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Differential Effects of the Tumor Promoter Phorbol-12-Myristate-13-Acetate on the Morphological and Biochemical Differentiation of N-18 Mouse Neuroblastoma Cells

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The phorbol ester tumor promoter phorbol-12-myristate-13-acetate (PMA) was found to have differential inhibitory effects on the expression of morphological and biochemical differentiation of N-18 mouse neuroblastoma cells. PMA completely inhibited neurite extension and associated growth characteristics and partially inhibited the increased expression of R_I cAMP-binding protein; PMA had no effect on the induction of acetylcholinesterase activity in cells prompted to differentiate either by treatment with 1 mM dibutyryl cAMP or by serum deprivation. 4-α-Phorbol-12,13-didecanoate, an inactive analogue of phorbol ester tumor promoter, was without effect. The implications of these findings concerning the mechanism of action of phorbol ester tumor promoters in the control of cell differentiation are discussed.

Mouse neuroblastoma cells grown in tissue culture can be induced to differentiate by treatment with 1 mM dibutyryl cAMP or by serum deprivation. Differentiation of these tumor cells is characterized by reduced growth rate, morphological appearance of neurites, and by expression of enzymes involved in neurotransmitter metabolism (Prasad and Kumar, 1974; Schubert et al., 1973).

Recent studies from a number of laboratories have provided evidence that, while there are significant variations and heterogeneity from cell type to cell type, there may be two common regulatory pathway in many cell types and tissues. Thus, the induction of inositol phospholipid breakdown and the activation of the Ca^{++}-activated and phospholipid-dependent protein kinase C promote activation of cellular function and proliferation, whereas the activation of adenyl cyclase and accumulation of cAMP often have the opposite effect (for review, see Nishizuka et al., 1984). Cases in point include the inhibition by tumor phorbol esters, agents that directly activate protein kinase C, of cAMP-induced neurite outgrowth in mouse neuroblastoma cells, and nerve growth factor-induced differentiation of embryonic chick sensory and sympathetic ganglia (Ishii, 1978; Ishii et al., 1978). The effect of tumor promoters on the expression of biochemical differentiation phenotypes, however, was not examined in these studies.

We have been interested in studying the action of cAMP in the control of neuroblastoma cell differentiation. Our previous studies have demonstrated increases in the R_I cAMP-binding protein and acetylcholinesterase activity concomitant with neurite outgrowth in neuroblastoma cell cultures induced to differentiate by the addition of dibutyryl cAMP or by serum deprivation, a condition that increases cellular cAMP concentration (Chen et al., 1983; Liu et al., 1980, 1981). To understand better the regulation of neuroblastoma cell differentiation and the role of phorbol ester tumor promoters in this process, we examined the effects of phorbol-12-myristate-13-acetate (PMA), a potent tumor-promoting phorbol ester, on neurite outgrowth and on the increased expression of the R_I cAMP-binding protein and acetylcholinesterase activity in cultures of N-18 neuroblastoma cells induced to differentiate either by treatment with dibutyryl cAMP or by serum deprivation (hereafter referred to as 1% fetal calf serum induced). We report on the differential effects of PMA in inhibiting the expression of these differentiation phenotypes in the N-18 mouse neuroblastoma cells.

MATERIALS AND METHODS

Materials

All tissue culture supplies were obtained from GIBCO (Grand Island, New York). Phorbol-12-myristate-13-acetate (PMA) and 4-α-phorbol-12,13-didecanoate (4-α-PDD) were gifts from Dr. Peter Blumberg. Other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. 8-N_3-[^{32}P]cAMP was purchased from ICN (Irvine, CA), and radiochemical purity of the compound was checked by TLC analysis in a solvent system of butanol:acetic acid:water (5:2:3; v:v:v). [^{3}H]cAMP was obtained from either NEN (Boston, MA)

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or ICN (Irvine, CA). [1-^{14}C]Acetylcholine was from Amersham Corp. (Arlington, IL).

Cell Culture

The N-18 mouse neuroblastoma cells were grown as monolayer cultures in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS). Cells were plated at a density of 5×10^3–2×10^4 cells/cm^2 and were cultured under standard conditions as previously described (Liu et al., 1980, 1981). Cells were induced to differentiate either by treatment with 1 mM dibutyryl cAMP (Bt_2cAMP) or by serum deprivation (i.e., culturing in medium supplemented with 1% rather than 10% FCS). PMA and 4-α-PDD, when used, were added to the cell cultures 8–24 hr after plating the cells, and cells were cultured in the presence of the phorbol esters with or without dibutyryl cAMP for approximately 3–5 days until they were harvested at an early stationary phase of growth.

Cells, harvested at an early stationary phase of growth, were homogenized in a buffer containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA, and the homogenate was centrifuged at 100,000g to yield a cytosol and membrane fraction. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. For any given experiment, matching sets of plates grown under identical conditions were used for quantitating neurite outgrowth and for preparing cell extracts to assay for R_I cAMP-binding protein and acetylcholinesterase activities.

