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Synergistic Stimulation of Early Events and DNA Synthesis by Phorbol Esters, Polypeptide Growth Factors, and Retinoids in Cultured Fibroblasts

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12-O-Tetradecanoyl-phorbol-13-acetate (TPA), in the absence of serum, acts synergistically with a range of polypeptide growth factors to stimulate DNA synthesis in quiescent Swiss 3T3 cells. These growth factors include epidermal growth factor (EGF), insulin, and the peptide produced by BHK cells transformed by SV-40 virus (fibroblast-derived growth factor, FDGF). Retinoids also show mitogenic synergism with TPA or polypeptide growth factors. The spectrum of mitogenic synergisms displayed by TPA are similar to those of vasopressin, a pituitary peptide. However, TPA and vasopressin do not synergistically interact to stimulate DNA synthesis in quiescent 3T3 cells. This suggests that TPA and vasopressin act via an identical biochemical pathway. Several lines of evidence suggest rapid postreceptor convergence of the mitogenic mechanisms of action of the hormone and the tumor promotor. Thus, vasopressin and TPA both inhibit EGF binding to cellular receptors. Furthermore, TPA and vasopressin induce a similar array of early events in quiescent cells – most strikingly, identical stimulation of Rb^+ influx. Stimulation of ion flux is suggested as the possible convergence point of the pathway by which TPA and vasopressin act as mitogens.

Key words: Phorbol esters, retinoids, vasopressin, mitogens, uridine uptake, deoxyglucose uptake, ion fluxes

Normal, untransformed fibroblasts reduce their rate of entry into the S (DNA-synthesizing) phase of the cell cycle and accumulate in a highly viable state (called G_0, A, or R) under a large number of nonoptimal environmental conditions [1–4]. Under usual culture conditions, the limiting component is the concentration of serum present in the medium [5, 6]. Addition of serum to such quiescent cultures enhances the rates of protein and RNA synthesis and dramatically stimulates DNA synthesis and cell division. This large and reproducible transition of growth rate provides an experimental system for elucidating mechanisms of growth control.

Phorbol esters, a family of compounds with tumor-promoting activity, are strongly mitogenic to several types of quiescent cells in culture in the presence of limiting concentrations of serum [7]. The mitogenic potency is related to the potency of the phorbol ester as a tumor promoter in the two-stage system of skin carcinogenesis in mice [8]. 12-O-Tetradecanoyl-phorbol-13-acetate (TPA) is the most active tumor promotor and mitogen of the phorbol ester family.

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Recently we studied the mitogenic properties of TPA in quiescent cultures of fibroblastic cells. We found that the tumor promotor acts synergistically with other growth-promoting agents [9], rapidly stimulates an array of early metabolic changes, and causes a profound inhibition of the binding of radiolabeled epidermal growth factor (EGF) to cellular receptors [10]. The neurohypophyseal hormone vasopressin is also mitogenic for 3T3 cells [11]. We have found that many of its physiologic properties are similar to those of TPA. We propose that TPA modifies cell function by a mechanism similar to that of polypeptide hormones, like vasopressin.

INTERACTION OF TPA WITH DEFINED GROWTH FACTORS

Serum in the nutrient medium was shown to be an essential requirement for the mitogenic actions of TPA in cultures of mouse epidermal cells [12] and fibroblastic cells [7, 13]. The role of serum remained unresolved. We recently showed that TPA is mitogenic in the absence of serum if other growth factors are present [9]. Thus, quiescent cultures of Swiss 3T3 cells in the complete absence of serum are stimulated to undergo DNA synthesis by the addition of TPA together with either insulin, epidermal growth factor, or fibroblast-derived growth factor (FDGF), a polypeptide isolated from medium conditioned by BHK cells transformed by SV-40 virus [14] (Table I). The synergistic stimulation of DNA synthesis by TPA and defined growth factors can be demonstrated by measurements of total ^{3}H-Thymidine (^{3}H-TdR) incorporation into acid-insoluble material or by determination of the percentage of autoradiographically labeled nuclei incorporating ^{3}H-TdR (see Dicker and Rozengurt [9], and Table I).

The interaction between the tumor promotor and EGF or insulin is particularly striking, because neither of these substances produces a substantial stimulation of DNA synthesis by itself. In the presence of a fixed concentration of insulin, TPA stimulates DNA

TABLE I. Mitogenic Synergisms Between TPA and Growth Factors

		DNA synthesis			
		^{3}H-TdR incorporation[a]		Autoradiographically labeled nuclei[b]	
Additions	μg/ml	No TPA	100 ng/ml TPA	No TPA	100 ng/ml TPA
None		1	2	1	1
EGF	0.01	2	34	1	22
Insulin	1	2	95	1	70
FDGF	0.6	1	56	ND[c]	ND
FDGF	1.2	3	105	3	67
FDGF	2.4	46	97	ND	ND

[a]Cumulative ^{3}H-TdR incorporation, as measured in Rozengurt and Heppel [24], was determined over a 40-h exposure of quiescent cells to the additions indicated. Results are expressed as a percentage of incorporation produced by serum (10%) in the same experiment.

