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Early Inhibition of Thymidine Uptake and Stimulation of Cell Proliferation in Rat Embryo Fibroblasts Treated with Tumor-Promoting 12-*O*-Tetradecanoyl-Phorbol-13-Acetate*

C. ROCHETTE-EGLY, I. CHOUROULINKOV and M. CASTAGNA
Institut de Recherches Scientifiques sur le Cancer B.P. No. 8, 94800-*Villejuif, France*

Abstract—*The effects of the tumor-promoting phorbol diester* 12-O-*tetradecanoyl-phorbol*-13-*acetate* (*TPA*) *on* [^{3}H] *thymidine* (*TdR*) *uptake and incorporation, induction of ornithine decarboxylase activity and on phosphate uptake and metabolism were studied in secondary cultures of rat embryo cells. Treatment with TPA or refeeding with serum-containing fresh medium elicited a stimulation of* [^{3}H] *TdR incorporation and cell division in both resting and growing cells. By comparing the effects of both mitogenic treatments applied separately or together during the first induced mitotic cycle, it was possible to identify the TPA-associated alterations which were not related to the proliferative response. Phosphate uptake and metabolism as well as ornithine decarboxylase activity and thymidine triphosphate synthesis were similarly affected by either treatment. In contrast, TPA caused an early and transient inhibition of* [^{3}H] *TdR uptake under conditions where a medium change produced a stimulation. The non-promoter derivative,* 4-O-*methyl-phorbol*-12-13-*didecanoate, did not significantly alter* [^{3}H] *TdR uptake. Results suggest that the membrane changes reflected by the observed inhibition of* [^{3}H] *TdR uptake may be relevant to tumor promotion.*

INTRODUCTION

THE INITIATING and promoting stages of carcinogenesis classically defined in studies on mouse skin [1–4], have recently been shown to occur in cell cultures. The promoting activity of TPA was effective in chemically [5, 6], as well as in physically [7, 8] transformed cells. Previous studies had already shown that TPA enhanced the phenotypic expression of transformation in a mixed cell culture system [9]. Recently the effect of TPA on the expression of transformation-associated properties of virally-transformed cells was confirmed [10]. The mechanisms by which TPA acts are still unknown. Extensive biochemical studies have found that most TPA effects relate to the cell membrane. The membrane-mediated effects include an increase in Mg^{2+}, Ca^{2+}, and Na^+, K^+ -ATPase activities [11, 12], phospholipid synthesis [13, 14], as well as changes in glucose uptake [15] and surface protein composition [16]. The biological activity of TPA is generally associated with a mitogenic effect [17–20]. However, several authors [18, 20] have claimed that hyperplasia and tumor promotion are independent phenomena, suggesting that events related to cell proliferation should be distinguished from the bulk of TPA-induced alterations in order to detect those which may be relevant to tumor promotion.

The present communication reports experiments which were undertaken in order to study further the early effects of TPA. For a comparison purpose, those alterations accompanying the cell entrance into the mitotic cycle in response to the addition of serum, a mitogenic treatment which does not lead to tumor promotion, were investigated in parallel studies.

The experiments were conducted on secon-

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Abbreviations: TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; MePDD, 4-*O*-methyl-phorbol-12-13-didecanoate; PBS, phosphate-buffered saline; TdR, thymidine; dTTP, deoxythymidine triphosphate; ODC, ornithine decarboxylase.

dary cultures of rat embryo cells in which the tumor-promoting activity of TPA has been demonstrated after initiation by polycyclic hydrocarbons [5]. The reported results provide evidence that the tumor promoter induces an inhibition of [^{3}H] TdR uptake unrelated to the initiation of cell proliferation whereas the stimulation of phosphate uptake and metabolism as well as dTTP synthesis and the induction of ODC appear to be events associated with the proliferative effect.

MATERIALS AND METHODS

Chemicals

TPA and MePDD, kindly donated by Professor E. Hecker of the Deutsches Krebsforschungszentrum (Heidelberg, Germany), were used as solutions in acetone. 6-[^{3}H] Thymidine (spec. act. 1 Ci/mmole) and [^{32}P] phosphoric acid without carrier (spec. act. 10 mCi/ml) were obtained from the Commissariat à l'Energie Atomique, Saclay, France; DL-[^{14}C] ornithine monohydrochloride (spec. act. 50 mCi/mmole) and the scintillation mixture used for the radioactivity countings (Scintix) were purchased from the Radiochemical Centre, Amersham, Bucks, U.K.

