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EFFECT OF PHORBOL AND BRYOSTATIN I ON CHONDROGENIC EXPRESSION OF CHICK LIMB BUD, IN VITRO

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SUMMARY

The ability of phorbol esters to promote tumor formation and alter cell differentiation has been attributed to its action on a number of critical cellular functions, in particular, on protein phosphorylation, through the activation of protein C kinase. The present paper describes the effects of PMA (phorbol 12-myristate 13 acetate) on in vitro chondrogenesis in non-passaged, embryonic limb bud cells, relative to the effects of Bryostatin I. This compound also activates C kinase and binds competitively to the phorbol ester receptor, yet does not affect cell differentiation. Levels of PMA as low as 10^{-7} M markedly reduced cartilage formation in 4-day cultures, as indicated by nodule count and Alcian blue staining for chondroitin sulfate. Coadministration of Bryostatin I at equimolar concentration prevented the PMA inhibitory effect on chondrocytic expression. This confirms other findings that phorbol activation of C kinase cannot exclusively account for the activity of phorbol on cell expression, i.e., that other pathway(s) must also be involved. Altering the time of PMA exposure demonstrated that PMA inhibited chondrocyte phenotypic expression, rather than cell commitment: early (0-48 h) exposure to PMA (during chondrocytic commitment in vitro) had little inhibitory effect on the staining index, whereas, exposure from 49-96 h (presumably post-commitment) and 0-96 h had moderate and strong inhibitory effects, respectively, on cartilage synthesis. Further research on the phorbol/Bryostatin I interaction should add to our knowledge of the control processes involved in tumor promotion and cell differentiation.

Phorbol esters, a group of potent co-carcinogens derived from Croton oil, have been extensively studied for their role in tumor promotion. In addition, the effects of phorbols on in vitro cell differentiation have been shown to be highly varied in a number of cell types in vitro (see 1 for review). PMA has been shown to induce the expression of a differentiated macrophage-like phenotype and inhibit proliferation among HL60 leukemia cells (2). In contrast, myoblasts exposed to phorbol become "partially" differentiated. That is, although they do not withdraw from the cell cycle (3) nor

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form myotubes, they produce the gene products meromyosin and desmin (4). Similarly, neuroblastoma cells remain morphologically immature in the presence of phorbol, yet produce the differentiated gene product acetylcholinesterase (5).

A number of critical cellular functions appear to be altered by phorbol esters. These compounds have been demonstrated in one system or another to induce gene amplification (6), ornithine decarboxylase (6), sister chromatid exchange (7), and increased synthesis of superoxide anion radicals (8). In addition, they have been shown to inhibit cell-cell communication (9) and high affinity epidermal growth factor binding sites (10). While numerous other effects have also been reported (see 2), current focus is on the action of phorbol on protein phosphorylation. The phorbol receptor is known to be a calcium-, lipid-dependent protein kinase (protein C kinase) which may phosphorylate proteins indiscriminately once activated. If this is indeed the primary cellular response to phorbol treatment, then phorbol-dependent gene expression should be a function of the altered activity of the proteins present in the system being studied rather than a more specific action on some metabolic pathway or other cell function.

There is, however, recent evidence by Kraft et al. (2) which indicates that the primary effect of phorbol on differentiation of HL60 cells may not be due to C kinase activation. Their evidence is based on studies utilizing the compound Bryostatin I, one of several recently isolated Bryostatins with suspected antineoplastic activity (11-16). This drug, a macro-cyclic lactone structurally unrelated to phorbols, binds competitively to the phorbol ester receptor, activates C kinase, yet has no apparent effect on cell differentiation among HL60 cells. When phorbol is administered with competitive levels of Bryostatin I, the cells continue to proliferate instead of forming the typical phorbol-induced, macrophage-like phenotype. This evidence tentatively suggests that phorbol-induced differentiation in this passaged cell line may be due to events other than C kinase activation. The purpose of the present paper is to describe the effects of phorbol on in vitro chondrogenic expression among non-passaged, embryonic limb bud cells and to relate these effects to the action of Bryostatin I.