[b]Autoradiography was performed as in Rozengurt and Heppel [24] after a 40-h exposure of quiescent cells to ^{3}H-TdR and the additions indicated. Results are expressed as a percentage of the fraction of labeled nuclei produced by serum in the same experiment. Serum (10%) produced 80–90% autoradiographically labeled nuclei in these experiments.

[c]ND, not done.

synthesis in a dose-dependent manner (Fig. 1). The lowest concentration of TPA needed to show synergism consistently with 1 μg/ml insulin was 3 ng/ml. In contrast to the effect of TPA, phorbol itself, tested either in the absence or presence of insulin, was completely inactive in stimulating DNA synthesis. The close analogue, 4-O-methyl-12-O-tetradecanoyl-phorbol-13-acetate, was slightly mitogenic (18% of the maximal effect of TPA with insulin) at high concentrations (1 μg/ml) and in the presence of 1 μg/ml insulin. Phorbol is inactive as a tumor promotor while 4-O-methyl-12-O-tetradecanoyl-phorbol-13-acetate is a weak tumor promotor. Thus in our system the ability of phorbol esters to stimulate DNA synthesis in the presence of defined growth factors correlates well with the tumor-promoting potential of phorbol esters.

There is a striking synergistic interaction between growth-promoting polypeptides and TPA in stimulating quiescent cultures to recommence cell proliferation (Table II). In these experiments, the stimulating agents were added to medium containing depleted serum (see legend to Table II). Neither TPA nor insulin added to such cultures caused a substantial increase in growth, while together they caused a threefold increase in cell number. Similarly, although EGF and insulin together caused a threefold increase in cell number, the combination of the tumor promotor with both insulin and EGF resulted in a fivefold increase in cell number.

Hayashi and Sato [15] have demonstrated that several tumor epithelial cell lines grow in the absence of serum if growth factors are added to the medium. However, no fibroblastic cell retaining the ability to reversibly arrest in the G_0G_1 phase of the cell cycle has been reported to be able to proliferate in the absence of serum. Swiss 3T6 cells are fibroblastic cells that possess the reversible G_0G_1 arrest point [16]. Table III shows that 3T6

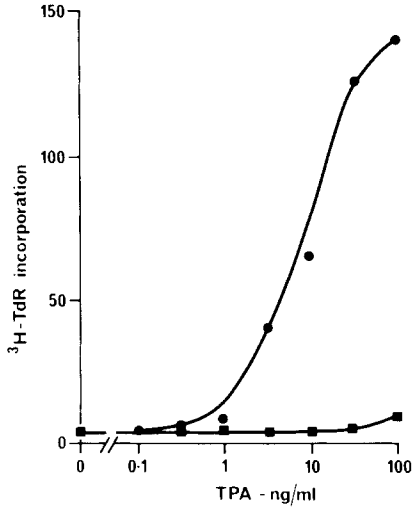

Fig. 1. Stimulation of ^{3}H-TdR incorporation by TPA and insulin into quiescent 3T3 cells. Cumulative ^{3}H-TdR incorporation (cpm × 10^{-3} per plate), measurement and conditions as in Rozengurt and Heppel [24], was determined over a 40-h exposure of quiescent cells to TPA in the absence (■) or presence (●) of 1 μg/ml insulin. Ten percent fetal bovine serum induced ^{3}H-TdR incorporation equal to 156 × 10^3 cpm per plate. As in all ^{3}H-TdR incorporations described in this review, quiescent cultures produced as in Rozengurt and Heppel [24] were washed twice in serum-free medium immediately prior to the experiment and experimental incubations were performed in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Waymouth medium.

TABLE II. TPA, EGF, and Insulin Stimulation of 3T3 Cell Proliferation

Addition	Cell number increase per 30-mm plate $\times 10^{-5}$ after 5 days	
	No TPA	200 ng/ml TPA
None	0	0.2
10 ng/ml EGF	0.5	1.5
1 μg/ml insulin	0.9	4.8
10 ng/ml EGF + 1 μg/ml insulin	3.9	8.8

Quiescent, confluent cultures of Swiss 3T3 cells were produced as in Rozengurt and Heppel [24]. Medium from parallel cultures was removed and one-third its volume of Waymouth's medium was added to replace low-molecular-weight nutrients that might have become depleted. TPA, insulin, and EGF were then added to give final concentrations as indicated. For each culture, two aliquots of cell suspension, produced by thorough trypsinization, were counted on a Coulter counter. Initial cell density was 2.3×10^5.

TABLE III. Proliferation of 3T6 Cells in Serum-Free Medium

Additions	Cells per 50-mm dish $\times 10^{-5}$
None	0.2
100 ng/ml TPA	2.0
1 μg/ml insulin	2.8
100 ng/ml TPA + 1 μg/ml insulin	10.8
0.5% fetal bovine serum	15.0

A 3T6 cell suspension was produced with crystalline trypsin, which was then neutralized with an excess of soya bean trypsin inhibitor. The cells were plated at 0.25×10^5 cells per 50-mm dish, in the absence of serum, in 1:1 Eagle's/Waymouth's plus 1.6 μM $FeSO_4$. The additions were made as above. Cultures were refed on days 4 and 8. On day 12 cultures were trypsinized and aliquots of the cell suspension were counted on a Coulter counter. The effect of 0.5% serum is shown for comparison.

cells can proliferate in serum-free medium if growth factors are present. Some proliferation of these cells occurs in the presence of insulin or TPA; however, a strikingly synergistic increase in proliferation occurs in the presence of both these factors. It should be noted that these experiments were performed with cells plated in the absence of serum (see footnote to Table III). Thus proliferation is indeed occurring in the complete absence of serum, and not owing to a residue left over from plating in serum.