Secondary culture and treatment of rat embryo cells

Seventeen-day-old rat embryos were used for establishing the primary cultures according to a previously described technique [10]. On the sixth day of cultures, the cells were trypsinized and seeded at a density of 2×10^5 cells per ml in 60 mm plastic Petri plates containing 5 ml of Dulbecco's modified Eagle medium (Catalog No. H 16 from Gibco, Paisley, Scotland) supplemented with 10% calf serum and incubated at 37°C in a humidified atmosphere containing 10% CO_2.

Five days after plating, the cells had become confluent ($3.5–4 \times 10^6$ cells and 1.5–2 mg of protein per dish). On day 6 the cells were treated with phorbol esters (TPA or MePDD) either by direct addition of the compound to the dishes without a medium change or by replacement of the used medium with 10% serum-supplemented fresh medium containing freshly-dissolved phorbol esters. The final concentration of phorbol ester was 0.1 μg/ml. Controls were either untreated or underwent a medium change at the time of the addition of phorbol esters. The final concentration of the solvent in the culture medium was 0.1%, a concentration which did not affect cell growth.

Actively multiplying cultures were obtained by seeding the cells at a density of 1×10^5 cells per ml in 5 ml of Dulbecco's growth medium containing 10% fetal calf serum. Sixty hours after plating, the log-phase cells ($0.8–1 \times 10^6$ cells and 0.3–0.4 mg of protein per dish) were treated similarly to confluent cells.

^{3}H TdR uptake and incorporation into DNA

At appropriate intervals after treatment, the rate of [^{3}H] TdR incorporation into DNA was determined by adding 10 μCi of [^{3}H] TdR to the culture medium and incubating for a further 30 min incubation (unless otherwise indicated) at 37°C. Then, the radioactive medium was aspirated, the culture rinsed thrice with 5 ml of pre-warmed PBS containing 1.6 μM unlabeled TdR and finally 2 ml of ice-cold 10% trichloroacetic acid were added. The acid-precipitable fraction was allowed to precipitate for 15 min at 0°C. Then the acid-soluble fraction was collected, and the acid-precipitable material which coated the bottom of the Petri dish was washed thrice with ice-cold 10% trichloroacetic acid and dissolved overnight in 0.6 N sodium hydroxide at 37°C. The precipitation-dissolution step was repeated once, and the final sodium-hydroxide solution analyzed for protein content by the method of Lowry *et al.* [21]. The liquid scintillation mixture (Scintix) was added to aliquots of the acid-soluble and sodium-hydroxide fractions for radioactive counting in an Intertechnique scintillation counter. In order to measure the formed dTTP, the incubation in the presence of [^{3}H] TdR was stopped by adding ice-cold 5% perchloric acid. Then the acid-precipitable fraction was allowed to precipitate for 15 min at 0°C. The acid-soluble fraction was neutralized with ice-cold KOH–$KHCO_3$, lyophilized and taken up in 200 μl of distilled water. Deoxythymidine triphosphate was separated from deoxythymidine mono- and diphosphates by descending chromatography on 3 MM Whatmann paper with a solvent mixture composed of isobutyric acid, H_2O, concentrated NH_4OH and 0.1 M EDTA, pH 4.5 (100: 49.8: 2.8: 1.6). The nucleotides spots were located with u.v. light, cut out and counted in the presence of Scintix. The rates of [^{3}H] TdR uptake, [^{3}H] dTTP synthesis and [^{3}H] TdR incorporation into DNA were expressed as dpm/min/mg of protein.

Phosphate uptake studies

At appropriate intervals after treatment, $^{32}PO_4$ at 5 μCi/ml of culture medium was added to duplicate cultures which were then incubated for 30 min at 37°C. The cells were washed twice and the acid-soluble fraction extracted as previously described for [^{3}H] TdR uptake. Then, inorganic phosphate was rapidly separated from phosphorylated molecules of the acid-soluble fraction, according to the technique described by Marsh [22]. Radioactivities of aliquots of organic and inorganic phosphate were counted in an Intertechnique scintillation counter. Phosphate uptake was expressed as ng P present into the acid-soluble fraction per min and per mg of protein. Phosphate metabolism was expressed as ng P incorporated into acid-soluble molecules per min and per mg of protein.

