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SUPPRESSION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY IN MURINE MELANOMA CELLS BY 12-O-TETRADECANOYLPHORBOL-13-ACETATE

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Summary - The effect of the potent tumor promoter 12-O-tetradeconylphorbol-13-acetate (TPA) on the cyclic AMP metabolism of B16 mouse melanoma cells was examined. TPA (10^{-7}M) slightly increased the growth rate and inhibited melanin production by these cells. Although TPA had little effect on basal or hormone stimulated cyclic AMP levels, it did significantly suppress cyclic AMP-dependent protein kinase activity from treated cells in a dose-dependent fashion. Other phorbol ester and non-phorbol ester tumor promoters also suppressed cyclic AMP-dependent protein kinase activity while the non-promoter, phorbol, did not alter cyclic AMP-dependent protein kinase activity.

There have been several reports that the potent tumor promoter TPA has various effects upon cyclic AMP metabolism when applied to mouse skin. These effects include the lowering of basal cyclic AMP levels (1), interference with the ability of isoproterenol to stimulate cyclic AMP formation (2), and induction of low-affinity cyclic nucleotide phosphodiesterase activity (3). Recently Rochette-Egly et al (4) have demonstrated that TPA can alter the basal levels of cyclic AMP and inhibit the ability of catecholamines and prostaglandin E_1 to stimulate cyclic AMP accumulation in cultured rat embryo fibroblasts (5). Since Mufson et al (6) have found that TPA can interfere with the expression of melanogenesis in B16 melanoma cells and since we (7) and others (8,9) have demonstrated that cyclic AMP may be involved in regulating melanogenesis we decided to explore the possibility that TPA may affect the cyclic AMP metabolism of B16 melanoma cells

Materials and Methods

B16-F_1 cells were obtained through the courtesy of Dr. I. J. Fidler, Frederick Cancer Research Center, Frederick, MD. These cells were routinely grown in Minimal Essential Medium with Earle's salts, L-glutamine (2mM), sodium pyruvate (1mM), non-essential amino acids, vitamin solution and 10% heat-inactivated fetal bovine serum

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(Microbiological Associates), adjusted to a final pH of 7.4. For all studies B16 cells were seeded onto 90mm tissue culture dishes at a density of 2 x 10^5/dish. Twenty-four hrs. after seeding the attached cells were refed with medium containing either various concentrations of phorbol esters, or solubilization vehicle (0.01% DMSO). At various time periods following the addition of phorbol esters, plates were harvested and examined for cell counts (hemacytometer), melanin production and protein kinase activity. The melanin content of both cells plus medium was determined by the method of Oikawa and Nakayasu (10). Protein kinase activity was assayed according to the method of Corbin et al (11). Briefly this involved washing the cells 3x with 0.05M phosphate buffer (pH 6.8), scraping the cells from the dish with a rubber policeman and sonicating the resultant suspension for 30 sec. at setting #3 in a model WI85 sonifier (Heat Systems, Plainview, N.Y.). The reaction mixture consisted of 0.05M phosphate buffer (pH 6.8), 0.2mM γ-^{32}P ATP (180 CPM/pmole), 0.5mg histone (type II-A) ± 2μM cyclic AMP and 20μl of cell homogenate. Following a 10 min. incubation at 30° C, 50μl of the reaction mixture was pipetted onto Whatman 3-MM filter paper discs (2.3mm diameter, Whatman, Inc., Clifton, N.J.). The discs were dropped into ice-cold 10% trichloroacetic acid (TCA) and washed in sequence with 10% TCA, 95% ethanol, and ether. After drying, the filters were placed in scintillation vials and counted. All experimental samples were corrected for endogenous phosphorylation, ie the amount of phosphorylation in the absence of histone or the absence of cyclic AMP.

All phorbol esters were obtained from P-L biochemicals (Kenankee, Wis.) while saccharin and iodoacetate were obtained from Aldrich Chemical Co. (Milwaukee, Wis.)

Results

Fig. 1 illustrates our initial findings concerning the effect of 10^{-7}M TPA on B16-F_1 growth and melanin production. Within 24 hrs. after the addition of TPA to the cells there is a small increase in the growth rate. This small increase is maintained throughout the duration of the experiment (6 days). In contrast to the small increase in the growth rate, there is almost a total suppression of melanin production in TPA treated cultures. This suppression is maintained for the duration of the experiment.