The above results show that TPA apparently behaves as a growth factor in participating in complex synergistic interactions with growth-promoting polypeptides and, indeed, in stimulating cell proliferation. Thus, TPA may well be acting through the same type of mechanism as that used by peptide growth factors. This possibility receives further support in the next section, where we discuss a novel synergism between TPA, hormones, and a group of nonpeptide growth factors, the retinoids.

RETINOIDS AND TPA

Vitamin A and its derivatives have been reported to be inhibitors of cell proliferation [17] and also inducers of cell differentiation [18]. These properties are the reverse of TPA, which enhances cell proliferation and in many systems blocks cell differentiation [19]. Indeed retinoids have been shown to specifically reverse the effects of TPA, in several cell types. Thus retinoids block the mitogenic activity of TPA in lymphocytes [20] and the induction of ornithine decarboxylase produced by TPA in epithelial cells [21]. However, retinoic acid has been reported to minic TPA in increasing synthesis of plasminogen activator in chicken fibroblasts [22]. We therefore investigated the effects of retinoids on the stimulation of DNA synthesis produced by TPA, serum, or purified growth factors in 3T3 cells [23].

The combination of retinoic acid plus TPA synergistically produces 20–30% of the ^{3}H-TdR incorporation induced by saturating amounts of bovine serum (Table IV). Retinoic acid also drastically increases the amount of ^{3}H-TdR incorporation induced by EGF and TPA (Table IV and Fig. 2). Retinoic acid potentiates the mitogenic potency of TPA and EGF in a concentration-dependent manner. The minimal concentration of retinoic acid at which stimulation is consistently seen is 0.01 μM and maximal effects occur at about 1 μM. At 10 μM retinoic acid becomes strongly cytotoxic to 3T3 cells in the absence of serum.

Retinoic acid also exerts its stimulatory effects in the absence of TPA. Retinoic acid at 1 μM causes a considerable increase in ^{3}H-TdR incorporation induced by low concentrations of serum in cultures of quiescent Swiss 3T3 cells (Table IV). Likewise, retinoic acid markedly increases ^{3}H-TdR incorporation induced by low concentrations of FDGF (Table IV). TPA also enhances the mitogenic potency of FDGF (Table IV). In the presence of both TPA and retinoic acid, FDGF becomes a very potent mitogen at low concentrations. The enhancement of the potency of mitogens by retinoic acid was also found when the percentage of labeled nuclei incorporating ^{3}H-TdR was measured by autoradiography [23]. Retinol, retinal, and retinyl acetate all have stimulating effects on ^{3}H-TdR incorporation similar to those produced by retinoic acid (unpublished results).

Retinoic acid possesses growth-promoting activity when cell proliferation is monitored over a period of several days. Experiments performed in the presence of 2% serum

TABLE IV. Retinoic Acid Enhancement of the Mitogenic Potency of Growth Factors

Additions	^{3}H-TdR incorporation	
	No retinoic acid	Retinoic acid
None	1	1
TPA	1	25
EGF + TPA	18	68
Insulin + TPA	46	118
FDGF	3	40
FDGF + TPA	44	120
Fetal bovine serum	35	98

Concentrations of EGF, retinoic acid, insulin, TPA, fetal bovine serum, and FDGF were 0.01 μg/ml, 1 μM, 1 μg/ml, 0.1 μg/ml, 6% (v/v), and 1.2 μg/ml, respectively. ^{3}H-TdR incorporation was measured over 40 h as in Rozengurt and Heppel [24]. ^{3}H-TdR incorporation is expressed as a percentage of the incorporation induced by 10% fetal bovine serum.

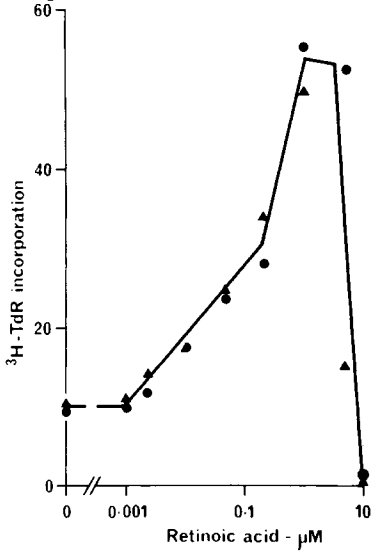

Fig. 2. Potentiation by retinoic acid of ^{3}H-TdR incorporation induced by EGF and TPA or EGF and vasopressin in quiescent 3T3 cells. Cumulative ^{3}H-TdR incorporation (cpm × 10^{-3} per plate) was determined over a 40-h exposure of cell cultures to retinoic acid with 10 ng/ml EGF and 100 ng/ml TPA (•) or with 10 ng/ml EGF and 10 ng/ml vasopressin (▲). Conditions and measurements were as in Rozengurt and Heppel [24]. EGF, TPA, vasopressin, or insulin alone induced incorporation of under 2×10^3 cpm per plate. Fetal bovine serum (10%) induced incorporation of 155×10^3 cpm per plate in this experiment.

in the medium (which does not support growth of Swiss 3T3 cells) demonstrate that retinoic acid causes a twofold to threefold increase in cell number. Furthermore, retinoic acid combined with either TPA, EGF, insulin, or FDGF synergistically caused a greater increase in cell number [23].

