α -PHR	130	150
Anthralin	264	415

* For TPA, results are means $\pm$ S.E.M. for ten cultures. For the other stimulants the values are means for two cultures.

thine together had no effect above that of 3-butyl-1-methylxanthine alone.

The goat anti-(apoprotein III) antiserum diluted 1:40 neutralized a standard crude thromboplastin preparation (Bjørklid *et al.*, 1973) from human brain as well as the procoagulant activity in stimulated monocytes (Prydz & Allison, 1978). After preincubation with unstimulated monocytes, the antibody titre remained unchanged. After preincubation with TPA-stimulated monocytes with high thromboplastin activity, the anti-(apoprotein III) titre was decreased by about 85%, showing that apoprotein III antigen had appeared in the cells.

Effect of TPA on release of cellular enzymes

Cytotoxic and general activating effects of TPA were monitored by testing the release of lactate dehydrogenase, lysozyme and β -glucuronidase. TPA at concentrations of $1\mu\text{g/ml}$ and above generally caused a slight increase in cytoplasmic lactate dehydrogenase release to the medium (165% of activity in control culture media). Lysozyme release was greatly enhanced in the presence of TPA, and release of β -glucuronidase was likewise enhanced (Table 3). Release of both enzymes was of the same order of magnitude as their release from monocytes stimulated with immune complexes, which among the other known thromboplastin inducers is the most potent in stimulating lysosomal-enzyme release in the doses used. Release of both enzymes was rapid, and maximal activities were recorded after 5 h. Cycloheximide and actinomycin D had essentially no effect on the release of these enzymes. The ability

to stimulate release of lysozyme and β -glucuronidase correlated well with the tumour-promoting activity and thromboplastin-inducing ability of the different phorbol analogues (Table 3).

Discussion

Weinstein *et al.* (1977) have recently reviewed the action of tumour-promoting agents in cell cultures. Phorbol esters have a variety of biological effects in different cell types both *in vivo* and *in vitro*.

The present work establishes that TPA, a potent promoter of mouse skin carcinogenesis (Boutwell, 1974; Berenblum, 1969) at nanomolar concentrations elicits a dramatic increase of thromboplastin activity in human monocytes. Also PDD and PDA are potent inducers, whereas 4 α -PHR and 4 α -PDD are not. The latter two are inactive as tumour promoters (Boutwell, 1974; Diamond *et al.*, 1978). TPA and PDD, which are potent tumour promoters, also induced a 15–20-fold increase in thromboplastin activity. Thus there is in general a good correlation between the ability to increase the thromboplastin activity and the activity of the various compounds as tumour-promoting agents on mouse skin. PDA, which is claimed to be a weaker promoter (Boutwell, 1974; Diamond *et al.*, 1978) is an exception in that it was highly active in our system. Such discrepancies between systems *in vitro* and *in vivo* were reported previously by Wigler *et al.* (1978) and Mondal *et al.* (1976) and merit further investigation. Anthralin, a structurally unrelated, non-phorbol promoter had a clear effect on thromboplastin activity, although smaller than that of the phorbol esters. A more detailed study of other promoting agents is required to see whether induction of thromboplastin synthesis is a valid screening system for promoters.

The effects of inhibitors of macromolecular synthesis (actinomycin D, α -amanitin and cycloheximide) indicate that induction of thromboplastin activity by TPA involves synthesis *de novo* of apoprotein III or of an hitherto unknown activator substance, rather than simply intracellular accumulation or enhanced expression. The induction requires both transcription and translation, since it is totally inhibited by actinomycin D as well as by cycloheximide.

Increased amounts of apoprotein III antigen were found in homogenates of TPA-stimulated monocytes by an antibody-neutralization test, and this constitutes further evidence for an increased synthesis *de novo* of apoprotein III. The increase thus represents another example of altered gene expression by these tumour promoters.

The activity of the phorbol esters most likely relates to their numerous effects at the plasma membrane, as evidenced by increased phospholipid

synthesis (Wertz & Mueller, 1978), increased membrane fluidity (Weinstein *et al.*, 1979), increased uptake of 2-deoxyglucose (Driedger & Blumberg, 1977), inhibition of binding of epidermal growth factor to its receptors in HeLa cells (Lee & Weinstein, 1978) and changes in cell morphology (Driedger & Blumberg, 1977) and in cell-surface glycoproteins (Weinstein *et al.*, 1977). They further inhibit metabolic co-operation in co-cultures of epidermal and 3T3 cells (Murray & Fitzgerald, 1979) and in Chinese-hamster cells (Yotti *et al.*, 1979). Although the mechanism of action of phorbol esters is unknown, it appears likely that they bind to cell-surface receptors and initiate one or a small number of primary responses that give rise to multiple secondary responses. Specific binding of phorbol 12,13-dibutyrate to chick-embryo fibroblasts has recently been shown by Driedger & Blumberg (1980).