MATERIALS AND METHODS

Chemicals

Benzamide (BAM), phorbol 12-myristate 13-acetate (PMA), and Alcian blue were purchased from Sigma Chemical Co., St. Louis, Missouri. Bryostatin I was isolated from the bryozoan Bugula neritina in the lab of G. Pettit.

Cell Culture

Limb bud cells were obtained from specific pathogen free (SPF) Cofal negative White Leghorn chicken eggs (Larson Lab-Vac Eggs, Inc., Gowrie, Iowa). Wings and legs were removed from stage 23-24 embryos (17) and the cells dissociated enzymatically for 30 minutes at 37°C. The digestion mixture, which consisted of 0.125% trypsin, 0.1% collagenase and 0.005% DNase in Hank's balanced salt solution, was prepared immediately before use. To facilitate digestion, cells were mixed on a Vortex mixer at 5 minute intervals throughout the incubation period. After digestion cells were further dissociated mechanically by trituration, washed twice in growth medium [Ham's F12 supplemented with 5% fetal calf serum (K.C. Biological, Inc., Lenexa, Kansas), 3 mM BAM (18), and penicillin/streptomycin/amphotericin B], filtered through two 20 micron mesh Nitex filters, and resuspended at the final plating density (2.5×10^6 to 2×10^7 cells/ml). Twenty microliters of cell suspension were placed in the center of each well of a 24 tissue cluster

plate and the cells allowed to attach to the substratum at 37°C for two hours. To each culture was added one ml of growth medium (with or without the added test compound(s)). Cultures were grown in a water-jacketed incubator containing an atmosphere of 5% CO_2-95% air. Cells were fed fresh medium/test compound at 48 hours and the cultures terminated for assay after 96 hours total incubation.

Cell Proliferation and Cartilage Production

At termination of the experiment, colonies were assayed for cell proliferation and cartilage synthesis. Three colonies from each treatment group were dissociated from the enclosing cartilage matrix using the enzymatic digestion mixture and counted on a Coulter counter. Cell size distributions were also determined using a Coulter channelyzer. Cartilage production was assayed as a function of Alcian blue staining. At termination of the culture period, 4 colonies from each treatment group were fixed for 30 minutes in 95% ethanol, followed by 30 minutes in 10% formalin. The sulfated glycosaminoglycan component of the cartilage matrix was then stained with Alcian blue (1%, pH 1.0) for 1 hour, and the colonies thoroughly rinsed in distilled water followed by 3% acetic acid. Dye was extracted in 4 M guanidine and absorbance measured at 620 nm (19).

RESULTS

Phorbol on Chondrogenesis

Levels of PMA as low as 10^{-7} M caused a marked reduction in cartilage formation (both number of nodules produced and total dye bound to matrix), while 10^{-8} M produced little, if any, inhibition (Fig. 1). Subsequent experiments utilized PMA at 10^{-7} M concentration (10^{-6} M when studying the effect of timing of PMA exposure). Cell counts after 4 days incubation demonstrated that PMA was not toxic at the levels tested.

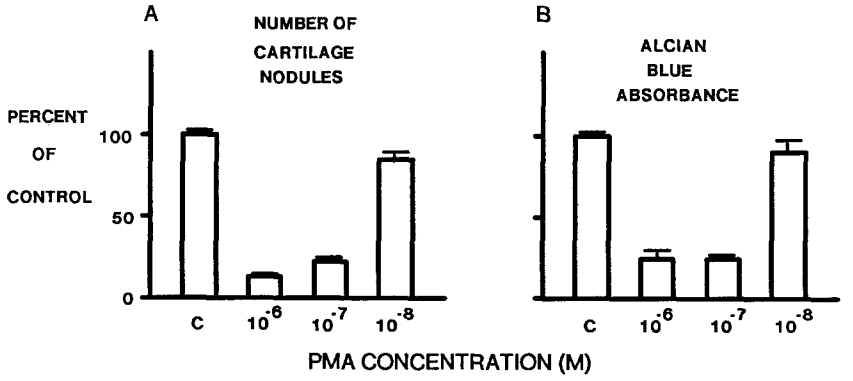

FIG. 1

Effect of PMA concentration (M) on chondrogenesis in 4-day cultures. Data given as mean percent control value (± SE) for (A) number of Alcian blue stained nodules from three colonies per treatment group, and (B) Alcian blue absorption by the sulfated cartilage matrix of four colonies per treatment group.