These results demonstrate that retinoids markedly enhance the mitogenic potency of a range of growth factors including TPA. Since high concentrations of FDGF [14] or the combination of insulin, EGF, and vasopressin [11] added in the absence of retinoids produce nearly maximal stimulation of DNA synthesis in Swiss 3T3 cells, retinoids are not required as essential nutrient molecules in this system. Their effects are probably due to a hormone-like, regulatory modulation of the mitogenic response. Intracellular retinoid-binding proteins have recently been isolated from many tissues, prompting the suggestion that these proteins mediate the biologic effects of retinoids, in a way similar to the role of steroid hormone receptors in responsive cells. Whatever the mechanism of action of retinoids, the fact that they potentiate the mitogenic activity of TPA as they do with other growth factors supports the notion that TPA performs its mitogenic actions via pathways like those used by other growth factors. Through which specific hormonal pathway does TPA exert its mitogenic effects? In the next section we produce strong evidence indicating that TPA activates the same mitogenic mechanisms as those stimulated by a defined peptide hormone, vasopressin.

SIMILARITIES IN THE MITOGENIC ACTIONS OF TPA AND VASOPRESSIN

Recent work from this laboratory has indicated that ionic fluxes may play a role in modulating the mitogenic response [24, 27]. These observations prompted us to test whether hormones or ionophores that stimulate ion fluxes are also able to regulate cell proliferation. We found that vasopressin, a neurohypophyseal nonapeptide that promotes Na^+ transport in several tissues, is a potent mitogen for Swiss 3T3 cells [11]. The hormone causes a shift of the dose response for the effect of serum on ^{3}H-TdR incorporation by quiescent cells. In the absence of added serum, the effect of vasopressin is greatly potentiated by insulin, EGF, and FDGF. Furthermore, we recently found that the mitogenic effect of vasopressin and EGF can be further enhanced by retinoic acid. Figure 2 shows the dose response of retinoic acid in the potentiation of EGF and vasopressin stimulation of ^{3}H-TdR incorporation in 3T3 cells. The dose response and extent of the enhancement by retinoic acid are similar to those seen in the presence of EGF and TPA (Fig. 2). Thus vasopressin appears to be able to replace TPA under these conditions. Nor is the equivalence of the mitogenic properties of vasopressin and TPA confined to the above situation. Table V illustrates that the phorbol ester and the nonapeptide produce identical stimulation of ^{3}H-TdR incorporation in the presence of several mitogens or combination of mitogens. Figure 3 shows that the time course of stimulation of ^{3}H-TdR incorporation caused by TPA and insulin is superimposeable with that caused by vasopressin and insulin. Further both vasopressin and TPA produce similar potentiation of the mitogenic potency of low concentrations of serum or FDGF (data not shown).

The similarities in the mitogenic properties of TPA and vasopressin suggest that these chemically diverse factors are stimulating DNA synthesis via identical pathways. If so, TPA and vasopressin should not synergistically increase each other's mitogenic activity. Results in Figure 4 demonstrate that this is indeed the case. TPA and vasopressin together cause no synergistic stimulation of DNA synthesis. That this is not because these molecules inter-

TABLE V. Similarity of Mitogenic Properties of TPA and Vasopressin

Additions	^{3}H-TdR incorporation		
	No addition	TPA	Vasopressin
None	1	2	1
EGF	1	14	10
EGF + retinoic acid	1	50	45
Insulin	1	60	58
Insulin + retinoic acid	2	105	93
EGF + insulin	21	93	98

Concentrations of EGF, retinoic acid, insulin, TPA, and vasopressin were 0.01 μg/ml, 1 μM, 1 μg/ml, 0.1 μg/ml, and 10 ng/ml, respectively. ^{3}H-TdR incorporation into acid-insoluble material was measured over 40 h as in Rozengurt and Heppel [24]. ^{3}H-TdR incorporation is expressed as a percentage of the incorporation induced by 10% fetal bovine serum.

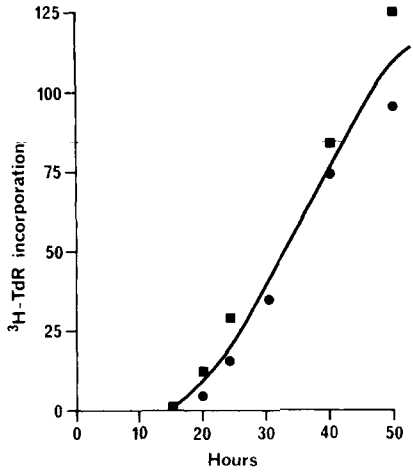

Fig. 3. Time course of ^{3}H-TdR incorporation into quiescent 3T3 cells stimulated by TPA and insulin or vasopressin and insulin. Cumulative ^{3}H-TdR incorporation (cpm × 10^{-3} per plate) was determined at various times after the addition of 100 ng/ml TPA and 1 μg/ml insulin (▪) or 10 ng/ml vasopressin and 1 μg/ml insulin (•) to cell cultures. Conditions and ^{3}H-TdR incorporation measurements were as in Rozengurt and Heppel [24]. Insulin, TPA, or vasopressin alone induced ^{3}H-TdR incorporation of under 5 × 10^3 cpm per plate at 50 h. Fetal bovine serum (10%) induced incorporation of 239 × 10^3 cpm per plate at 50 h.

