Effects of Retinoic Acid and Juvenile Hormone on the Induction of Ornithine Decarboxylase Activity by 12-O-Tetradecanoylphorbol-13-acetate


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ABSTRACT

The tumor-promoting agent 12-O-tetradecanoylphorbol-13-acetate (TPA), a highly active comitogen in phytohemagglutinin-treated bovine lymphocytes, induces an 11-fold increase in ornithine decarboxylase activity over cultures treated with the lectin alone. This synergistic action of TPA could be antagonized by the simultaneous addition of the acyclic sesquiterpene, insect juvenile hormone III. Retinoic acid (vitamin A acid), an inhibitor of the tumor-promoting action of TPA in mice, was also an effective antagonist but required administration to lectin-activated lymphocytes 1 hr prior to TPA. These data suggest that metabolic activation of retinoic acid is required in order to exert its antagonistic action. Comparison of the responses in the lymphocytes and mouse skin suggests that the lymphocytes provide an excellent system for studying the molecular processes through which phorbol esters and retinoids influence the growth and differentiation of both normal and premalignant cells.

INTRODUCTION

A number of chemicals act as effective comitogens in PHA-treated lymphocytes. TPA, the most active of a series of phorbol esters that promotes 2-stage carcinogenesis in mouse skin, is an example of a highly potent comitogen. When added to PHA-treated cultures of bovine lymphocytes at the 100 nM level, TPA causes a 2- to 3-fold increase in lymphoblast formation and stimulates the coupled synthesis of RNA, protein, and DNA (7). Recently, it has been shown that retinoic acid, as well as the structurally related insect juvenile hormones, can selectively antagonize the comitogenic action of TPA without affecting the responsiveness of the lymphocytes to PHA (5, 6). This interaction of phorbol esters and retinoic acid in the regulation of lymphocyte mitogenesis appears to have relevance to the function of these agents in tumor promotion since the comitogenic activity of the different phorbol diesters runs parallel to their tumor-promoting activity, and the comitogenic activity, like tumor promotion, is antagonized by retinoids.

The present study extends these correlations to include the effects of these agents on the induction of ODC activity (EC 4.1.1.17; L-ornithine carboxy-lyase) in lymphocytes. The stimulation of the rate of polyamine biosynthesis may be a prerequisite for the transition of a cell from a restricted to an active growth state (17). In the case of lymphocytes, the activity of ODC, the enzyme that catalyzes the first step in the biosynthesis of polyamines rises markedly in PHA-stimulated cells and remains at an elevated state for more than 2 days (3, 4).

As shown earlier in mouse skin, TPA induces a dramatic stimulation of epidermal ODC activity (12). Retinoic acid, which has prophylactic effects on skin tumors formed by the 2-stage process (1), is a specific inhibitor of TPA-induced ODC activity in mouse epidermis (18, 19). In the present report we show that TPA, acting as a comitogen, induces a rapid and transient increase in lymphocyte ODC activity and that this induction can be selectively inhibited by the appropriate administration of retinoic acid or juvenile hormone III. These data provide further evidence that PHA-stimulated lymphocytes constitute a useful system for exploring the molecular processes underlying the metabolic interaction of the tumor-promoting agent TPA and the antitumor-promoting agent retinoic acid.

MATERIALS AND METHODS

Materials. PHA-P, obtained as a vial of sterile powder from Difco Laboratories, Inc., (Detroit, Mich.), was diluted to 5 ml with sterile water. PHA was added to cell cultures at a final dilution of 1:10,000 of this stock solution. This point constitutes time zero in all experiments. TPA and phorbol were obtained from Consolidated Midland Corp., Brewster, N. Y. Retinoic acid (all-trans) and juvenile hormone III (10-epoxy-3,7,11-trimethyl-2,6-trans,trans-dodecadienoic acid, methyl ester) were purchased from Sigma Chemical Company (St. Louis, Mo.) and Calbiochem (San Diego, Calif.), respectively. Dimethyl sulfoxide (spectroscopic grade; Aldrich Chemical Co., Milwaukee, Wis.) was used as the vehicle for the phorbol esters and terpenoids; its final concentration in the medium never exceeded 0.55%. dl-[1-14C]Ornithine hydrochloride (specific activity, 49.9 mCi/mmole) was purchased from New England Nuclear, Boston, Mass.

Lymphocytes. Lymphocytes were isolated from the retropharyngeal lymph nodes of freshly slaughtered cattle. Isolation procedures were carried out at room temperature under sterile conditions as described previously (5). Lymphocytes were suspended at a concentration of 10 to 10^6 cells/ml in a modified Eagle's medium that contained 10% bovine serum (10) and were incubated overnight at 37°C in a spinner bottle with gentle stirring and a 5% CO_2-95% air atmosphere. The following day the lymphocyte concentration was diluted to 3 x 10^6 cells/ml and 5-m1 aliquots of the cell suspension were transferred to 12-ml conical centrifuge tubes and placed on a 4°C slant in a 37°C incubator.
Assay of ODC Activity. At appropriate times after treatment, lymphocytes were harvested and washed twice with ice-cold 0.9% NaCl solution. The cells were disrupted by alternate freezing and thawing for 4 times in 0.3 ml of 50 mM sodium phosphate buffer (pH 7.2) containing 0.1 mM pyridoxal phosphate and 0.1 mM EDTA, followed by centrifugation at 2000 x g for 15 min to obtain the soluble supernatant.