Ornithine decarboxylase assay

The assay involved measuring the release of $^{14}CO_2$ from DL-[1-^{14}C] ornithine as described by Russel and Snyder [23], including minor modifications. The growth medium was aspirated from the Petri dishes and the cells were washed twice with ice-cold PBS before being scraped from the dishes with a rubber policeman and collected in 1.5 ml of a mixture containing 10 mM KH_2PO_4 at pH 7.2, 0.75 M sucrose, 5 mM dithiothreitol, 0.1 ml EDTA and 0.2 mM pyridoxal phosphate. The cells were lysed by the freezing–thawing procedure and the supernatant, collected after a 10 min centrifugation at 4500 ***g***, was used as enzyme preparation. The reaction was carried out in plastic liquid scintillation vials sealed with a plastic cap which was equipped with a pin holding a 5 N potassium hydroxide-soaked disc of filter paper. The reaction which was initiated by adding [1-^{14}C] ornithine (spec. act. 0.2 mCi/mmole) was conducted in a 2 ml volume consisting of the above described incubation mixture and different dilutions of the enzyme preparation. The mixture was incubated at 37°C in a shaking water bath for 60 min, and the reaction was stopped by injecting 1 ml of 2 M citric acid through the plastic cap in order to release carbon dioxide. Shaking was continued for an additional 30 min to complete the CO_2 adsorption by the filter paper disc. Then the disc was removed, immediately immersed in a liquid scintillation mixture (Scintix) and counted for radioactivity in an Intertechnique scintillation counter. Ornithine decarboxylase activity was expressed as nmole of $^{14}CO_2$ released per mg of soluble protein per hr. The storage of the washed cells at −70°C before scraping from the dish did not affect the ornithine decarboxylase activity.

RESULTS

Growth curves

Secondary cultures of rat embryo cells plated at 0.5 or 1×10^6 cells per dish (5 ml of growth medium per dish) exhibited since day 1 a log-phase growth for 2–3 days followed by a stationary phase. (Fig. 1). During the log-phase the cell number increased with a population doubling time of 18 ± 1 hr, until a cell density of $3.4–4 \times 10^6$ cells per dish was reached at which point cell division ceased and the cultures became confluent. Cells reached the stationary phase at day 5 when plated at 1×10^6 cells per dish.

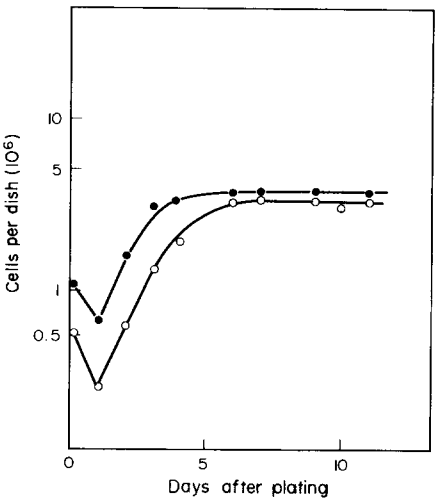

Fig. 1. Growth curves of secondary cultures of rat embryo cells seeded at 0.5×10^6 (—○—) *and* 10^6 (—●—) *cells per dish in* 5 ml *of growth medium.*

Figure 2 illustrates the effects of TPA at the concentration of 0.1 μg/ml on cell growth. When added to confluent cultures, simultaneously with a 10% calf serum-supplemented medium change, TPA released about 40% of cells from density-dependent inhibition (Fig. 2a). Furthermore, when TPA was added directly to the cultures without medium renewal, TPA was also able to trigger cell division, and the cell population reached a higher level of saturation. TPA, by itself, released cells from density-dependent inhibition at the same extent that did a medium change, that is about 15% of the cell population. Confluent cells are predominantly in G_1 [24] or blocked at one particular point in G_1, G_0 [25]. For the purpose of investigat-