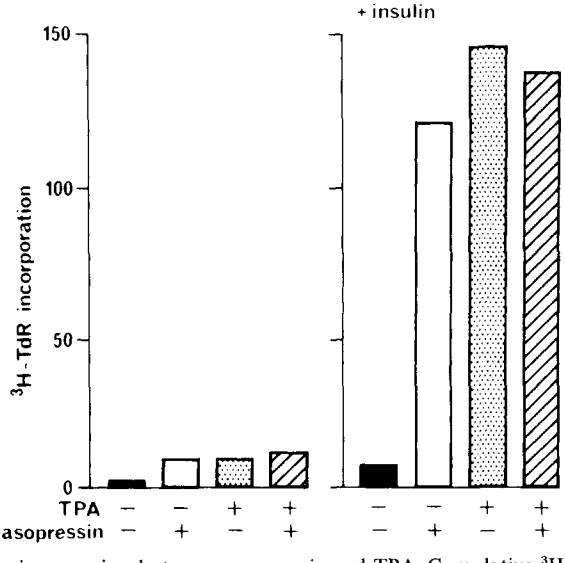

Fig. 4. Lack of mitogenic synergism between vasopressin and TPA. Cumulative ^{3}H-TdR incorporation (cpm × 10^{-3} per plate) was measured over a 40-h exposure of cell cultures to various combinations at 100 ng/ml TPA, 10 ng/ml vasopressin, and 1 μg/ml insulin. Conditions and measurements were as in Rozengurt and Heppel [24]. Fetal bovine serum (10%) induced incorporation of 156 × 10^3 cpm per dish in this experiment.

fere with each other's action is shown by the fact that saturating levels of TPA and insulin produce the same amount of ^{3}H-TdR incorporation in the presence or absence of vasopressin. The findings shown in Figure 4 are in sharp contrast to the results obtained with other mitogenic molecules like FDGF, EGF, or insulin, which interact synergistically among themselves as well as with either TPA or vasopressin.

These results strongly indicate that TPA and vasopressin act via an identical pathway. This need not mean they act through the same initial receptor. Indeed the fact that TPA is mitogenic in 3T6 cells [9], whereas vasopressin is not active in these cells (unpublished data), suggests that this is not the case. Rather it seems that the series of events initiated by TPA or vasopressin binding to a cell converge at some postreceptor locus. An approach to defining the molecular nature of this common pathway is to investigate metabolic effects of TPA and vasopressin that occur much earlier than induction of DNA synthesis. Attempts in this direction are discussed in the following sections.

TPA STIMULATION OF EARLY EVENTS

When quiescent 3T3 cells are stimulated by serum or by defined growth factors, a complex array of biochemical changes occur [28]. These may be divided into primary, rapid, protein and RNA synthesis independent changes, and subsequent changes that are dependent on the synthesis of macromolecules [28]. The early events include stimulation of uridine and deoxyglucose uptake, increase in ion fluxes, and increase in the rate of glycolysis. If TPA exerts its mitogenic effects in a manner similar to other growth factors, it might be expected to trigger the same early events.

URIDINE UPTAKE

Quiescent 3T3 cells show a severalfold increase in the rate of uridine uptake when stimulated by serum [29, 30], EGF [31], insulin [31], or FDGF [14]. In the case of all these agents the stimulation occurs as follows: 1) After addition of the stimulant there is a lag of 5–15 min during which the rate of uridine uptake remains unchanged [14, 29, 31]; 2) after this lag period the rate of uridine uptake rapidly increases until the stimulated rate is reached, and then the rate of uridine uptake remains steady at its new elevated level [29, 31]; 3) the increase in uptake occurs primarily via an increase in the rate of phosphorylation and not in the rate of transport across the cell membrane [30, 31].

Figure 5 shows that TPA stimulates uridine uptake after a lag period of 10 min, followed by a rapid increase in the rate of uridine uptake. This kinetic conforms with uridine uptake stimulation induced by polypeptide growth factors.

To distinguish whether TPA stimulates transport of uridine across the membrane or its subsequent intracellular phosphorylation, the following experiments were performed. In both experiments cultures were stimulated with TPA for 40 min prior to the measurements of uptake. Figure 5 shows that the initial rate of entry of ^{3}H-uridine into TPA-stimulated cells does not differ from that of the controls. However, after 6–8 sec the rate of uptake in nonstimulated cultures levels off markedly. These results suggest that the initial rate of uridine *transport* is not substantially different in stimulated and nonstimulated cultures and that TPA stimulates the intracellular *trapping* of ^{3}H-uridine. To directly substantiate this conclusion, acid-soluble pools were separated chromatographically into charged and noncharged moieties (Table VI). Incubation with TPA considerably changes the composition of the acid-soluble pools in 3T3 cells; it increases the radioactivity re-

