

Phorbol Ester-induced G₂ Delay in HeLa Cells Analyzed by Time Lapse Photography¹

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ABSTRACT

The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has been shown previously to mimic X-irradiation in altering cell cycle parameters in HeLa cells [Kinzel, V., Richards, J., and Stöhr, M., *Science* (Wash. DC), 210: 429-431, 1980]. These changes include a delay in G₂ phase from which cells recover in the presence of TPA, which suggests an involvement of cellular mediators. In order to obtain information on the onset and the duration of the G₂ delay, as well as the onset and rate of recovery, a time-lapse study has been carried out. The analysis of cells in prophase shows that at 10⁻⁶ M and 10⁻⁷ M concentrations, TPA and 12-*O*-retinoylphorbol-13-acetate (RPA) cause a G₂ delay which lasts on the order of 3.5 to 4 h. Below 10⁻⁷ M of RPA and below 10⁻⁸ M of TPA a clear-cut inhibition of HeLa cells in G₂ is no longer detectable by this method. These results for a given phorbol ester are dose dependent within a certain range but unlike the case of X-rays are not proportional to dose. Within the dose range studied the recovery rate follows the opposite order. At 10⁻⁶ M TPA and RPA an indication of a parasynchronous burst is observed. At smaller concentrations or with less biological activity of phorbol ester, the cell multiplication rate approaches that of the control or remains even smaller. Possible reasons are discussed. The determination of the transition points seems to indicate that the cellular events inhibited in G₂ occur shortly before visible prophase.

INTRODUCTION

The tumor promoter TPA³, a powerful mitogen in mouse skin (for review see Ref. 1) and in certain cultured cells (2, 3), exhibits an inhibitory action in proliferating cells *in vivo* (4) and *in vitro* (5, 6). Evidence has been presented which seems to indicate that the mitogenic activity of TPA and its interaction with replicating cells "cooperate" in effecting a particular step in multistage tumorigenesis in mouse skin (7, 8). Using HeLa cells as a replicating model system it has been shown that cell cycle alterations induced by TPA resemble those observed after X-irradiation (5, 6). Chromosome alterations induced by TPA in mouse keratinocytes during one cell cycle (9) may be the result of such radiomimetic cell cycle interference. The biological significance of the inhibitory action on proliferating cells of other mitogens such as epidermal growth factor (10, 11) or transforming growth factor-beta (for review see Ref. 12) is much less understood.

The mechanisms by which such receptor-mediated inhibition in the cell cycle is effected as well as those governing the X-ray-induced delay of G₂ are unknown. However, there is now a chance of finding common metabolic pathways involved in such receptor-mediated inhibition due to progress made in the past years in the understanding of transmembrane signalling processes (13, 14). Moreover, in the case of the G₂ delay the onset of the cellular response occurs much more rapidly than, for

instance, the earliest observable mitogenic effect measurable several hours later by thymidine incorporation. Precise timing of the inhibitory response with respect to its onset, duration, and recovery is desirable in order to correlate the inhibitory response with transmembrane signalling events. Therefore, a time lapse study was initiated on the action of TPA and RPA in G₂ phase of HeLa cells.

MATERIALS AND METHODS

The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate was a generous gift from Prof. Dr. E. Hecker (German Cancer Research Center); 12-*O*-retinoylphorbol-13-acetate was provided by Dr. B. Sorg (German Cancer Research Center); both compounds were kept in acetone as a 5 × 10⁻³ M stock solution at -70°C in the dark.

HeLa cells were cultivated routinely as monolayers in Eagle's minimal essential medium containing Earle's salts supplemented with 10% calf serum. Cells were kept in plastic bottles gassed with 95% air and 5% CO₂ in a humidified incubator at 37°C. Cells were free of Mycoplasma as checked routinely.

For experiments HeLa cultures were established in plastic flasks (2 × 10⁴ cells/cm²) 16 h prior to the experiment. The phorbol ester, TPA or RPA, was added in acetone (final concentration 0.2%) directly to the cultures which were kept carefully in slow motion in order to allow an even distribution of the compounds and to minimize detachment of mitotic cells. The compound was present during the entire experiment. In certain experiments, prior to addition of the compound, the cells engaged in mitosis were removed (15) before beginning of recording. For this purpose the medium was removed and the culture flask was tapped 3× on the bench. The culture was washed with complete medium and finally covered with fresh medium containing 10% calf serum.