Incorporation of 8-N_3-[^{32}P]cAMP and [^{3}H]cAMP

Photoaffinity labeling experiments with 8-N_3-[^{32}P]cAMP were performed as previously described (Liu et al., 1980, 1981) using 1 μM 8-N_3-[^{32}P]cAMP (specific activity, 4–8 Ci/mmol) and 100–200 μg protein. The pattern of radioactivity incorporated was determined by SDS-polyacrylamide gel electrophoresis and autoradiography. The amount of radioactivity incorporated into the 47,000-dalton R_I and the 52–54,000-dalton R_{II} was determined by liquid scintillation counting of excised gel slices.

The binding of [^{3}H]cAMP was determined by a filter binding assay method as described by Gilman (1970).

Acetylcholinesterase assay

Acetylcholinesterase activity present in extracts of the N-18 mouse neuroblastoma cells was determined by a radiometric assay method as previously described (Liu et al., 1980, 1981). The amount of [1-^{14}C]acetylcholine hydrolyzed was determined by liquid scintillation spectrometry. The results are expressed in millimoles of acetylcholine hydrolyzed per minute per milligram protein at 37°C with a substrate concentration of 1×10^{-4} M.

RESULTS

Figure 1 presents typical photomicrographs of control, 1 mM dibutyryl cAMP-treated, and 1% fetal calf serum-treated N-18 mouse neuroblastoma cells cultured in the absence or presence of 100 ng/ml of PMA. PMA inhibited the 1 mM dibutyryl cAMP-induced and 1% FCS-induced neurite outgrowth. Furthermore, the reduced growth rate and saturation density, normally seen in cultures treated with 1 mM dibutyryl cAMP or 1% FCS (Liu et al., 1980, 1981), were abolished by the addition of PMA to the cell culture. The saturation densities (i.e., number of cells/cm^2 at a stationary phase of growth) of the control, 1 mM dibutyryl cAMP-treated, and 1% FCS-treated N-18 neuroblastoma cells were 1.9×10^5, 0.8×10^5, and 0.65×10^5 cells/cm^2, respectively, when cultured in the absence of PMA; the values were 2.1×10^5, 1.75×10^5, and 1.68×10^5 cells/cm^2, respectively, when cultured in the presence of 100 ng/ml (1×10^{-7} M) of PMA. The corresponding doubling times of these cells, measured at the logarithmic phase of cell growth, were 19.5 hr (control), 24 hr (1 mM dibutyryl cAMP), and 25 hr (1% FCS) when cultured in the absence of PMA and were 19.5 hr, 21 hr, and 19.5 hr, respectively, when cultured in the presence of 100 ng/ml of PMA. These effects of cAMP and PMA on the cell growth characteristics of the N-18 mouse neuroblastoma cells are highly reproducible. Whether the effect of PMA on neuroblastoma cell growth is a direct consequence of mitogenic action (Driedger and Blumberg, 1977) of the tumor promoter or is secondary to the inhibition of neurite extension of these cells is not known.

Autoradiograms illustrating the effects of PMA on the dibutyryl cAMP-induced and 1% FCS-induced increase in R_I cAMP-binding protein are shown in Figure 2. Quantitation of the amounts of 8-N_3-[^{32}P]cAMP incorporated into the 47,000-dalton R_I and into the 52–54,000-dalton R_{II} present in the 100,000g supernatant (cytosol) and pellet (membrane) fractions are shown in Table 1; results on parallel studies of the amount of [^{3}H]cAMP reversibly bound are shown in Table 2. In agreement with our previous observations (Chen et al., 1983; Liu et al., 1980, 1981), the induction of differentiation of N-18 neuroblastoma cells with 1 mM dibutyryl cAMP or with 1% FCS is associated with a significant increase in cAMP-binding activity as measured either by the amount of 8-N_3-[^{32}P]cAMP incorporated into the 47,000-dalton R_I protein (Fig. 2; Table 1) or by the amount of [^{3}H]cAMP bound (Table 2). PMA reduced the magnitude of this dibutyryl cAMP-induced and 1% FCS-induced increase in R_I cAMP-binding activity from a three- to fivefold increase (in cells cultured in the absence of PMA) to a 1.5–2.5-fold increase (in cells cultured in the presence of PMA). The effect of PMA is specific for the induced R_I cAMP-binding activity. Under these experimental conditions, PMA had no consistent effect on the basal R_I cAMP-binding activity, and it did not significantly affect the R_{II} cAMP-binding activity. In this connection, it was noted that an inactive analogue of phorbol ester tumor promoter, 4-α-phorbol-12,13-didecanoate (4-α-PDD), had no effect on the dibutyryl cAMP-induced increase in R_I cAMP-binding activity. Similarly, the direct addition of PMA to the [^{3}H]cAMP- or 8-N_3-[^{32}P]cAMP-binding assay mixture had no effect.