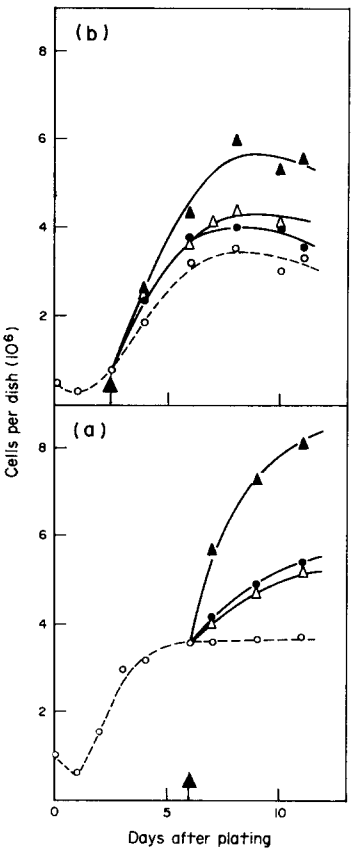

Fig. 2. Growth curves of rat embryo cells grown in the absence (○) and presence (—△—) of TPA (0.1 μg/ml) or refed with either control (—●—) or TPA-containing (—▲—) medium. (a) 10^6 cells were plated per dish and treated 6 days later. (b) 0.5×10^6 cells were plated per dish and treated at time 60 hr. Arrows indicate the time of treatment.

ing whether TPA displayed a cell cycle-specific action, similar experiments were carried out on growing cells which mostly traverse the cell cycle randomly although some may be reversibly blocked in a particular cell cycle phase.

TPA was shown to be also effective in stimulating the growth rate of growing cells (60-hr-old cultures) whatever the mode of TPA addition: alone or associated with a medium change (Fig. 2b). The number of TPA-responsive cells was lower than 20% without medium change and was significantly higher in combined treatment (Fig. 2b).

[^{3}H] *TdR Incorporation into DNA*

The effects of TPA on cell proliferation have been further studied by measurements of [^{3}H] TdR incorporation into DNA during the first mitotic cycle that the tumor promoter induced.

It has been observed that TPA stimulated TdR incorporation into confluent cells when added alone or associated with a medium change. As shown in Fig. 3 the change of 10% calf serum-supplemented medium caused a 4-fold stimulation of [^{3}H] TdR incorporation into the acid-precipitable material of confluent cells about 15 hr later. When TPA was included in the fresh medium an early 50% depression of [^{3}H] TdR incorporation was observed through the first 6 hr, which was followed by a 2-fold stimulation over the medium change controls by 15 hr. In addition, when TPA was added directly to confluent cultures an approximately 2-fold stimulation was observed subsequently to an earlier inhibition (Fig. 3b). The treatment with acetone (phorbol ester solvent) at the same volume than that used in phorbol ester treated cultures did not alter the rate of [^{3}H] TdR incorporation.

Treatment of 60-hr-old cultures with TPA-containing fresh medium also increased [^{3}H] TdR incorporation into acid-precipitable material but with a quite different pattern. As shown in Fig. 4, two waves of [^{3}H] TdR incorporation occurred successively. The first one which appears rapidly and represents a 5-fold increase in the rate of [^{3}H] TdR incorporation was observed during the first 3 hr. The second, which appears 15–18 hr later,