After addition of compound(s) the flasks were closed tightly and placed undisturbed on an inverted phase contrast microscope (Leitz Diavert, equipped with a 10× lens and a 8× ocular) in an incubator at 37°C. Recording started immediately. Every 10 min one picture was taken from the same field (containing between 700 and 1000 cells) for several hours. During the 10-min intervals the illumination of the instrument was turned off. Analysis from enlarged prints (24 × 30.5 cm) was carried out for each individual cell division during the time course of an experiment. The start of mitosis was defined as the point when the cell started to contract (indicated as "prophase") and the end, as the point when visible separation of daughter cells (indicated as "telophase") was seen.

In each experiment approximately 60 or more individual mitotic events were followed up.

The following parameters were analyzed: (I) total cell number per time point (*N*₀ resp. *N*); (II) number of mitotic events starting at a particular time (cells in prophase); (III) cumulative number of cells (*N*) plus cells in prophase (see II, *i.e.*, determination of cells which entered mitosis *after* onset of the experiment).

Parameters I, II, and III yield the basis for the comparison of different experiments (see below), for the determination of the G₂ delay, and for the calculation of the slope of recovery (see Table 1).

In a number of experiments the following parameters were analyzed in addition: (IV) total number of mitotic figures per time point (*e.g.*, Fig. 2B); (V) number of mitotic events finished at a particular time (cells in telophase; *e.g.*, Fig. 2A); (VI) duration of each individual mitotic event which started and finished during the experiment (the accuracy of these values is limited by the 10-min intervals of recording); *e.g.*, Fig. 1B and Table 2; (VII) cumulative number of cells (*N*) plus cells in telophase (see Parameter V); this type of analysis includes also

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³ The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; RPA, 12-*O*-retinoylphorbol-13-acetate.

cells which entered G₂ prior to the onset of the experiment (e.g. Fig. 2A).

In order to compare individual experiments cumulative countings were expressed in relative terms as the logarithm of the cell number plus number of cells in prophase (respectively telophase) at a given time divided by the number of cells at zero time indicated as $\log(N/N_0)$ (see for instance Ref. 16). Linear regression analysis was done by the use of the Hewlett-Packard SD-03A program on an HP-67 calculator.

The G₂ delay was determined from relative cell accumulation due to prophase cells after resumption of mitotic activity. It was defined as the intercept between zero time and the intersection of the abscissa with the regression line (for this purpose slight variations in the slope of the regression-line may introduce a certain degree of inaccuracy, but this was negligible with respect to the message expected from this study). In view of differences in the transition points (see Table 1) this method was chosen as the basis for comparison of different experiments.

RESULTS

The influence of TPA and RPA on the G₂ phase of HeLa cells was analyzed by time lapse photography. Figs. 1 and 2 show the effects of 10⁻⁷ M TPA added at zero time. Fig. 1A represents the number of cells in prophase at a particular time after the beginning of the experiment. For approximately 20 min cells continued to enter prophase, then they stopped. The recovery from G₂ inhibition was detectable approximately 4 h after addition of TPA. Restoration of mitotic activity took place in those cells previously inhibited by TPA (17). Mitotic duration was not significantly influenced by TPA (Fig. 1B).

The analysis of the rise in cell number due to cells in prophase in the same experiment is shown in Fig. 2A. Major resumption of the mitotic activity started at approximately 4 h after addition of TPA. A few cells may have entered prophase earlier. The G₂ delay was 239 min. The analysis of the relative rise in cell number due to cells in telophase is included in Fig. 2A. After approximately 1 h of delay the curve came to a plateau and started to rise by approximately 5.5 h.

The initial values derived from telophase figures (Fig. 2A) illustrate a possible reason for inaccuracy inherent in the analysis of cells already engaged in mitosis at the start of an experi-

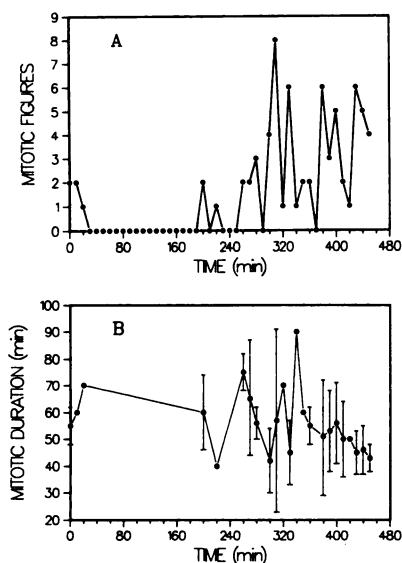


Fig. 1. Number of cells in prophase and mitotic duration after addition of 10⁻⁷ M TPA at zero time (experiment 180). A, number of cells in prophase at a particular time; B, duration of mitosis (\pm deviation respectively standard deviation where applicable) of the cells shown in A. For mean mitotic duration see Table 2.