The effect of increasing concentrations of PMA on the expression of the R_I and R_{II} cAMP-binding protein is shown in Figure 3. In this experiment, the addition of 1 mM dibutyryl cAMP to the N-18 neuroblastoma cell culture increased the amount of 8-N_3-[^{32}P]cAMP incorporation into the 47,000-dalton R_I from 2.35 pmol/mg protein to 8 pmol/mg protein. PMA partially inhibited this dibutyryl cAMP-induced R_I cAMP-binding activity in a dose-dependent manner. PMA [30 ng/ml (5.1×10^{-8} M] caused a significant inhibition of the dibutyryl cAMP-induced R_I cAMP-binding activity. At the highest concentration of PMA tested (300 ng/ml), the inhibition of

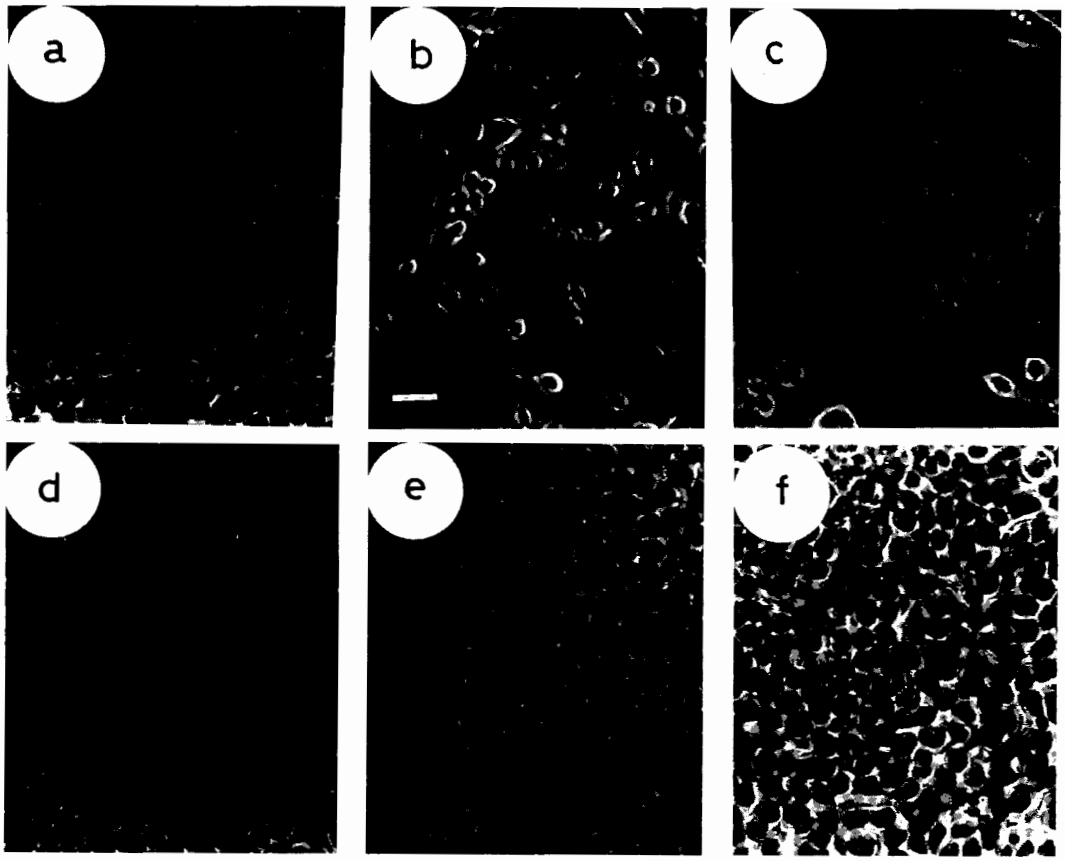

Fig. 1. Photomicrographs of control (a, d), 1 mM dibutyryl cAMP-induced (b, e), and 1% fetal calf serum-induced (c, f) N-18 mouse neuroblastoma cells cultured in the absence (a, b, c) and presence (d, e, f) of 100 ng/ml (1×10^{-7}M) of PMA. N-18 neuroblastoma cells were plated at a density of 1×10^4 cells/cm^2 in Dulbecco's modified Eagle medium supplemented with either 10% or 1% fetal calf serum according to methods described above. PMA (100 ng/μl stock solution in dimethylsulfoxide) and dibutyryl cAMP (150 mM stock solution in balanced salt solution) were added to designated plates of cells 12 hr after their plating to give respective final concentrations of 100 ng/ml and 1 mM, and cells were cultured in the presence of these agents until they reached an early stationary phase of growth approximately 3 days later. Representative fields of the cell cultures at an early stationary phase of growth were photographed. Bar = 50 μm.