Because stage 24 chick limb bud cells are thought to be undifferentiated at the time of initial plating, presumably becoming irreversibly committed to their differentiated state (chondrocyte or fibroblast) during the first 24-48 hours in culture, we examined the effect of the timing of phorbol exposure on cartilage synthesis. An experiment was performed in which cultures were exposed to PMA from 0-48, 49-96, 0-96 h of incubation (Fig. 2). The presence of phorbol caused a reduction of Alcian blue staining, with the 0-48 h group displaying the least effect, and the 0-96 h group the greatest. The moderate inhibition of staining in the 49-96 h group suggests that PMA reduces the degree of sulfated proteoglycan synthesis (the primary indicator of chondrogenesis) which is occurring during the period of exposure.

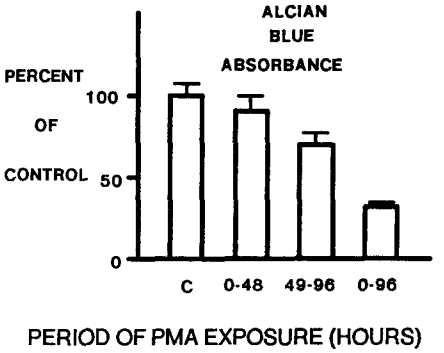

FIG. 2

Effect of the time of PMA exposure (10^{-6} M) on chondrogenesis in 4-day cultures, given at 0-48, 49-96, or 0-96 h. Data given as mean percent control value (± SE), as outlined in Figure 1B.

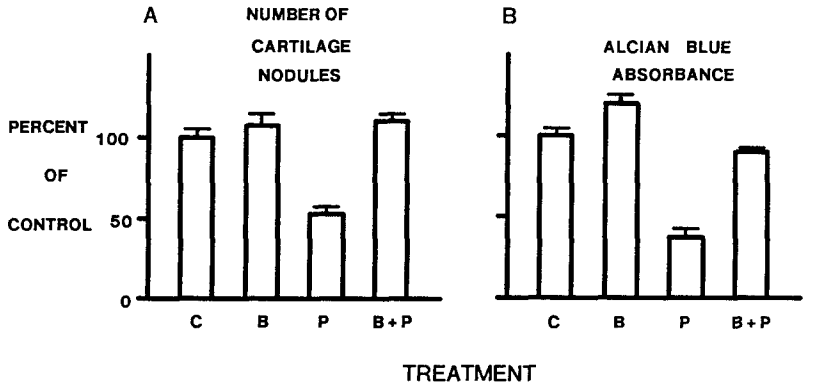

FIG. 3

Effect of PMA/Bryostatin I interaction on chondrogenesis in 4-day cultures: Bryostatin I (B) (10^{-7} M), PMA (P) (10^{-7} M), or Bryostatin I and PMA in combination (B + P), relative to control (C). Data given as mean percent control value (± SE) as outlined in Figure 1.

Phorbol-Bryostatin I Interactions

Bryostatin I has been reported to be a competitive inhibitor of the phorbol binding site. Bryostatin was added to cultures 90 minutes prior to addition of an equimolar amount of PMA (10^{-7} M), and the cultures maintained for 4 days prior to assay. Bryostatin by itself appeared to have a slight stimulatory effect on number of cartilage nodules formed and on total Alcian blue staining (Fig. 3). As noted above, 4 days of phorbol treatment inhibited both of these indices. The combination of PMA and Bryostatin returned these values to control levels, demonstrating that Bryostatin prevented the inhibitory effect of PMA on chondrocytic expression.