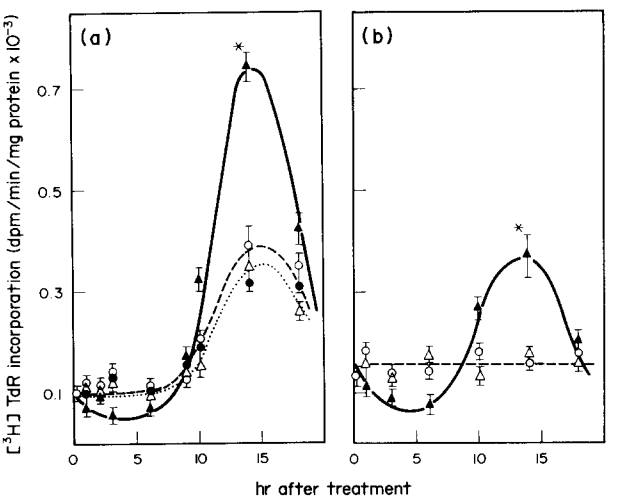

Fig. 3. Effect of phorbol esters (0.1 μg/ml) on [^{3}H] TdR incorporation into the TPA-precipitable material of confluent rat embryo cells. Six-day-old *cultures seeded at 10^6 cells per dish were treated at 0-time and cells were pulse-labeled with 1.6 μM of [^{3}H] TdR for 30 min at the indicated times before being processed as described in Materials and Methods. (a) Cells were treated with either TPA (—▲—) or MePDD (—△—) without medium change; control (—○ --). (b) Cells were refed with control (—○—) acetone-containing (—●--), MePDD-containing (—△—) or TPA-containing (—▲—) medium. Each value represents the mean ±S.E. of duplicate determinations on 4–6 separate dishes (2 experiments).*
$P < 0.001$ compared with the appropriate controls as determined by Student's t-test.

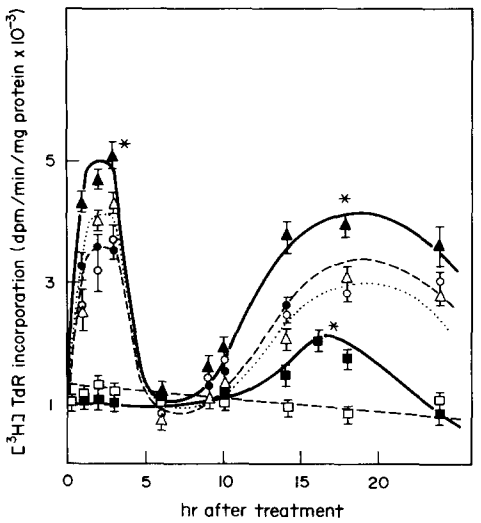

Fig. 4. Effect of phorbol esters (0.1 μg/ml) *on* [^{3}H] *TdR incorporation into TCA-precipitable material of growing rat embryo cells. Cells plated at* 0.5×10^6 *per dish were treated* 60 hr *later and labeled with* [^{3}H] *TdR as described under Fig.* 3. *Cells were refed with control* (—○—), *acetone-containing* (—●—), *MePDD-containing* (—△—), *TPA-containing medium* (—▲—); *TPA-treated cells without medium change* (—■—) *and untreated cells* (—□—). *Each value represents the mean* ± S.E. *of duplicate determinations on* 4–6 *separate dishes* (2 *experiments*). **P* < 0.01 *compared with the appropriate controls as determined by Student's* t-*test.*

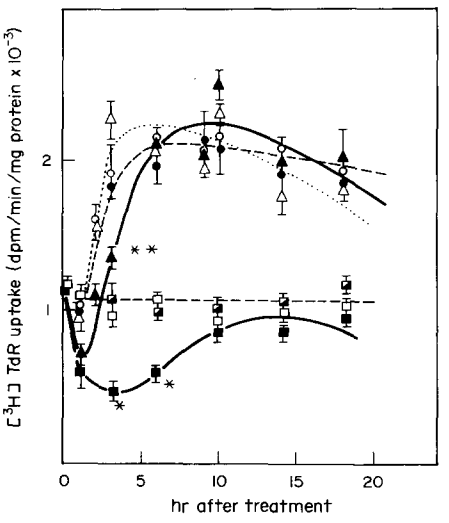

Fig. 5. Effect of phorbol esters (0.1 μg/ml), *fresh medium and fresh medium containing phorbol esters on* [^{3}H] *TdR uptake into rat embryo cells at confluency. Cells were treated and labeled as described under Fig.* 3. *Radioactivity of the acid-soluble fraction was determined as detailed in Materials and Methods and referred as* [^{3}H] *TdR uptake. Cells were untreated* (—□—) *or treated with TPA* (—■—), *MePDD* (—◪—), *fresh medium* (—○—), *fresh medium + acetone* (—●—), *fresh medium + TPA* (—▲—) *or fresh medium + MePDD* (—△—). *Each point is the mean activity* ± S.E. *of duplicate determinations on* 4 *dishes* (2 *experiments*). **P* < 0.001, *compared with the appropriate controls, as determined by Student's* t-*test.*

was a broader wave with a smaller magnitude. A similar pattern of variations was observed when cells underwent a medium change without being treated with TPA. Cells which received TPA without being refed with control medium did not display the early wave in [^{3}H] TdR incorporation as compared with the appropriate controls unless the difference in [^{3}H] TdR uptake, as shown later, was to be taken into account.