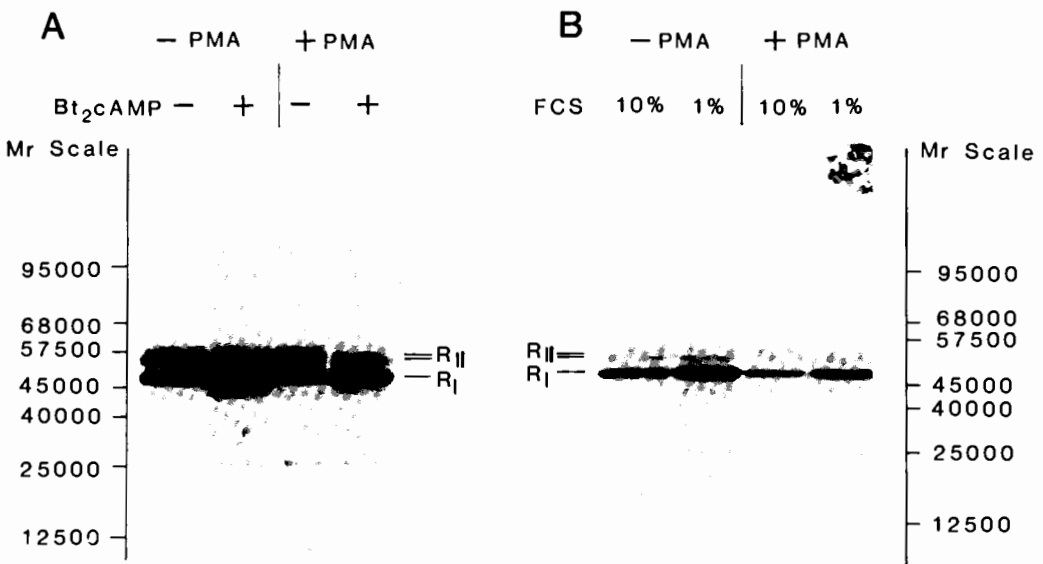

Fig. 2. Autoradiographs illustrating the pattern of 8-N_3-[^{32}P]cAMP incorporation into cytosol proteins of control, 1 mM dibutyryl cAMP-, and 1% fetal calf serum-induced N-18 neuroblastoma cells cultured in the absence and presence of 100 ng/ml of PMA. A) Control and 1 mM dibutyryl cAMP-treated neuroblastoma cells cultured in the absence and presence of PMA. B) Control (10% FCS) and serum-deprived (1% FCS) neuroblastoma cells cultured in the absence and presence of PMA. Cells were plated at a density of 8×10^3 cells/cm^2. Dibutyryl cAMP and PMA were added 15 hr after plating the cells, and cells were harvested 4 days later upon entering an early stationary phase of growth. The 8-N_3-[^{32}P]cAMP incorporation was carried out under standard conditions with 1 μM 8-N_3-[^{32}P]cAMP and 200 μg protein. The pattern of radioactivity incorporated into cytosol proteins was analyzed by SDS-polyacrylamide gel (5–15%) electrophoresis and autoragraphy. The positions on the gel of the 47,000-dalton R_I and the 52–54,000-dalton R_{II} are indicated.

TABLE 1. Effect of PMA on the R_I and R_{II} cAMP-binding activity of control and dibutryl cAMP-induced N-18 mouse neuroblastoma cells

	8-N_3-[^{32}P]cAMP incorporation (pmol/mg protein)			
	Without PMA		With 100 ng/ml PMA	
	Control	1 mM Bt_2 cAMP	Control	1 mM Bt_2 cAMP
Membrane				
R_I	0.51 ± 0.08	2.93 ± 0.52	0.76 ± 0.1	1.74 ± 0.5
R_{II}	0.5 ± 0.04	0.66 ± 0.07	0.49 ± 0.07	0.43 ± 0.038
Cytosol				
R_I	2.1 ± 0.36	7.9 ± 0.5	2.25 ± 0.1	4.73 ± 0.35
R_{II}	1.24 ± 0.11	1.48 ± 0.16	1.3 ± 0.06	1.29 ± 0.16

The conditions for cell culturing and for preparing cell extracts are described in detail in Materials and Methods. Five to eight 100-mm plates of cells were used for each of the conditions. The photoactivated incorporation of 8-N_3-[^{32}P]cAMP into the 47,000-dalton R_I and the 52–54,000-dalton R_{II} and the quantitation of radioactivity incorporated were carried out according to procedures previously described. Results, expressed in picomoles 8-N_3-[^{32}P]cAMP incorporated per milligram protein, represent the average ± standard error of the mean of four independent determinations.