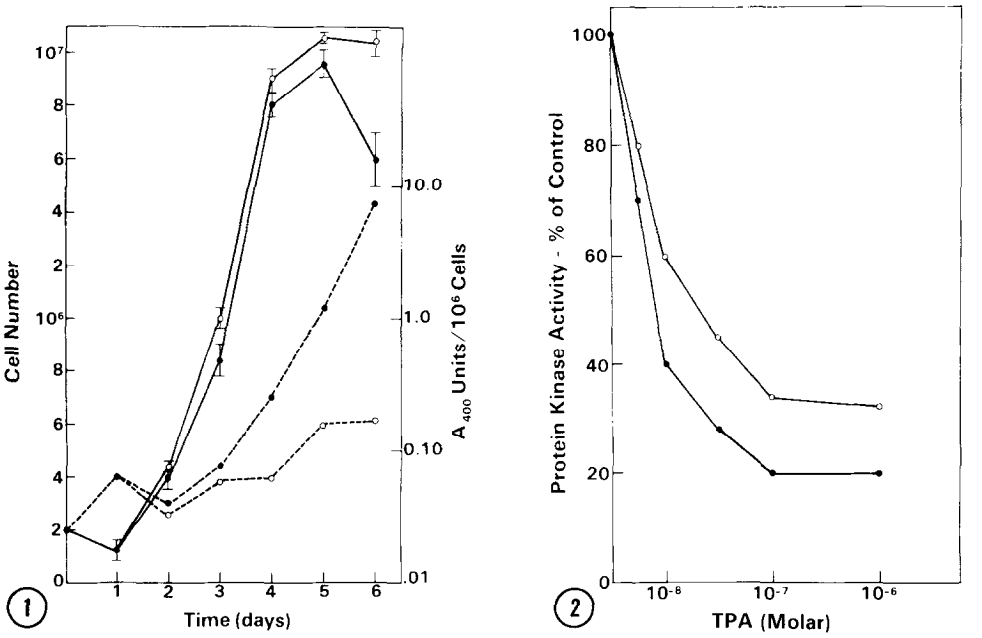

Fig. 1. The effect of TPA on cell growth and melanin production in B16-F_1 mouse melanoma cells. Cells were seeded at 2 x 10^5/90mm dish. One day after seeding, the cells were refed ± 10^{-7}M TPA. Control cells received DMSO (0.01%). At the indicated times cell number and melanin content was determined as described in the text. Cells were not refed during the duration of the experiment. . _ . control cell number; o _ o TPA-treated cell number. The bars above and below these points represent the SEM of triplicate plates. . --- . melanin content of control cells + medium; o --- o melanin content of TPA-treated cells + medium. These data points are the average of quadruplicate plates which were ± 15% of the mean.

Fig. 2. The effect of TPA on cyclic AMP-dependent protein kinase activity in B16-F_1 mouse melanoma cells. Cells were seeded at 2 x 10^5/90mm dish. The following day they were refed with various concentrations of TPA or solubilization vehicle (DMSO - 0.01%). After 48 hrs. of further incubation the cells were harvested and processed for protein kinase assay as described in the text. . _ . protein kinase activity in the absence of cyclic AMP in the reaction mixture. o _ o protein kinase activity in the presence of 10^{-6}M cyclic AMP. The protein kinase

Since the cyclic AMP system has been shown to play a significant role in the regulation of melanin production in B16 cells (7), and TPA has been reported to have effects on the cyclic AMP system, we were curious as to whether the effects of TPA on melanin formation might be the result of its effect on the cyclic AMP metabolism in B16-F_1 cells. Our initial experiments along this line revealed that TPA had little or no effect on basal or MSH stimulated cyclic AMP levels in B16-F_1 cells. However a further examination of the cyclic AMP system revealed a rather dramatic effect of TPA on cyclic AMP-dependent protein kinase activity. Fig. 2 illustrates a typical experiment in which B16-F_1 cells were treated for 48 hrs. with various concentrations of TPA. It can be seen that there is a dose dependent inhibition of both basal and cyclic AMP stimulated protein kinase activity, which is maximal at 10^{-7}M TPA. The I_{50} for TPA inhibition of protein kinase is approximately 7 x 10^{-9}M.

In order to determine the time required for the onset of protein kinase inhibition, measurements of protein kinase activity were conducted at 6, 24, 48, and 72 hrs. following the addition of 10^{-7}M TPA. The results of these experiments (Fig. 3) show that inhibition of protein kinase activity cannot be detected until 24 hrs. of incubation with TPA. The inhibition is maximal at 48 hrs. with little further inhibition at 72 hrs.

A variety of other known or suspected tumor promoters were tested at equimolar (10^{-7}M) concentrations for their effect on protein kinase activity from B16-F_1 mouse melanoma cells. After incubating these compounds with the cells for 48 hrs. and then assessing protein kinase activity, it was found (Table 1) that there is some correlation between the ability of a compound to suppress protein kinase activity and its reported ability to promote papilloma formation in mouse skin.

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activity is expressed as % of control which was derived by dividing the specific activity (pmoles ^{32}P transferred to histone/10^6 cells) of experimental samples by the specific activity of control samples. The experiment was repeated several additional times with similar results.