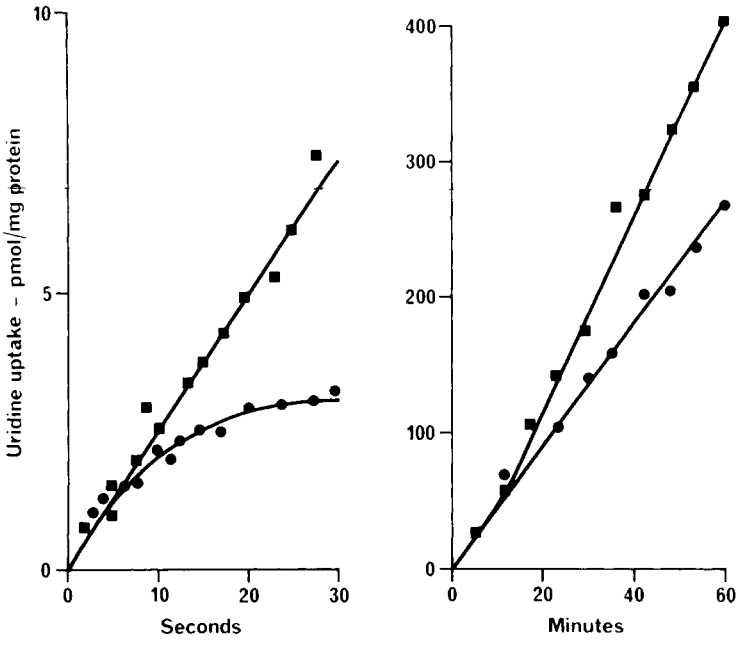

Fig. 5. TPA stimulation of uridine uptake. Left panel: Quiescent cultures of Swiss 3T3 cells were incubated with DMEM (•) or DMEM plus 100 ng/ml TPA (▪) for 40 min. ^{3}H-Uridine was then added at 10 μCi/ml, 28 Ci/mmole, for the times indicated. Cultures were then washed and ^{3}H-uridine uptake measured as in Rozengurt et al [30]. Right panel: Quiescent cultures of Swiss 3T3 cells were exposed to 1 μCi/ml, 1 Ci/mmole ^{3}H-uridine in DMEM (•) or DMEM plus 100 ng/ml TPA (▪) at 0 min. At the times indicated cultures were washed and accumulative ^{3}H-uridine uptake measured as in Rozengurt and Stein [29].

TABLE VI. ^{3}H-Uridine Uptake Stimulated by TPA and Serum

Additions	% ^{3}H-Uridine in phosphorylated pool after 6 sec	^{3}H-Uridine uptake (pmoles/mg protein)	
		6 sec	10 min
None	58	1.2	41
100 ng/ml TPA	82	1.2	102
10% fetal bovine serum	88	1.3	125

Quiescent cultures of Swiss 3T3 cells, produced as in Methods, were exposed to DMEM plus the additions indicated for 40 min. ^{3}H-Uridine was then added at 1 μCi/ml, 1 Ci/mmole or 10 μCi/ml, 28 Ci/mmole for 10 min and 6 sec uptake periods, respectively. Measurement of ^{3}H-uridine uptake, and separation of phosphorylated and nonphosphorylated pools, was determined as described previously [31].

covered in the nucleoside phosphate pool from 58% of total counts in unstimulated cultures to 82% in cultures stimulated by TPA. Serum, which increases the radioactivity in the phosphorylated pool to 88% of total counts, is shown for comparison. Clearly TPA stimulates the rate of conversion of ^{3}H-uridine to phosphorylated nucleosides. Thus, TPA stimulates uridine uptake in quiescent 3T3 cells in the same fashion as other growth factors.

DEOXYGLUCOSE UPTAKE AND GLYCOLYSIS

When quiescent 3T3 cells are stimulated by serum, an increase in the rate of deoxyglucose uptake occurs in two distinct phases [14, 28, 32–34]: 1) In the first 30–60 min after serum addition the rate of deoxyglucose uptake increases severalfold, independently of protein synthesis; 2) by 6 h after serum stimulation the rate of deoxyglucose uptake dramatically increases to at least ten times that of quiescent cultures. This second phase of increase is inhibited by cycloheximide [32, 33].

The two-phase stimulation of deoxyglucose uptake by serum is demonstrated in Figure 6. The figure also demonstrates that either TPA or insulin alone causes an increase in the rate of deoxyglucose uptake after 45 min of incubation. They do not, however, cause a further increase after 6 h incubation. Thus TPA or insulin alone activates the first but not the second phase of the stimulation of deoxyglucose uptake. However, TPA and insulin added together do cause a second phase of stimulation of sugar uptake (Fig. 6). This finding suggests that ability to induce the cycloheximide-sensitive phase increase might be indicative of the mitogenic potential of a growth factor or combination of growth factors. Thus, neither TPA nor insulin alone causes substantial DNA synthesis when added to quiescent 3T3 cells, nor do they produce the second phase of deoxyglucose uptake stimu-