TABLE 2. Effect of PMA (100 ng/ml) on the [^{3}H]cAMP-binding activity of control and 1 mM dibutryl cAMP- and 1% fetal calf serum-induced N-18 neuroblastoma cells

	[^{3}H]cAMP binding (pmol/mg protein)		
	Control	1 mM Bt_2cAMP	1% FCS
Without PMA	3.13	8.4 (2.7)	6.9 (2.2)
With PMA (100 ng/ml)	2.8	3.9 (1.4)	3.6 (1.3)

Cells were plated at a density of 1×10^4 cells/cm^2. Dibutryl cAMP and PMA were added to designated plates of cells 12 hr after plating, and cells were harvested approximately 3.5 days after plating. The [^{3}H]cAMP-binding activity present in the 100,000g cytosol fraction was determined by a filtration assay as described in Materials and Methods. Results are corrected for nonspecific binding, as determined from the amount of radioactivity bound in the presence of a 100-fold excess of cAMP. Results are the average of two separate determinations (with SD < 10%) and are representative of three separate sets of experiments. The numbers in parentheses indicate the fold of increase in [^{3}H]cAMP binding activity over that of the control.

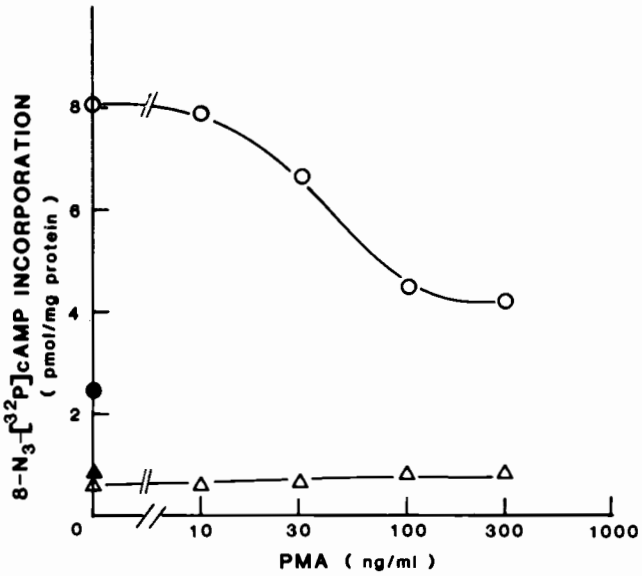

Fig. 3. Effects of increasing concentrations of PMA on the R_I and R_{II} cAMP-binding activity of N-18 neuroblastoma cells induced with 1 mM dibutyryl cAMP. Cells were plated at a density of 1.1×10^4 cells/cm^2. Dibutyryl cAMP (1 mM) and various concentrations of PMA were added to designated cultures 12 hr after plating. Groups of five to eight 100-mm plates of cell were used for each of the culturing conditions. Cells were harvested 3 days after plating upon reaching an early stationary phase of growth. Cytosol extracts were prepared and used to assay for the R_I (○) and R_{II} (△) cAMP-binding activity by the incorporation of 8-N_3-[^{32}P]cAMP. Results are representative of four separate experiments. (In this experiment, the amounts of 8-N_3-[^{32}P]cAMP incorporated into the R_I and R_{II} cAMP-binding proteins present in extract of the control neuroblastoma cell culture, i.e., without dibutyryl cAMP, were 2.35 (●) and 0.95 (▲) pmol/mg protein, respectively; PMA had little or no effect on this basal cAMP-binding activity.)

the dibutyryl cAMP-induced expression of R_I cAMP-binding activity remained incomplete. PMA caused a similar dose-dependent inhibition of neurite extension in dibutyryl cAMP-induced N-18 mouse neuroblastoma cells (data not shown), although, in contrast to the incomplete suppression of the increase in R_I cAMP-binding activity, the inhibition of neurite outgrowth was virtually complete at the highest concentration of PMA used (300 ng/ml). Similar results on the inhibition of neurite outgrowth by increasing concentrations of PMA have been reported by Ishii et al. (1978).

Increases in the R_I cAMP-binding protein and acetylcholinesterase activity have been used as two biochemical indices to assess neuroblastoma cell differentiation (Chen et al., 1983; Liu et al., 1980, 1981). The effects of various concentrations of dibutyryl cAMP on induction of the R_I cAMP-binding protein and acetylcholinesterase activity and the effect of 100 ng/ml (1.7×10^{-7} M) PMA on these increases are shown in Figure 4. Dibutyryl cAMP gave a dose-dependent increase in R_I cAMP-binding and acetylcholinesterase activities in both the cytosol and membrane fractions of the N-18 mouse neuroblastoma cells; these increases were significant even at the lowest concentration of dibutyryl cAMP tested (0.3 mM). The addition of 100 ng/ml of PMA to these cultures partially inhibited the dibutyryl cAMP-induced increase in R_I cAMP-binding activity and had little or no effect on the increase in acetylcholinesterase activity at all concentrations of dibutyryl cAMP used.