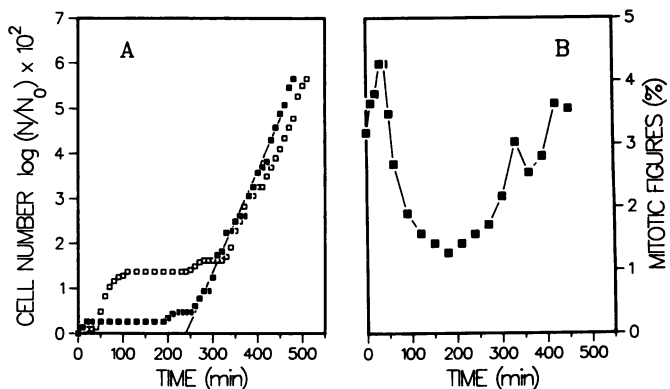


Fig. 2. Cumulative number of cells and mitotic activity after addition of 10⁻⁷ M TPA (experiment 180). A, cumulative number of cells due to prophases (●) and telophases (□); for details see "Materials and Methods" (for reasons of clarity in parts of the curves every second value has been omitted). Included is the regression line for the respective prophase increase. B, mitotic activity (including all stages of mitosis) in presence of 10⁻⁷ M TPA analyzed as described earlier (5, 6).

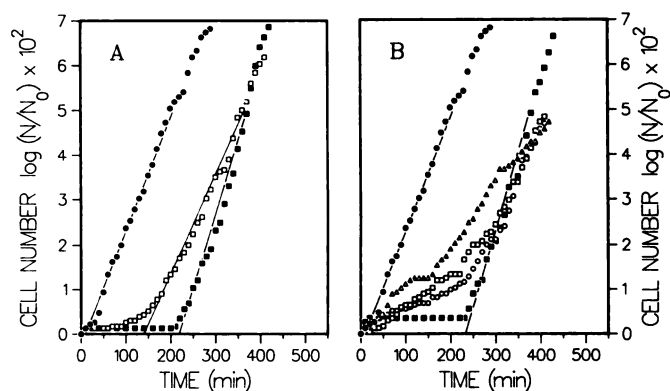


Fig. 3. Cumulative number of prophase cells in presence of different concentrations of phorbol esters added at zero time. A, 0.2% acetone, experiment 203 (●); 10⁻⁶ M TPA, experiment 113 (□); 10⁻⁶ M TPA, experiment 208 (■). Included are the regression lines. B, 0.2% acetone, experiment 203 (●); 10⁻⁸ M RPA, experiments 143 a-c (○, □, △); 10⁻⁸ M RPA, experiment 218a (■). In parts of the curves every second value was (partly alternately) omitted.

ment. After an initial lag period of almost 40 min there was a steep increase in the total number within the subsequent 30-min period. A possible explanation may be given by the plot of total mitotic activity from the same experiment in Fig. 2B. The initial increase of overall mitotic activity was probably due to reattachment of a few metaphase cells which came off the monolayer due to the handling of the culture at the beginning of the experiment. The time course of the curve in Fig. 2B demonstrates that this method of analysis is less powerful for the determination of the duration of the TPA-induced G₂ delay and the recovery from it than that represented in Fig. 2A by the prophase values.

The influence of TPA in G₂ has been shown to be dose dependent in a certain range but not proportional to dose, unlike the G₂ response to X-rays (18, 19). The analysis of the G₂ delay confirms these findings. At 10⁻⁶ and 10⁻⁷ M concentration TPA caused a comparable delay which was on the order of 3.5 to 4 h (Table 1, Fig. 3A); 10⁻⁸ M TPA was less active but still induced a delay of more than 2 h (Fig. 3A). At 3 × 10⁻⁹ and 10⁻⁹ M (not shown) concentration TPA exhibited no activity detectable by this methodology (Table 1). The synthetic phorbol ester RPA, which is as mitogenic in mouse skin as TPA but an order of magnitude less promoting, has also been shown to be less effective in G₂ phase in HeLa cells (19). At 10⁻⁶ and 10⁻⁷ M concentrations RPA caused a G₂ delay of approximately 3.5

Table 1 Summary of G₂-delay time (respectively slope of retardation), slope of recovery, and transition point