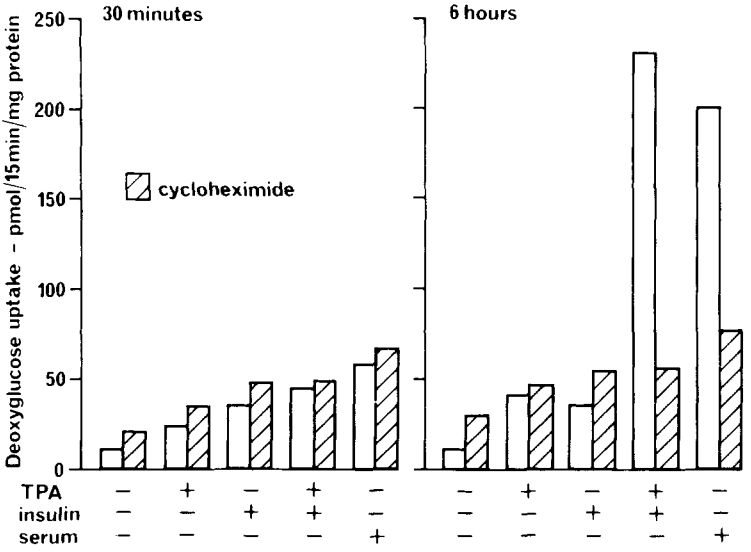

Fig. 6. TPA stimulation of deoxyglucose uptake. Left panel: Quiescent Swiss 3T3 cells were washed three times in DMEM minus glucose, then incubated with DMEM minus glucose in the presence (striped bars) or absence (plain bars) of 10 μg/ml cycloheximide plus the mitogens as indicated. After 30 min ^{3}H-2-deoxyglucose was added at 1.25 μCi/ml, 2.5 μM and uptake measured as in De Asua and Rozengurt [32] during the subsequent 15 min. Right panel: Quiescent cultures of Swiss 3T3 cells were incubated in DMEM in the presence (striped bars) or absence (plain bars) of 10 μg/ml cycloheximide plus the mitogens as indicated. After 5.5 h the cultures were washed three times in DMEM minus glucose and exposed to DMEM minus glucose with (striped bars) or without (plain bars) 10 μg/ml cycloheximide plus the mitogens as indicated. After 30 min ^{3}H-2-deoxyglucose was added at 1.25 μCi/ml, 2.5 μM and uptake was measured as in De Asua and Rozengurt [32] during the subsequent 15 min. For both panels concentrations of mitogens were 100 ng/ml, 1 μg/ml, 10% v/v for TPA, insulin, and fetal bovine serum, respectively. The serum was previously dialyzed against saline.

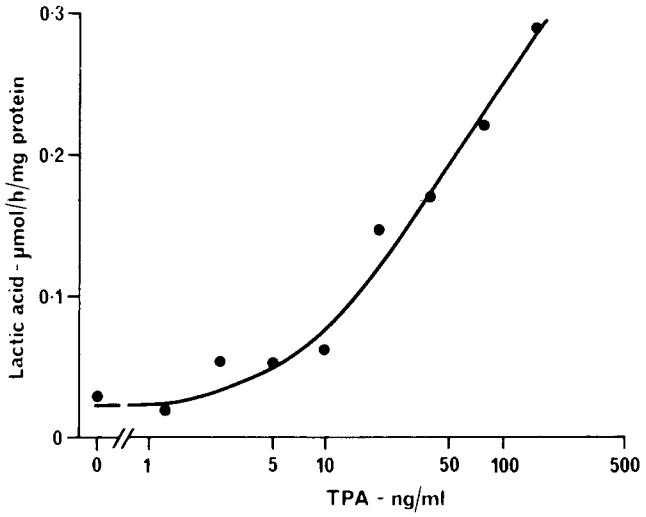

Fig. 7. TPA stimulation of lactic acid production. Quiescent cultures of 3T3 cells were exposed to TPA and cumulative lactic acid concentration in the medium was measured after 4 h. Conditions, medium, and assay for lactic acid production was as in Diamond et al [35].

lation. Serum, or TPA and insulin, induce both the second phase of deoxyglucose uptake and DNA synthesis.

Addition of serum or growth factors like EGF, insulin [35, 36], or FDGF (unpublished results) causes a stimulation of the glycolytic flux in quiescent 3T3 cells. Figure 7 shows that TPA also causes a concentration-dependent increase in glycolysis, as measured by the rate of lactic acid production. The stimulation of lactic acid production has been studied at high, saturating concentrations of extracellular glucose (25 mM), so the rate-limiting step in lactic acid production lies in the glycolytic pathway rather in the transport of glucose across the cell membrane [35]. TPA can synergistically increase lactic acid production still more in the presence of EGF [10].

Na^+, K^+ PUMP

One of the earliest changes produced subsequently to the addition of serum to quiescent cells is a stimulation of Rb^+ (a K^+ tracer) influx across the membrane [14, 24, 25, 27]. This increased flux represents an activation of the Na^+,K^+ pump, since it is inhibitable by ouabain. TPA similarly causes a rapid, ouabain-inhibitable increase in influx of Rb^+ when added to quiescent cells [37, 38].

Vasopressin, in general, induces an array of early events similar to those caused by TPA [27, 38, and unpublished results]. The similarity of their stimulation of Rb^+ uptake is most striking, especially considering that EGF and insulin show little enhancement of Rb^+ influx [24]. Both vasopressin and TPA produce an increase in the V_{max} of ^{86}Rb uptake without altering the affinity of the uptake system for K^+ [38]. This again indicates a rapid, postreceptor convergence of the mitogenic mechanisms of action of TPA and vasopressin.