For most of the experiments presented in this study, a single dose of PMA was added simultaneously with dibutyryl cAMP 12–24 hr after subculturing the neuroblastoma cells. Experiments were also carried out to examine the effects of refeeding cells daily with medium containing fixed amounts of dibutyryl cAMP and PMA. No difference in results was observed between cells treated with a single dose of dibutyryl cAMP and PMA at the beginning of the culturing period versus cells that were refed daily with fresh medium that contained fixed concentrations of the drugs, suggesting that the lack of inhibition by PMA on the dibutyryl cAMP-induced AChE activity cannot be attributable to metabolic inactivation of PMA.

DISCUSSION

The results presented in this study demonstrate the differential inhibition by PMA of the dibutyryl cAMP-induced and 1% FCS-induced increases in neurite outgrowth, R_I cAMP-binding protein, and acetylcholinesterase activity. Differential suppression of the different aspects of myogenesis by PMA has previously been reported (Cohen et al., 1977; Dlugosz et al., 1983). Thus,

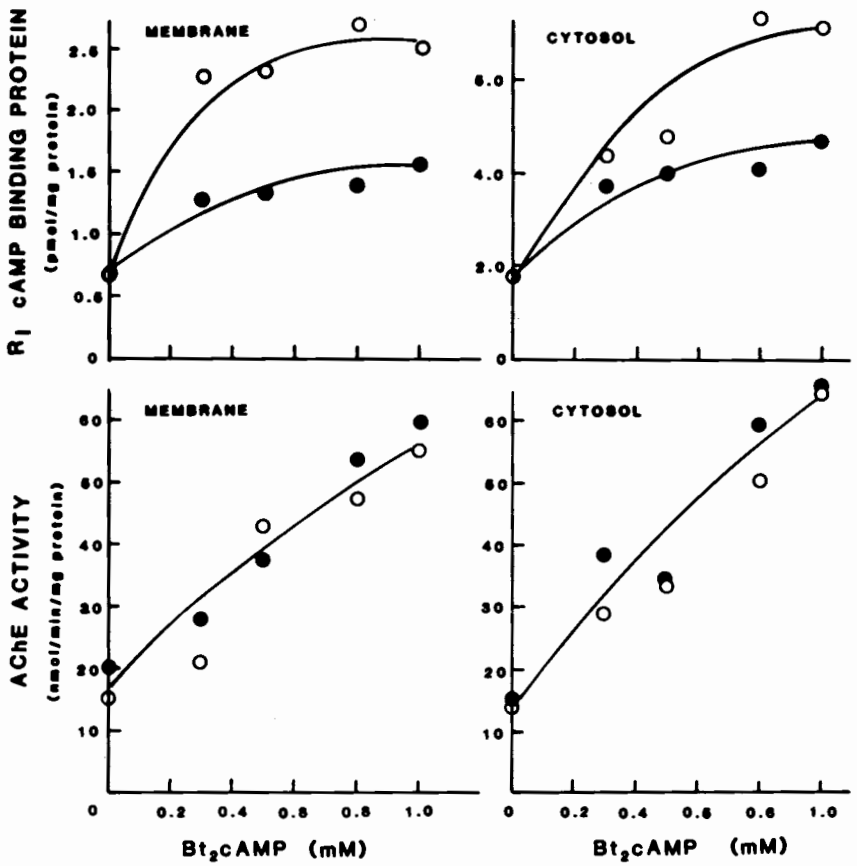

Fig. 4. Induction of the R_I cAMP-binding protein and acetylcholinesterase activity by increasing concentration of dibutyryl cAMP and the effect of PMA (100 ng/ml) on this induction. Cells were plated at a density of 9×10^3 cells/cm^2. Various concentrations of dibutyryl cAMP either with (●) or without (○) 100 ng/ml PMA were added to cells 12 hr after their plating. Groups of five to eight 100-mm plates of cells were used for each of the culturing conditions. Cells were harvested 4 days after plating upon entering an early stationary phase of growth. R_I cAMP-binding protein and acetylcholinesterase activity present in the 100,000g supernatant (cytosol) and pellet (membrane) fractions of the cell homogenate were assayed according to methods described above. Results are representative of four separate determinations.

the fusion of mononucleated myoblast to form myotubes is exquisitely sensitive to suppression by PMA, while the synthesis and accumulation of two muscle-specific proteins (desmin and muscle-specific light meromysin) appear relatively insensitive. Furthermore, PMA apparently permits the myogenic cells to initiate expression of muscle-specific proteins without having the cells withdrawn from the cell cycle.