Experiment	Compound (concentration, [M])	G ₂ delay (min), ^a respectively slope (asterisks) (period analyzed, min) ^{b,c}	Slope (period analyzed, min) ^{b,d}	Transition point (min) ^e
208a	TPA [10 ⁻⁶]	218	3.28 (200-470)	≤20 (4)
208b	TPA [10 ⁻⁶]	212	3.34 (200-470)	≤10 (6)
175	TPA [10 ⁻⁷]	241	2.27 (250-480)	≤20 (2)
180	TPA [10 ⁻⁷]	239	2.26 (250-480)	≤30 (5)
113	TPA [10 ⁻⁸]	140	2.22 (160-410)	≤20 (5)
216	TPA [10 ⁻⁸]	128	2.82 (120-470)	≤50 (11)
199	TPA [3 × 10 ⁻⁹]	0	1.86 (0-440)	
217	TPA [3 × 10 ⁻⁹]	0	2.42 (0-290)	
218a	RPA [10 ⁻⁶]	233	3.43 (230-470)	≤30 (8)
218b	RPA [10 ⁻⁶]	174	3.26 (170-470)	≤30 (8)
165a	RPA [10 ⁻⁷]	235	2.38 (220-440)	≤50 (4)
165b	RPA [10 ⁻⁷]	216	2.53 (220-450)	≤30 (5)
219a	RPA [3 × 10 ⁻⁸]	0.50* (0-150)	2.76 (160-350)	ND ^f
219b	RPA [3 × 10 ⁻⁸]	0.97* (0-150)	2.63 (160-350)	ND ^f
143a	RPA [10 ⁻⁸]	0.70* (0-210)	1.78 (220-380)	ND ^f
143b	RPA [10 ⁻⁸]	0.45* (0-220)	2.06 (230-360)	ND ^f
143c	RPA [10 ⁻⁸]	1.03* (0-140)	1.32 (150-400)	ND ^f
(Mean of five experiments)	Acetone (0.2%)		2.37 ± 0.52 (0-300)	

^a For details see "Materials and Methods."

^b Slope given by $\frac{\text{Cell number } [\log(N/N_0) \times 10^3]}{\text{Time } [\text{min} \times 10^{-2}]}$.

^c Slope of initially retarded prophase activity.

^d Slope after resumption of mitotic activity.

^e Time elapsing between addition of compound and zero prophase activity lasting for at least 30 min; number of cells which enter division during this period are given in parenthesis.

^f ND, not determinable.

Table 2 Mitotic duration

Experiment	Compound (concentration [M])	Duration of mitosis in min ± SD (number of mitotic events)		
		During the 1st hour	Over the entire experiment	Graphically determined ^a
112	Acetone (0.2%)	67 ± 25 (24)	66 ± 19 (124)	ND ^b
180	TPA [10 ⁻⁷]	60 ± 7 (5)	53 ± 17 (71)	55
113	TPA [10 ⁻⁸]	58 ± 10 (4)	63 ± 17 (73)	65
165a	RPA [10 ⁻⁷]	70 ± 8 (4)	67 ± 20 (58)	70
143a	RPA [10 ⁻⁸]	61 ± 16 (14)	56 ± 21 (67)	ND ^b

^a By overlapping the prophase curve with the telophase curve (e.g., see Fig. 2A) and reading the difference between intersections of the regression lines with the abscissae.

^b ND, not determinable.

h (Table 1, Fig. 3B). However, at 10⁻⁸ and even 3 × 10⁻⁸ M RPA no clear-cut G₂ delay was observed (Table 1, Fig. 3B). The three experiments using 10⁻⁸ M RPA are shown in Fig. 3B. The variation of the results is not surprising in view of the fact that the dose-response curve of RPA is rather steep at this concentration (19). The blockage appears to be "leaky." Therefore, instead of the G₂ delay, which cannot be determined in these experiments, slopes of initially retarded prophase activity are given in Table 1. Moreover, in all three experiments with 10⁻⁸ M RPA the cell number continued to diverge from the control (Fig. 3B). The steep, probably parasynchronous increase in cell number seen at 10⁻⁶ M TPA and 10⁻⁶ M RPA (Fig. 3, Table 1) disappeared either due to a smaller concentration (e.g., 10⁻⁸ M TPA; Fig. 3A) or due to less biological activity of a given phorbol ester (e.g., 10⁻⁸ M RPA; Fig. 3B; for slopes see Table 1). Whether these results reflect only a quantitative difference or another quality in the action of phorbol esters is discussed.

The last column of Table 1 contains the transition points expressed in min, i.e., the period required to reach zero prophase activity. The values reflect the time point (before visible prophase) after which the culture was refractory to inhibition in G₂ by a particular treatment and continued in the cycle. The fidelity of the analysis is again limited by the 10-min periods. The transition point varies in the case of TPA between ≤10

and ≤50 min, in the case of RPA between ≤30 and ≤50 min. The number of cells which entered prophase during these periods is given in parentheses.