EFFECTS OF TPA ON EGF BINDING

The finding that TPA potentiated the mitogenic potency of various purified polypeptides led us to test the effects of TPA on the binding of growth factors to cell surface receptors. We found, surprisingly, that TPA causes a potent, dose-dependent inhibition of ^{125}I-EGF binding [10]. TPA causes a tenfold decrease in the affinity of EGF receptors without a change in the number of receptors. We also produced evidence showing that TPA initially binds to a cell by a site other than the EGF receptor. That TPA exerts its mitogenic effects via a pathway not involving the EGF receptor is shown by the synergism of TPA and EGF in stimulation of DNA synthesis. EGF is able to initiate a biologic response when TPA inhibits its binding presumably because of the availability of "spare receptors" for the peptide. It is known that the maximal response to EGF can be obtained at concentrations at which only a small fraction of EGF receptors are occupied.

The similarity of the mitogenic properties of TPA and vasopressin prompted the testing of the effects of vasopressin on EGF binding. Brown and Rozengurt [39] found that vasopressin inhibits EGF binding to Swiss 3T3 cells. The effect is specific since oxytocin, a peptide 10^3 times less potent than vasopressin in stimulating DNA synthesis, is also much less effective than vasopressin in inhibiting EGF binding. Thus TPA and vasopressin exert a similar effect on a membrane protein (the EGF receptor) soon after their addition to cell cultures. These findings provide further support to the idea that the pathways of action of these two mitogens converge very soon after they bind to 3T3 cells.

CONCLUSIONS

The biochemical mechanism of action of tumor promotors has aroused much interest. Knowledge of such mechanisms would indicate the best strategy for countering their effects. The basis of the relationship between the mitogenic and tumor-promoting properties of the phorbol esters is at present unknown. However, the two properties are not dissociable in the various members of the phorbol ester series. Thus elucidation of the means by which the esters exert their mitogenic effects should help in the understanding of their tumor-promoting properties.

We have shown that TPA, in the absence of serum, acts synergistically with a range of polypeptide growth factors to stimulate DNA synthesis in quiescent 3T3 cells. We also demonstrated a mitogenic synergism between TPA and a group of nonpeptide molecules, the retinoids. Likewise, the retinoids increased the mitogenic potency of polypeptide growth factors. These similarities suggested that TPA acted via mechanisms akin to those used by other growth factors. A similar conclusion has been reached by Weinstein and his co-workers [40, 41] on other grounds. They further advanced the possibility that, at least partly, the biologic effects of TPA could be mediated by the EGF receptor-effector triggering system. This possibility seems unlikely, because EGF and TPA synergistically stimulated DNA synthesis in quiescent cells [9] and because TPA interacts with EGF receptors in a different manner from native EGF [10]. Thus, the important question is "Which specific hormonal path of action does TPA use to exert its mitogenic effect?" The answer appears to be that of vasopressin, since TPA and vasopressin have nearly identical patterns of mitogenic synergism with other factors, and they show no synergism with each other. This is clearly summarized in Table VII, which shows that 9 of 10 combinations between

TABLE VII. Mitogenic Interactions Between Various Growth Factors in Quiescent Swiss 3T3 Cells

Fixed concentration	Variable concentration					
	FDGF[a]	EGF	Insulin	Vasopressin	TPA	References
FDGF[a]		++	++	++	++	9,11,14
EGF	++		+	+	+	9,11,42
Insulin	++	+		++	++	9,11,14,42
Vasopressin	++	+	++		0	11
TPA	++	+	++	0		9

Symbols: ++, Interaction of these factors induces ^{3}H-TdR incorporation of more than 40% of that caused by 10% fetal bovine serum; +, interaction of these factors induces ^{3}H-TdR incorporation of 10–40% of that caused by 10% fetal bovine serum; 0, no synergistic interaction.
[a]Concentrations of FDGF causing ^{3}H-TdR incorporation of less than 5% of that produced by 10% fetal bovine serum.

FDGF, EGF, insulin, vasopressin, and TPA act synergistically in stimulating DNA synthesis, while TPA and vasopressin do not potentiate the effect of each other.

We do not believe that TPA and vasopressin bind to the same cell surface receptor, because they show different cellular specifities in their biologic actions. Thus TPA and vasopressin seem initially to bind to different sites and their mitogenic train of events converge subsequently. That this convergence occurs soon after the initial binding is suggested by the ability of both TPA and vasopressin to rapidly inhibit EGF binding to its receptors. To further elucidate the nature of this convergence, we studied the stimulation of early events by TPA. The tumor promotor was shown to activate in quiescent cells a wide range of early events similar to those induced by other growth factors. Vasopressin also induces these early events. The stimulation of Rb^+ influx by TPA and vasopressin is strikingly similar. We conclude from the results given here that TPA's mechanism of action as a mitogen is of the same general nature as that of polypeptide growth factors. In particular the events that lead from vasopressin or TPA binding with a cell to their subsequent mitogenic effects seem to converge into the same pathway. Whether this convergence may have its basis in the identical stimulation of ion fluxes into quiescent cells by vasopressin and TPA is an important question that warrants further experimental work.

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