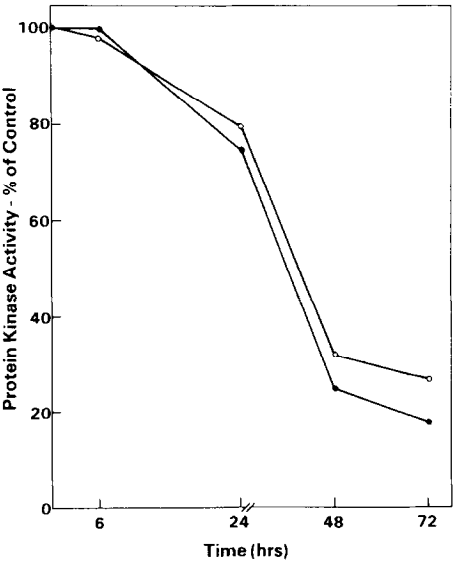

Fig. 3. Time dependent decrease in protein kinase activity subsequent to TPA addition to B16-F_1 cells. Cells were seeded at 2 x 10^5/90mm dish. One day later the cells were refed with 10^{-7}M TPA or solubilization vehicle (DMSO - 0.01%). At the indicated time points cells were processed for protein kinase assay as described in the text. Protein kinase activity is expressed as a ratio between specific activity of experimental samples and control samples x 100. . — . protein kinase activity in the absence of added cyclic AMP. o — o protein kinase activity + 10^{-6}M cyclic AMP.

Discussion

There have been a variety of reports concerning the action of TPA on the cyclic AMP system (1-5). To our knowledge this is the first study to show an effect of TPA and other tumor promoters on the activity of cyclic AMP dependent protein kinase. We verified the observations of Mufson et al (6) that TPA can inhibit density dependent melanogenesis in B16 cells and that the ability of various phorbol esters to inhibit melanogenesis correlated somewhat with their reported ability to act as promoters in the mouse skin assay system. In contrast to the latter study we did not find that the TPA treated cells "escaped" the inhibition of melanogenesis. This may be the result of our using shorter

Table 1. The relative inhibition of B16-F_1 protein kinase by various types of tumor promoters.

Additive	% Inhibition of Protein Kinase		Biological Activity
	Basal	+ Cyclic AMP	
None	0	0	------
TPA	56	32	++++
Phorbol	10	0	+/-
Myristic Acid	29	0	------
Saccharin	55	54	?
Iodoacetic Acid	57	66	+++
Phorbol 12, 13 dibenzoate	58	41	+++/++
Phorbol 12, 13 diacetate	16	47	++

All compounds were incubated with B16-F_1 cells for 48 hrs. at a concentration of 10^{-8}M. Basal protein kinase activity refers to phosphorylation of histone in the absence of cyclic AMP, while + cyclic AMP refers to phosphorylation of histone in the presence of 10^{-6}M cyclic AMP. The biological activity of the various compounds is based upon their relative ability to promote the formation of papillomas in the mouse skin assay as reported by other investigators.

time intervals to study TPA action and if our cells were incubated long enough they may indeed "escape" TPA restriction of melanogenesis.

The mechanism whereby tumor promoters can suppress protein kinase in B16 melanoma cells is not clear. Suppression does not occur immediately, but requires more than 6 hrs. This suggests, but does not prove, that TPA may be suppressing protein kinase activity by interfering with transcription or translation of RNA coding for protein kinase, or alternatively may be stimulating the production of a protein kinase inhibitory molecule, such as the protein described by Walsh et al (12). While it appeared in some experiments that the inhibition of protein kinase activity assayed in the absence of cyclic AMP was greater than activity assayed in the presence of cyclic AMP, (Fig. 2), data from other experiments (Fig. 3) showed similar degrees of enzyme suppression whether assayed

in the absence or presence of cyclic AMP. In other experiments (data not presented) we found that the K_{act} of protein kinase from control and TPA treated cells by cyclic AMP was nearly identical ($\sim 2 \times 10^{-7}$M), but that the V_{max} was considerably lower in preparations from TPA treated cells. This would suggest, at least superficially, that there was no alteration in the regulatory subunit of protein kinase from cells incubated with TPA.

Tumor promoters have been shown to be potent inhibitors of differentiation in several experimental systems (13-16). Cyclic AMP on the other hand has been demonstrated to promote differentiated functions in several cell systems including B16 melanoma cells (17,18,8). Therefore, it is conceivable that tumor promoters may exert some of their actions on differentiation by interfering with the cyclic AMP system. In this report we have found that tumor promoters suppress in a concentration dependent manner, cyclic AMP dependent protein kinase activity, an enzyme known to be involved in regulating melanogenesis (19). The ability of a variety of phorbol and non-phorbol tumor promoters to suppress protein kinase activity correlated well with their ability to inhibit melanin production. These findings are particularly interesting in light of our recent study demonstrating that retinoic acid enhanced melanogenesis (20), inhibited growth, and stimulated cyclic AMP dependent protein kinase activity in B16 melanoma cells (21). Other investigators using a different strain of murine melanoma cells have found that TPA antagonized the effects of retinoic acid on melanogenesis (22). Such antagonism between TPA and retinoic acid on differentiation in other cell systems has been reported (23,24). In light of our findings on the opposing actions of TPA and retinoic acid on protein kinase activity, it is tempting to speculate that in the B16 murine melanoma system the antagonism between these two compounds on differentiation may be mediated by their influence on protein kinase activity.

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