The analysis of the influence of phorbol esters in G₂ phase of HeLa cells published earlier (5, 6, 18, 19) has been carried out under the assumption (supported by preliminary evidence) that mitotic duration was not significantly altered by such treatment. For the mode of analysis used here it was important that cells which were in mitosis at or entered mitosis after addition of the phorbol esters finished cell division undisturbed (see also Figs. 1B and 2B). The data on mitotic duration during the first hour of recording (Table 2) support this notion.

DISCUSSION

The analysis of the influence of phorbol esters in G₂ phase of HeLa cells by time-lapse photography is shown to yield information on time course, onset, and duration of the G₂ delay as well as on onset and rate of recovery from inhibition. The method appears to be more selective than the counting of all mitotic events (e.g., Refs. 5 and 6) or the determination of physically detachable cells (15, 20).

The data obtained on the time delay allow the calculation of equipotent X-ray doses. Based on the data reported with HeLa cells (16, for this purpose approximately 60-min division time has to be added to the data presented here) TPA 10⁻⁷ M is equivalent to approximately 2.1 Gy and TPA 10⁻⁸ M to 1.4 Gy. This comparison reinforces the conclusion derived earlier (19), namely that the response, unlike the case of irradiation, was not proportional to the dose. TPA and RPA at 10⁻⁶ M and 10⁻⁷ M concentrations appear to be equally potent. However, in the case of RPA the clear-cut G₂ delay in HeLa cells disappears between 10⁻⁷ M and 3 × 10⁻⁸ M concentration, whereas in the case of TPA this occurs between 10⁻⁸ and 3 × 10⁻⁹ M concentration.

The degree of recovery from G₂ delay indicated by the slope (Table 1) exhibits an interesting pattern: the more effective the phorbol ester used or the higher the concentration applied, the

larger the multiplication rate after the delay period, indicating a parasynchronous effect in that respect, *e.g.*, at 10⁻⁶ M TPA and RPA. The reason for the lack of complete recovery of the multiplication rate seen at low concentrations of phorbol esters, *e.g.*, at 10⁻⁸ M RPA, is not clear. It appears to reflect a difference in the cellular response to high *versus* low phorbol ester concentrations. Two or more phasic dose-response relations in the action of phorbol esters have indeed been observed, as in studies on the incorporation of choline into phospholipids (21) and on the release of arachidonic acid and the production of prostaglandins (22). At 10⁻⁸ M TPA, for instance, the respective cellular response occurs within an ascending part of the dose-response curve whereas at 10⁻⁷ and 10⁻⁶ M TPA it is seen in a descending part. There is evidence which seems to indicate that phorbol esters act in the G₂ phase of the cell cycle by mimicking diacylglycerol (23). If this is the case, then the reason for the differential response may be due to the fact that phorbol esters at low concentration cover potential diacylglycerol binding sites, whereas at high concentrations of phorbol esters appreciable amounts of cellular diacylglycerols are liberated (24, 25).⁴ These in turn may compete with phorbol esters for the binding sites yielding a qualitatively smaller response due to the differential metabolic stability of phorbol esters *versus* diacylglycerols. Therefore, at low concentrations the cells may respond mainly to the phorbol ester as such, whereas at high concentrations a response to cellular diacylglycerol may also come into play. The cells may recover differently from the response to cellular diacylglycerol than from the inhibition induced by phorbol ester. In support of such a hypothesis are data by Wolfman and Macara (26) showing that increased cellular diacylglycerol levels may modulate responsiveness of cells to phorbol esters. The recovery from G₂ delay in the presence of TPA is likely to be due to temporarily appearing mediators (22, 24, 25) and/or to receptor down regulation rather than to TPA degradation. HeLa cells have been shown not to metabolize TPA to any great extent (27); in addition, supernatants from cells treated with TPA for several hours induce the same G₂ delay (and subsequent recovery) in fresh cultures.⁴

The determination of the onset of inhibition in G₂ shows that a complete inhibition in G₂ can be effected within 10 min (*Experiment 208b*, Table 1). Therefore, the variance observed in the transition points in G₂ from ≤10 to ≤50 min may indicate (a) that the cellular events affected may occur shortly (≤10 min) before visible prophase, and (b) that in different experiments the time required to reach either the necessary intracellular concentration of the phorbol ester and/or to build up enough cellular mediator in response to phorbol ester, presumably diacylglycerol (see above), may vary among the experiments. If these assumptions are correct, the stimulation of protein kinase C by phorbol ester and/or diacylglycerol and subsequent protein phosphorylation may be involved in preventing cell progress into prophase.

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⁴ Unpublished observation.