An important question in understanding the tumor-promoting activity of phorbol esters is whether these agents have specific effects in the control of cell growth and differentiation. It has been proposed that the tumor-promoting activity of various phorbol esters may be related and perhaps attributable to their inhibitory action on the terminal differentiation of target cells (for reviews see Diamond et al., 1978; Weinstein and Wigler, 1977). However, the results presented in this study and results from the following lines of experiments provide evidence suggesting that the hypothesis needs to be evaluated in the context of the specific cell type under consideration. These results include 1) PMA apparently has diametrically opposite effects in regulating the differentiation of related cell types in culture (for example, the inhibition of mouse neuroblastoma cell differentiation [Ishii et al., 1978] versus the stimulation of human neuroblastoma cell differentiation [Spinelli et al., 1982; Spinelli and Ishii, 1983], the inhibition of mouse melanoma cell differentiation [Mufson et al., 1979] versus the stimulation of human melanoma cell differentiation [Huberman et al., 1979], and the inhibition as well as stimulation of differentiation in various virus-transformed proerythroid cells in vitro [Miao et al., 1978]); 2) the effects of PMA in modulating cell differentiation is readily reversible; and 3) there is no correlation between the tumor promotional and antipromotional activities of various compounds with their corresponding effects on cell differentiation (Mondal and Heidelberger, 1980).

As an alternative working hypothesis, it seems likely that tumor-promoting phorbol esters may have selective effects on the expression of a differentiation phenotype either by perturbing the plasma membrane or by modulating the activity of specific metabolites of the target cell. The rapidity of the effects of PMA in inhibiting neurite outgrowth of neuroblastoma cells in culture, in inhibiting fusion of myoblast to form myotubes (Cohen et al., 1977; Dlugosz et al., 1983), and in causing rapid and reversible morphological changes of chick embryo chondroblasts and fibroblasts (Lowe et al., 1978) is evidence suggestive of a direct effect of PMA at the level of the cell surface or cytoskeleton structure. A direct and

reversible effect of PMA on the actin-containing elements of the cytoskeleton of chick embryo fibroblasts has previously been demonstrated (Rifkin et al., 1979). Recently reported rapid and significant effects of PMA on Ca^{++} transport and metabolism (Brostrom et al., 1982; Schimmel and Hallam, 1980) also support the contention that PMA may modulate the structure and function of cell surface and cytoskeleton.

In attempting to delineate the biochemical events underlying the action of cAMP and PMA in the regulation of neuroblastoma cell differentiation, it is noteworthy that protein phosphorylation plays an important role in the action of both cAMP and PMA. There is abundant evidence that the action of cAMP in eukaryotic cells is achieved through activation of cAMP-dependent protein kinase and phosphorylation of specific substrate proteins (for review see Krebs and Beavo, 1979). More recent studies have provided evidence that phorbol ester tumor promoters directly and permanently activate a Ca^{++}-activated and phospholipid-dependent protein kinase C and that this enzyme is most likely a receptor protein for tumor-promoting phorbol esters (for a review see Nishizuka et al., 1984). In human platelets, the activation of protein kinase C, either by a receptor-induced turnover of inositol phospholipid or by the direct action of phorbol ester tumor promoter, has been shown to be correlated to phosphorylation of a 40,000-dalton protein and to the release of serotonin from platelets. Furthermore, this chain of events, including inositol phospholipid turnover, diacylglycerol formation, phosphorylation of the 40,000-dalton protein, and the release of serotonin, is inhibited concurrently and progressively by increasing concentrations of dibutyryl cAMP or of prostaglandin E_1, an agent that activates adenyl cyclase in platelets (for review see Nishizuka et al., 1984). It is likely that this transmembrane signal transduction pathway of receptor occupancy, activation of protein kinase C, and phosphorylation of specific substrate proteins may subserve the action of phorbol ester tumor promoters and agents that increase inositol phospholipid turnover in the control of various biological processes in a number of different cell types.

Research over the past 20 years has underscored the importance of protein phosphorylation as a biological control mechanism (for reviews see Greengard, 1978; Liu, 1984). It seems likely that protein phosphorylation may be a crossover point in the chain of events initiated by cyclic nucleotides, by Ca^{++}, and by increased inositol phospholipid turnover. In this connection, it would be of interest to identify those proteins in the N-18 mouse neuroblastoma cells whose phosphorylation may be regulated by cAMP and by PMA and to determine if phosphorylation of any of these proteins may subserve the regulation of expression of the various differentiation phenotypes.

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LITERATURE CITED

Bostrom, M.A., Brostrom, C.O., Brotman, L.A., Lee, C.-S., Wolff, D.J., and Geller, H.M. (1982) Alterations of glial tumor cell Ca^{2+} metabolism and Ca^{2+}-dependent accumulation by phorbol myristate acetate. J. Biol. Chem., *257*:6758–6765.

Chen, K.Y., Nau, D., and Liu, A.Y.-C. (1983) Effects of inhibitors of ornithine decarboxylase on the differentiation of mouse neuroblastoma cells. Cancer Res., *43*:2812–2818.

Cohen, R., Pacifici, M., Rubinstein, N., Biehl, J., and Holtzer, H. (1977) Effect of a tumour promoter on myogenesis. Nature, *266*:538–540.

Diamond, L., O'Brien, T.G., and Rovera, G. (1978) Tumor promoters: Effects on proliferation and differentiation of cells in culture. Life Sci., *23*:1979–1988.