The phorbol diester, MePDD, which lacks tumor-promoting activity, did not affect [^{3}H] TdR incorporation in either confluent or growing cells, regardless of the renewal of growth medium (Figs. 3 and 4).

Uptake studies

A medium change caused a rapid stimulation of [^{3}H] TdR uptake in cultures of rat embryo fibroblasts. Results obtained at confluency are depicted in Fig. 5. The effect peaked 6 hr later and continued through the duration of the experiment (18 hr). By contrast, in TPA-treated confluent cells where the medium was not changed, a rapid and marked inhibition of [^{3}H] TdR uptake occurred up to 6 hr, which returned to the basal levels by 10 hr at the onset of the DNA synthesis. The inhibitory effect of TPA on the rate of thymidine uptake has been observed independently of the incubation time, as shown in Fig. 6. In order to examine whether TPA affected the thymidine transport and/or subsequent metabolism, the level of [^{3}H] thymidine triphosphate has been studied in relation to time. The results presented in Table 1

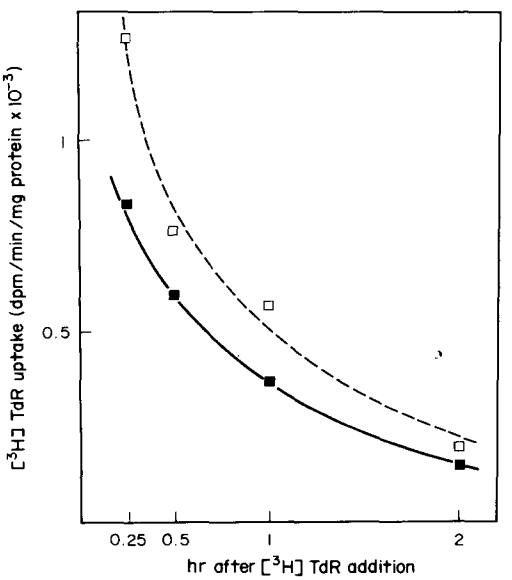

Fig. 6. Rate of [^{3}H] *TdR uptake into confluent cultures of rat embryo cells treated* 1 hr *before the addition of labeled precursor with* 0.1 μg/ml *of TPA* (—■—) *and acetone controls* (- - - -□- - - -). [^{3}H] *TdR uptake was measured as described under Fig.* 5. *Results are the average of* 4 *determinations.*

show that the level of radioactivity incorporated into dTTP was lower in TPA-treated cells for the first 7 hr, likely reflecting the decreased [^{3}H] TdR uptake into the cells. Then TPA elicited an increased dTTP synthesis which accompanied the increased DNA synthesis suggesting that TPA inhibits the resting or growing cells whether it was combined or not with a medium change (Figs. 5 and 7).

The effect of TPA on phosphate uptake in confluent rat embryo cells proliferatively activated by refeeding with control medium has been examined. The increase of phosphate

Table 1. *Effect of TPA on the incorporation of* [^{3}H] *TdR into* [^{3}H] *dTTP*

Time after treatment (hr)	[^{3}H] dTTP (dpm/min/mg protein) Controls	TPA	% of Controls
0.5	81±2	68±4	84
1	116±11	100±8	86
2	93±5	56±3	60
7	117±12	65±5	56
9	127±5	224±9	176
10	253±7	483±13	191
11	167±16	367±17	220
16	116±8	241±17	208

Six-day-old cultures of rat embryo cells were treated with acetone or phorbol esters. At specified times after treatment, the cells were incubated for 30 min in the presence of [^{3}H] TdR and separated from other [^{3}H] metabolites as indicated in Materials and Methods. Results are mean values±S.E. of 4 determinations.

thymidine transport rather than subsequent metabolism.