Ca²⁺ is held to be a key intracellular messenger in many cellular processes. Whitfield *et al.* (1973) suggested that alterations in Ca²⁺ fluxes might be the primary event in the mitogenic responses of lymphocytes to TPA. Schimmel & Hallam (1980) also demonstrated rapid alterations in both Ca²⁺ content and transport in cultured differentiated chick myoblasts on treatment with TPA. Smith & Iden (1979), on the other hand, have shown that TPA-stimulated release of lysozyme from human neutrophils is independent of extracellular Ca²⁺. We were unable to demonstrate alterations in Ca²⁺ influx in monocytes during incubation with TPA, and exogenous Ca²⁺ is probably not necessary for the thromboplastin response to occur. This does not exclude mobilization of internal Ca²⁺ pools as part of the chain of events that occur after TPA stimulation, but it appears that various cell types may respond differently with respect to Ca²⁺ movements over the plasma membrane after TPA treatment.

Vitamin A and its analogues (retinoids), which have been shown to inhibit the tumour-promoting action of TPA in mouse epidermis (Bollag, 1972) caused a moderate decrease in the induced thromboplastin activity.

Dexamethasone inhibits mouse skin carcinogenesis when applied simultaneously with croton oil (Belman & Troll, 1972). It also inhibits the TPA-induced production of plasminogen activator in certain tumour-cell cultures (Wigler *et al.*, 1975) and in normal activated macrophages (Vasalli *et al.*, 1976). In our system, however, dexamethasone had no inhibitory effect on the thromboplastin increase. Lack of effect of dexamethasone is also seen when the ionophore A23187 or immune complexes are used as stimulators (Prydz & Lyberg, 1980); all three agents have in common that they elicit a relatively delayed thromboplastin response (see Table 1) and have a partial or absolute requirement

for serum factors for full response. Serum factors are also necessary for stimulation of Balb/c-3T3 cell growth by TPA (Frantz *et al.*, 1979). In contrast, dexamethasone had a significant inhibitory effect on the thromboplastin response induced by the fast-acting serum-independent agents like lectins, endotoxin and PPD (Prydz & Lyberg, 1980).

Initially, TPA increased the cyclic AMP concentration in monocytes. A 3–4-fold increase was seen, with maximum values appearing between 20 and 50 min in different experiments. Basal values were reached again after 90–120 min. A similar, somewhat protracted, increase of cyclic AMP has been described in rat-embryo fibroblasts (Rochette-Egly *et al.*, 1979). TPA rapidly alters cyclic nucleotide metabolism in myoblasts (Schimmel & Hallam, 1980). If this cyclic AMP increase is a positive internal signal for the protein-synthesis-dependent thromboplastin response, the effect of the phosphodiesterase inhibitor 3-butyl-1-methylxanthine becomes somewhat paradoxical. Although increasing cellular cyclic AMP concentrations (Beavo *et al.*, 1979), 3-butyl-1-methylxanthine has a universal effect, of varying strength, in decreasing the induced thromboplastin activity, regardless of the stimulatory agent used (T. Lyberg & H. Prydz, unpublished work), and this is also true for TPA. Exogenous dibutyryl cyclic AMP, however, had no significant inhibitory effect on the thromboplastin increase induced by TPA. One explanation of this discrepancy may be that the effect of 3-butyl-1-methylxanthine is unrelated to its effect on the concentration of cyclic AMP (Lehnert, 1979), or 3-butyl-1-methylxanthine may exert its effect on a compartmental pool of cyclic AMP. An initial decrease in cyclic AMP concentration during the very first minutes of TPA action has not been ruled out by these investigations. It seems more likely, however, that the cyclic AMP increase is secondary to other events related to TPA action, and in fact is a negative signal hampering and delaying the thromboplastin response.

We suggest that all the different stimulatory agents trigger a common pathway leading to thromboplastin synthesis. In the case of TPA, immune complexes and ionophore A23187 the process may be delayed because it may not proceed before the cyclic AMP 'brake' is released. The dexamethasone effect on the lectin-, endotoxin- and PPD-induced thromboplastin increase may thus be mediated via an increase of cyclic AMP (Deporter *et al.*, 1979).

Systems *in vitro* responding to tumour promoters may facilitate the identification of such agents and their mode of action. The results presented here indicate that induction of thromboplastin synthesis in monocytes may be a useful marker for tumour promoters. In addition, the induction by TPA of

apoprotein III synthesis may throw further light on the thromboplastin response.

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