Dlugosz, A.A., Tapscott, S.J., and Holtzer, H. (1983) Effects of phorbol 12-myristate 13-acetate on the differentiation program of embryonic chick skeletal myoblasts. Cancer Res., *43*:2780–2789.

Driedger, P.E., and Blumberg, P.M. (1977) The effect of phorbol diesters on chicken embryo fibroblasts. Cancer Res., *37*:3257–3265.

Gilman, A.G. (1970) A protein binding assay for adenosine 3′:5′-cyclic monophosphate. Proc. Natl. Acad. Sci. U.S.A., *67*:305–312.

Greengard, P. (1978) Phosphorylated proteins as physiological effectors. Science, *199*:146–152.

Huberman, E., Heckman, C., and Langenbach, R. (1979) Stimulation of differentiated functions in human melanoma cells by tumor-promoting agents and dimethyl sulfoxide. Cancer Res., *39*:2618–2624.

Ishii, D.N. (1978) Effect of tumor promoters on the response of cultured embryonic chick ganglia to nerve growth factor. Cancer Res., *38*:3886–3893.

Ishii, D.N, Fibach, E., Yamasaki, H., and Weinstein, I.B. (1978) Tumor promoters inhibit morphological differentiation in cultured mouse neuroblastoma cells. Science, *200*:556–559.

Krebs, E.G., and Beavo, J.A. (1979) Phosphorylation-dephosphorylation of enzymes. Annu. Rev. Biochem., *48*:923–959.

Liu, A.Y.-C. (1984) Modulation of the function and activity of cAMP-dependent protein kinase by steroid hormones. Trends Pharmacol. Sci., *5*:106–108.

Liu, A.Y.-C., Chan, T., and Chen, K.Y. (1981) Induction of the regulatory subunit of type I adenosine cyclic 3′:5′-monophosphate-dependent protein kinase in differentiated N-18 mouse neuroblastoma cells. Cancer Res., *41*:4579–4587.

Liu, A.Y.-C., Fiske, W.W., and Chen, K.Y. (1980) Regulation of cyclic adenosine 3′:5′-monophosphate-binding protein in N-18 mouse neuroblastoma cells. Cancer Res., *40*:4100–4108.

Lowe, M.E., Pacifici, M., and Holtzer, H. (1978) Effects of phorbol-12-myristate-13-acetate on the phenotypic program of cultured chondroblasts and fibroblasts. Cancer Res., *38*:2350–2356.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193*:265–275.

Miao, R.M., Fieldsteel, A.H., and Fodge, D.W. (1978) Opposing effects of tumour promoters on erythroid differentiation. Nature, *274*:271–272.

Mondal, S., and Heidelberger, C. (1980) Inhibition of induced differentiation of C3H/10T clone 8 mouse embryo cells by tumor promoters. Cancer Res., *40*:334–338.

Mufson, R.A., Fisher, P.B., and Weinstein, I.B. (1979) Effect of phorbol ester tumor promoters on the expression of melanogenesis in B-16 melanoma cells. Cancer Res., *39*:3915–3919.

Nishizuka, Y., Takai, Y., Kishimato, A., Kikkawa, U., and Kaibuchi, K. (1984) Phospholipid turnover in hormone action. Recent Prog. Horm. Res., *40*:301–345.

Prasad, K.N., and Kumar, S. (1974) Cyclic AMP and the differentiation of neuroblastoma cells in culture. In: Control of Proliferation in Animal Cells. B. Clarkson and R. Baserga, eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 581–594.

Rifkin, D.B., Crowe, R.M., and Pollack, R. (1979) Tumor promoters induce changes in the chick embryo fibroblast cytoskeleton. Cell, *18*:361–368.

Schimmel, S.D., and Hallam, T. (1980) Rapid alteration in Ca^{++} content and fluxes in phorbol 12-myristate 13-acetate treated myoblasts. Biochem. Biophys. Res. Commun., *92*:624–630.

Schubert, D., Harris, A.J., Heinemann, S., Kodokoro, Y., Patrick, J., and Steinbach, J.H. (1973) Differentiation and interaction of clonal cell lines of nerve and muscle. In: Tissue Culture of the Nervous System. G. Sato, ed. Plenum Press, New York, pp. 55–86.

Spinelli, W., and Ishii, D.N. (1983) Tumor promoter receptors regulating neurite formation in cultured human neuroblastoma cells. Cancer Res., *43*:4119–4125.

Spinelli, W., Sonnenfeld, K.H., and Ishii, D.N. (1982) Effects of phorbol ester tumor promoters and nerve growth factor on neurite outgrowth in cultured human neuroblastoma cells. Cancer Res., *42*:5067–5073.

Weinstein, I.B., and Wigler, M. (1977) Cell culture studies provide new information on tumour promoters. Nature, *270*:659–660.