Opposite TPA- and medium-change-mediated effects on thymidine uptake were additive in cells undergoing the combined treatment and resulted in minor alterations of the [^{3}H] TdR uptake during the first hours. These results which suggest that the transient inhibition of [^{3}H] TdR uptake may account for the early depression of [^{3}H] TdR incorporation into DNA observed in TPA-treated cells (Figs. 3a and b), will be discussed later on.

The inhibitory effect of TPA on [^{3}H] TdR uptake was also observed in growing cells. Results, as depicted in Fig. 7, have shown that during the first hours following its addition, TPA prevented the increased [^{3}H] TdR uptake resulting from refeeding with fresh medium. However, it should be noted that the compound did not significantly affect the small increase in precursor uptake at the onset of the second wave of DNA synthesis. Similarly to effects seen at confluency, TPA added without renewing the medium, also caused a marked depression of [^{3}H] TdR uptake. The inactive derivative MePDD had no effect on [^{3}H] TdR uptake in either uptake is considered as one of the earliest events leading to the cell proliferation [24]. Results given in Table 2 indeed show that TPA-containing medium caused the stimu-

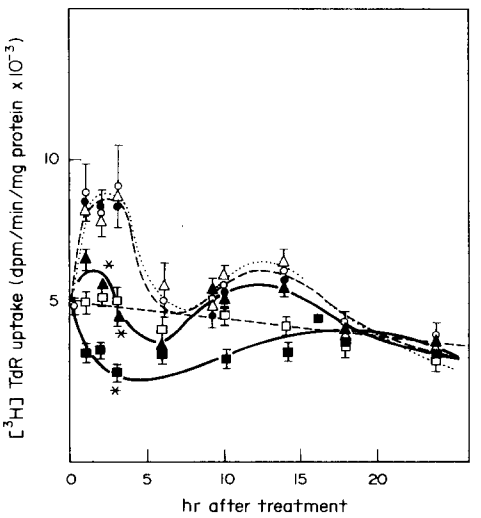

Fig. 7. *Effect of phorbol esters* (0.1 μg/ml), *fresh medium and fresh medium containing phorbol ester on* [^{3}H] *TdR uptake into growing rat embryo cells. Cells are treated* 60 hr *after seeding and labeled as described under Fig.* 3 (*see Fig.* 4 *for key to symbols*). *Each point is the mean activity*±S.E. *of duplicate determinations on* 4 *dishes* (2 *experiments*) **P*<0.01 *compared with the appropriate acetone controls, as determined by Student's* t-*test.*

Table 2. Effects of TPA on phosphate uptake and incorporation into acid-soluble molecules

Treatment	Phosphate uptake	Phosphate metabolism
	(ng P min/mg protein)	
None	81.7±2.5	56.3±2.3
Fresh medium	144.1±7	110 ±7.1
Fresh medium +acetone	146 ±6.3	112.8±3.1
+TPA	210.5±7.4*	150.6±5.0*
+MePDD	155.9±6.1	108.1±6.1

The existing medium of six-day-old cultures of rat embryo cells was replaced by serum-supplemented fresh medium with or without 0.1 μg/ml of phorbol ester or acetone. The amount of phosphate incorporated into the acid-soluble fraction was measured 6 hr later as described in Materials and Methods. All values are the mean±S.E. of duplicate determinations on four separate dishes (2 experiments).
*$P<0.01$ compared with the appropriate acetone controls, as determined by Student's *t*-test.

lation of both phosphate uptake and incorporation into the small organic molecules. The effect occurred very rapidly and reached a peak of a 3-fold stimulation 6 hr later which was markedly greater than those resulting from refeeding with acetone or MePDD-containing fresh medium.

Ornithine decarboxylase activity

The combined treatment of either confluent or growing cells with TPA and medium change, resulted in a rapid rise in ODC activity which was at the peak values approximately 12 times greater than that of the controls (Table 3). When applied separately both mitogenic treatments also stimulated ODC activity and the bulk of the induced activities was additive in cells undergoing the combined treatment. However, it should be mentioned that the effect of TPA was greater than that induced by refeeding with control medium. The greatest effect of TPA on ODC induction in rat embryo cells may be somewhat related to results obtained in previous studies on mouse skin [26] and cultured cells [19, 27] which showed a large and specific effect of TPA on ODC activity suggesting the possible