DISCUSSION

Phorbol esters are a unique group of compounds which display varied and unpredictable biological activity. Dependent on the cell type under study, these compounds may either induce or inhibit the development of a differentiated phenotype (1). It has been assumed by a number of researchers that the unpredictable nature of the cellular response is due to the ubiquitous fashion in which phorbol-activated protein kinase C phosphorylates cellular proteins, possibly modifying regulatory proteins in an unusual fashion or sequence. Attractive as it may be, this theory has yet to be proven, and it cannot account for the observed interaction of phorbol with Bryostatin I in a passaged cell line (HL60) recently documented by Kraft et al. (2). Since transformed cell lines do not behave in the same fashion as non-transformed cells, particularly concerning regulatory events, we looked at the effect of Bryostatin I on embryonic chick limb bud cells. This cell type provides a good model for studying factors involved in cell commitment and expression, since the ultimate fate (myocytic, chondrocytic or fibroblastic) of the limb bud cells can be readily modified *in vitro*.

It has been reported that one of the primary effects of PMA on chondrocytic expression is to reduce the amount of sulfated proteoglycan produced (3). The inhibition of Alcian blue staining among PMA-treated cells in the present study is in agreement with these findings. Since chondroitin sulfate makes up the majority of the sulfated product in cartilage, PMA is almost certainly affecting this compound. The well-formed matrix seen among PMA-treated cells, and the rapid recovery of cells exposed to PMA from 0-48 h to near control values by 96 h, may indicate that the observed inhibition of staining was not due to a total lack of extracellular matrix materials. Likely the lack of staining was caused by a depletion of the sulfated proteoglycan portion of the matrix. Previous work (3, 20) suggests that this depletion is due to synthesis inhibition of a portion of the proteoglycan that is present.

We believe that the effect of the phorbol is not to inhibit the commitment of mesenchyme cells to chondrocytes, but to prevent complete phenotypic expression when phorbol is present. This view is supported by several observations. PMA-treated limb bud cells were morphologically indistinguishable from control cartilage cells, displaying the characteristic rounded cell shape and extensive enclosing extracellular matrix. Exposure to PMA from 0-48 h, the "window" of chondrocytic commitment in culture, should have irreversibly affected expression if commitment were blocked, preventing Alcian blue staining even after the removal of the PMA. However, this early exposure group showed a rapid recovery of staining to near control values by day 4, suggesting a transient role for the phorbol. Indeed, PMA also inhibited staining when present from 49-96 h, presumably after the cells had undergone chondrocytic commitment. The reversible nature of the phorbol effect suggests that these cells were fully differentiated, rather than partially differentiated.

It is interesting to note that cultures exposed to PMA from 0-96 h synthesized significantly less Alcian blue staining matrix than cultures exposed from 49-96 h. The increased staining observed in this latter group probably has two causes. Any proteoglycan synthesized prior to 49 h will be present in the 49-96 h group, but not the 0-96 h group. Also, it is highly unlikely that addition of PMA at 49 hours will immediately or completely "shut down" any proteoglycan synthesis which is occurring, potentially allowing further elaboration of the matrix which is already present.

Thus far, in the few reports on the biological response to the newly isolated compound Bryostatin I, its mechanism of action has been studied relative to phorbol activity among transformed cell lines (2, 21). This earlier work demonstrated that Bryostatin I competed with phorbol for C kinase receptor binding sites, stimulating C kinase activity. In the case of 3T3 cells arrested in the G_1/G_0 phase of the cell cycle, Bryostatin I mimicked the mitogenic effect of phorbol (21). In contrast, HL60 cells cotreated with phorbol and Bryostatin I were prevented from acquiring the differentiated phenotype typically associated with phorbol treatment, suggesting that the activity of phorbol must be related to other factors as well as C kinase activation (2).

Phorbol/Bryostatin I interactions seen in the present study support the contention that phorbol esters exert their effects on cell expression via some pathway other than C kinase activation. If C kinase activation was the sole pathway by which gene expression was altered, Bryostatin I would not inhibit the phorbol-induced phenotype, as both compounds activate this enzyme. However, since binding of Bryostatin I to the phorbol receptor did prevent alteration of cartilage production, it is likely that PMA exerts its effect on chondrocytic expression via this receptor, although the effect of Bryostatin I on other cellular processes affected by phorbol esters has yet to be determined. As these effects are elucidated, the unique ability of the Bryostatins to counteract some of the observed effects of phorbols will undoubtedly aid in the study of control processes involved in both tumor promotion and cytodifferentiation.

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