ODC activity in the clear supernatant was determined by measuring the release of $^{14}$CO$_2$ from DL-[1-$^{14}$C]ornithine hydrochloride. The assay mixture contained 35 mM sodium phosphate (pH 7.2), 0.2 mM pyridoxal phosphate, 4 mM dithiothreitol, 0.4% EDTA, 0.1 mM L-ornithine containing 0.5 µCi of DL-[1-$^{14}$C]ornithine hydrochloride, and 100 µl of lymphocyte extract in a final volume of 0.25 ml. After incubation at 37° for 60 min in 15-ml Corex centrifuge tubes equipped with rubber stoppers and center well assemblies (12), the reaction was stopped by addition of 0.5 ml of 2 M citric acid. The incubation was continued for at least another 1 hr to ensure complete absorption of $^{14}$CO$_2$ by the ethanolamine and methoxymethanol (0.2 ml; 2:1) contained in the center well. Finally, the center well containing the ethanolamine:methoxymethanol was transferred to a vial containing 10 ml of toluene based scintillation fluid and 2 ml ethanol, and radioactivity was measured in a Packard Tri-Carb liquid scintillation counter with an 80% efficiency (18). Assays were always carried out in duplicate; blank assays contained no enzyme or boiled enzyme.

ODC activity was linear with assay times up to 60 min, and increasing extract volumes were prepared from up to 6 x 10$^6$ lymphocytes. In practice incubations were carried out for 60 min, and assay tubes contained extract from less than 5 x 10$^6$ lymphocytes. The apparent K_m's for ornithine, both for the enzyme induced by PHA and by PHA and TPA, were 100 µM.

RESULTS

Induction of ODC Activity by TPA. We have shown previously in bovine lymphocytes that the potent tumor-promoting agent TPA acts synergistically with a suboptimal level (1:10,000) of PHA to enhance thymidine incorporation into DNA (stimulation of 6-fold over PHA alone) (5). Utilizing identical culture conditions and lectin concentration, we now find that 10 mM TPA causes a 12-fold induction of ODC activity when assayed at the point of maximal induction. As is shown in Chart 1, maximal ODC activity was observed 18 hr after TPA treatment, and the enzyme activity returned to near the PHA base-line level beyond 30 hr. This pattern of rapid and transient stimulation of ODC activity by TPA is also seen in mouse epidermis (12) but is dependent on the concomitant activation of the lymphocytes with PHA. Suboptimal levels of PHA used in these experiments induce only a slight and gradual persistent increase in enzyme activity over a 48-hr period (Chart 1).

The dose-response curve for this synergistic action of TPA in the induction of ODC activity is shown in Chart 2. Maximal induction occurs with 100 nM TPA, a concentration that is also optimal for induction of DNA synthesis. Phorbol, the free alcohol obtained from TPA which is inactive both as a tumor-promoting agent and a comitogen, did not increase the ODC activity over the basal level with PHA treatment alone.

The results of a time-course experiment in which lymphocytes were cultured in the presence of PHA and to which TPA was added at intervals from 0 to 16 hr later are shown in Chart 3A. ODC activity was assayed in all cultures at 18 hr. PHA plus TPA; O, TPA alone; A, PHA plus phorbol. Values are the means of triplicate cultures; bars, S.E.

The results of a time-course experiment in which lymphocytes were cultured in the presence of PHA and to which TPA was added at intervals from 0 to 16 hr later are shown in Chart 3A. ODC activity was assayed in all cultures at 18 hr. It would appear that the addition of TPA must be made nearly concurrently with the lectin treatment to obtain the maximal enhancement; however, because this curve appears to be the mirror image of the ODC time course shown in Chart 1, the possibility arises that TPA, although requiring an initial PHA activation of the lymphocyte, can induce
ODC activity in a time-independent fashion. In a test of this possibility TPA was given at various intervals after PHA, but in this case activity was assayed 18 hr post-TPA addition to allow for maximal induction of the enzyme. This experiment (data not shown) revealed that delayed addition of TPA by 1 hr did not effect the induction event; however, when TPA was added 6 hr after PHA only two-thirds of the ODC activity was observed. Thus, while not showing the same strict temporal dependence as is observed for TPA synergism in the induction of DNA replication (5), the delayed addition of TPA relative to PHA does result in a significant reduction in ODC induction. For further evaluation of the time kinetics of the inductive event by a different approach, TPA and PHA were added to cultures concurrently and at various intervals; thereafter the media were changed adding back only PHA (Chart 3B). This experiment shows that exposure of the lymphocytes for 10 min to a level of 10 nM TPA was sufficient for the subsequent full induction of ODC activity. The likely possibility that not all of the TPA is washed out by 10-fold dilution of the TPA will lower its level to an inactive concentration. The possibilities remain therefore that either the TPA is somehow sequestered by the cells for later action or the TPA acts immediately. The latter possibility is favored by the observation that TPA stimulates the capping of lymphocytes within 10 min (C. H. Kwong and G. C. Mueller, unpublished data).