Table 3. TPA-induced ornithine decarboxylase activity

Treatment	Ornithine decarboxylase activity (nmole CO_2/min/mg protein)	
	Confluent cells	Growing cells
None	0.32±0.09	0.68±0.03
Acetone	0.34±0.02	0.55±0.03
TPA	2.56±0.29*	2.93±0.15*
MePDD	0.33±0.18	0.52±0.03
Fresh Medium	1.72±0.28	1.76±0.32
Fresh Medium +acetone	1.75±0.12	1.85±0.16
+TPA	3.93±0.38*	3.97±0.40*
+MePDD	1.94±0.35	1.71±0.09

Sixty-hour or six-day-old cultures of rat embryo cells were treated with acetone, with TPA or MePDD (both at a final concentration of 0.1 μg/ml) or underwent a medium renewal either separately or concomitantly. One hour later, the cells were harvested for ODC determinations. Each value is the mean ±S.E. of duplicate determinations on 3 dishes.
*$P<0.001$ compared with the appropriate acetone controls as determined by Student's *t*-test.

relevance of this enzyme induction to tumor promotion. At the same level of the concentration, MePDD did not induce ODC activity in either resting or growing cells and did not affect the rise in activity following a medium change.

DISCUSSION

The experiments reported here show that TPA induced the stimulation of DNA synthesis and increased the number of proliferating cells in rat embryo fibroblasts, possibly by increasing the probability of transition from a resting to a cycling state. TPA was also co-mitogenic for cells stimulated to proliferate by a medium change, regardless of the growth state of the culture. TPA did not shift the point at which the cells began to incorporate [^{3}H] TdR and both mitogenic treatments induced one burst of DNA synthesis in confluent cells whilst an early wave followed by a late wave of DNA synthesis occurred in the growing cells. It is suggested that the early peak of DNA synthesis observed in the latter cells may infer the presence of a population committed to DNA synthesis at the G_1-S boundary.

The TPA-mediated proliferative response has been reported in a number of studies on mouse skin as well as on cell cultures although the effects were somewhat different in respect of the examined cell type. A transient inhibition of cell proliferation measured by the overall [^{3}H] TdR incorporation and/or the number of DNA synthesizing cells has been reported to occur before the burst of DNA synthesis in mouse skin [14, 20, 28], epidermal cells [27, 29], fibroblasts [30], erythroleukemic cells [31] and lymphoblastic cells [32]. The extent of the growth inhibitory effect varied greatly in regard of the cell type and its occurrence seems to depend on the actual growth rate in malignant epidermal cells [29]. The present experiments have only shown an early drop of the overall [^{3}H] TdR incorporation in resting cells. Simultaneously the decreased [^{3}H] TdR incorporation was associated with a decreased [^{3}H] TdR uptake and a subsequent decrease of labeled dTTP, suggesting that decreased [^{3}H] TdR incorporation into DNA may result of the inhibition of [^{3}H] TdR uptake rather than reflect a reduced DNA synthetic rate. A similar artifactual inhibition was reported by Balmain and Hecker who observed that the initial depression of the incorporation of [^{3}H] thymidine into DNA was not associated with a decrease in DNA synthesis since the amount of DNA, as directly measured, increased at the same time [14].

TPA-induced stimulation of phosphate uptake and induction of ODC activity which have been presently reported confirmed previous studies [19, 23, 26, 27, 33]. However, it should be noted that in serum and TPA-stimulated cells, phosphate uptake and metabolism as well as ODC activity, increased in a similar fashion. There was a rather good correlation between the increase of these parameters and the magnitude of the proliferative response, even though the TPA-mediated stimulation of ODC activity was slightly higher than that resulting from a medium change.

In contrast, TPA and serum had opposite early effect on [^{3}H] TdR uptake suggesting that an early increased TdR uptake was not a necessary link in the sequence of events leading up to DNA synthesis. The early inhibition of [^{3}H] TdR uptake following TPA treatment seems unrelated to the dTTP synthesis as well as to the subsequent burst of DNA synthesis. The TPA-mediated inhibition of thymidine uptake, which was not observed with a non-promoter TPA derivative, reflects some alterations at the membrane level probably unrelated to the growth control mechanisms.

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