Antagonism of the TPA-mediated Induction of ODC Activity by Retinoic Acid and Juvenile Hormone III. Retinoic acid, which is required for growth and maintenance of differentiation of epithelial tissue (16) and which has been shown to protect against cancer induction in the mouse skin tumorigenesis system (1), is a specific inhibitor of TPA-induced ODC activity in mouse epidermis (18, 19). Additionally, retinoic acid and its related congener, juvenile hormone III, are highly effective antagonists of phorbol ester-mediated comitogenesis in the lymphocyte system (5, 6). For these reasons we were prompted to examine the effects of these antagonists on TPA-mediated induction of ODC activity in lymphocytes. As shown in Chart 4, juvenile hormone III is an effective inhibitor, the dose-response curve being identical with that seen for the inhibition of TPA comitogenesis (6). In contrast retinoic acid at concentrations (e.g., 100 nM) well above those required to inhibit TPA comitogenesis (dose causing 50% inhibition = 5 × 10⁻⁷ M) (5) had no inhibitory effect on ODC activity. However, an inhibitory response could be obtained with retinoic acid by culturing lymphocytes in the presence of PHA and retinoic acid for 1 hr prior to addition of TPA. This enhancement of retinoic acid activity may reflect a requirement for metabolic activation of retinoic acid acid prior to exerting its antagonistic action. The parallel nature of the dose-response curves for juvenile hormone III and preincubated retinoic acid suggests that the juvenile hormone III molecule may be similar to an intermediary form of retinoic acid.

**DISCUSSION**

It has been proposed that lymphocytes in the G₁ state are restrained by membrane-mediated reactions that regulate the availability or activity of inducing principles that cause a concerted expression of the genes, leading to the synthesis of specific proteins that are required for DNA synthesis and cell replication. Based on the observations that phorbol esters have dramatic effects upon membrane metabolism (14, 15), it is our working hypothesis that TPA acts early...
and directly on the cell membrane to alter its capacitor-like state and release these inducers to effect the expression of certain genes or influence the character of chromatin in cells engaged in nuclear replication (9, 11). We consider that the induction of ODC by TPA for a required biosynthesis of polyamines may be one aspect of this transition.

Previously, we have shown that retinoic acid and juvenile hormone III can selectively prevent the induction of DNA synthesis in small lymphocytes by the comitogenic combination of PHA and TPA when added concomitantly (5, 6). Both of these agents inhibit the TPA-mediated induction of DNA synthesis in the cultures without affecting other aspects of the TPA-promoted blastogenic response such as the increased overall synthesis of RNA and protein, which leads to cell hypertrophy. Under similar circumstances in this study, juvenile hormone III antagonized the TPA-mediated induction of ODC activity in a dose-dependent manner, whereas retinoic acid was virtually ineffective. This suggests that retinoic acid blocks lymphocyte mitogenesis by interfering in some process other than the synthesis of polyamines as might result from a reduction in DNA activity.

Retinoic acid, however, was able to block the TPA-mediated induction of ODC activity as seen in mouse epidermis if the retinoic acid was preincubated for 1 hr with PHA-treated lymphocytes prior to the addition of TPA (Chart 4). This observation suggests that retinoic acid may not be the active antagonist and that a metabolic activation may be necessary. In this regard PHA may serve to activate the appropriate metabolizing enzymes. In polymorphonuclear leukocytes both mitogenic lectins and TPA rapidly stimulate oxygen metabolism in the plasma membranes of these cells (2, 13). The production of superoxide anion is strikingly enhanced within 15 min after TPA addition. This highly reactive species can be readily converted into metabolic intermediates (8) such as singlet oxygen, hydroxyl radicals, or hydrogen peroxide which in turn may be involved in the activation of retinoic acid through conversion to an intermediate that has properties in common with juvenile hormone, which was more active in the present experiments. Evidence in support of this hypothesis will be presented elsewhere. At this point in our investigations, it appears that retinoic acid may be a poor inhibitor of the induction of ODC activity in the lymphocyte system because the TPA-inductive event proceeds very rapidly (Chart 3B) and may outpace the activation of the enzymes that metabolize retinoic acid to the active form. In summary our studies of the interaction of TPA and juvenile hormone (or retinoic acid) in PHA-stimulated small bovine lymphocytes suggest that TPA, acting at the cell membrane, indirectly activates the expression of many genes in this highly repressed cell and that juvenile hormone and retinoic acid (or an activated form) block certain of these inductions selectively. The molecular processes that are involved in these induction events are the subject of current study.

Their identification is especially prompted by the possibility that the same mechanisms may operate in the promotion and restraint of tumorigenesis.

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REFERENCES


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