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INHIBITION OF PHORBOL ESTER ACTION IN LYMPHOCYTES BY 5,6-EPOXY-β-IONONE[1]

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SUMMARY

The tumor promoting agent, 12-O-tetradecanoylphorbol-13-acetate (TPA) is both a highly active stimulator of phospholipid metabolism and a potent comitogen in bovine lymphocytes. Retinoic acid and the short chain analog, β-ionone, antagonize TPA action, but exhibit broad dose-response curves suggesting a requirement for metabolic activation of these inhibitors. In the present study 5,6-epoxy-β-ionone has been synthesized as a model metabolite and shown to be a more effective inhibitor of TPA action than the parent compound, β-ionone. The data are in accord with the concept that activation of both β-ionone and retinoic acid may proceed by epoxidation.

INTRODUCTION

Previous studies from this laboratory have established that low levels of phorbol esters stimulate choline-phospholipid metabolism in bovine lymphocytes (1) and act along with phytohemagglutinin as comitogens to trigger the replication of these cells (2,3). The activity of different phorbol esters for both responses parallels the tumor-promoting activity of these agents in mouse skin (1,3,4). In addition, it was shown that retinoic acid, an agent which antagonizes the tumor-promoting effects of phorbol esters in mouse skin (5), also prevents their induced metabolic responses in lymphocytes (3). An unusual aspect of retinoic acid action, however, is the broad dose-response curve (1,3); this feature has suggested the possibility that metabolic activation of retinoic acid may be required. A clue

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to the nature of such a process came from surveying a series of structurally related compounds. Methyl-10,11-epoxyfarnesoate (insect juvenile hormone III) which has an epoxide group at a position which corresponds to the double bond in the ionone ring of retinoic acid, was found to mimic retinoic acid in the lymphocyte system and to have a much steeper dose-response curve (6). This result has prompted an investigation of the possible role of epoxidation in the activity of retinoic acid and related compounds. The present report describes the synthesis and testing of the 5,6-epoxide of β-ionone, a short chain analog of retinoic acid. It has been found that the introduction of the epoxide group strikingly increases the activity of β-ionone as an antagonist of phorbol ester action in lymphocytes. The implications of this finding for the control of the tumor-promoting action of phorbol esters by retinoids are discussed.

MATERIALS AND METHODS

Reagents

Phytohemagglutinin-P (PHA), obtained as a sterile powder from Difco Laboratories (Detroit, Mich.), was reconstituted with 5 ml sterile water and added to cell cultures at a dilution of 1:10000 of this stock. TPA, a generous gift from Dr. R. K. Boutwell, was used at a concentration of 10 nM. β-Ionone and dimethyl sulfoxide (spectrophotometric grade) were purchased from Aldrich Chemical Company (Milwaukee, Wis.). Dimethyl sulfoxide was used as the solvent for delivery of TPA, β-ionone and 5,6-epoxy-β-ionone to cell cultures, and in all cases was present at a final concentration of 0.55%. Radioisotopes, [^{3}H]thymidine (64.7 Ci/mmole) and [^{3}H-Me]choline (69.5 Ci/mmole) were obtained from New England Nuclear (Boston, Mass.).

Synthesis of 5,6-epoxy-β-ionone

To 2 mmoles (385 mg) of β-ionone in 15 ml dry ethyl ether were added 2 mmoles of monoperphthalic acid (determined by iodometric titration) in 5 ml ether. The reaction vessel was flushed with dry nitrogen and kept at room temperature for 2 days. At this point the precipitated phthalic acid was removed by filtration, and the ethereal solution was washed once with dilute aqueous sodium bicarbonate and twice with double distilled water. After drying over sodium sulfate, the ether was removed via rotary evaporator. The resulting clear syrup crystallized spontaneously to yield 278 mg of the crude product melting at 44-47°.

After recrystallization from light petroleum (bp 40-60°), the product melted at 49-50° [lit. (7) mp 46-48°] and showed an absorption maximum in n-hexane at 341 nm [lit. (7) λmax = 340 nm]. The infrared absorption spectrum of this product (1% w/w in KBr) contained a strong band at 1672 cm^{-1} indicating the presence of the ketone moiety and a moderate absorption at 1635 cm^{-1} which is consistent with the presence of one olefinic double bond

conjugated with the carbonyl group. The 60 MHz proton magnetic resonance spectrum of the compound in $DCCl_3$ with tetramethylsilane as internal standard confirmed the assigned structure: δ6.6 (2H, AB, $|\nu_A-\nu_B|$ = 45 Hz, $|J|$ = 16 Hz trans olefinic H's on C-7 and C-8); δ2.3 (3H, S, H_3C- on C-9); δ1.2 - 2.0 (6H, m, $-CH_2-$'s at C-2, C-3 and C-4); δ1.15 (6H, S, H_3C's on C-1) and δ0.9 (3H, S, H_3C- on C-5).

Preparation of lymphocytes and assay procedures

Lymphocytes were prepared as described previously (2) from bovine retropharyngeal lymph nodes obtained from Oscar Mayer and Company (Madison, Wis.). The cells were maintained in Eagle's HeLa medium supplemented with 10% bovine serum and 50 μg gentamicin sulfate per ml (BEHM) for one day prior to use.

In the assay of either DNA synthesis (2,3) or choline incorporation (1), 5-ml aliquots of cell suspension containing 15 x 10^6 lymphocytes were planted in 12-ml conical centrifuge tubes and incubated at 37° on a slant.

When [^{3}H-Me]choline incorporation was to be monitored, the lymphocytes were first collected by centrifugation at 150 x g for 10 min and resuspended in Dulbecco's phosphate buffered saline (8) containing 5.6 mM glucose. Except when otherwise indicated, this medium was not supplemented with serum. β-Ionone and its epoxide were added to cell cultures 0.5 h before the addition of 2 μCi of [^{3}H-Me]choline/culture and TPA. After a 1 h labeling period, cells were harvested and the phospholipids were extracted and purified as described elsewhere (9,10). Radioactivity in the dried phospholipid extracts was determined by liquid scintillation spectrometry.

In the mitogenesis assay, the lymphocytes were kept in BEHM throughout and were labeled with [^{3}H]thymidine during the interval of 48-50 h after the concurrent addition of PHA, TPA and β-ionone or its epoxide. Incorporated [^{3}H]thymidine was then assayed using a standard Whatman GF/C filter disc procedure (11).

RESULTS AND DISCUSSION

To test the hypothesis that epoxidation of the double bond in the β-ionone ring may represent a step in the metabolic activation of retinoic acid and related compounds for the antagonism of phorbol ester action, we have compared β-ionone and 5,6-epoxy-β-ionone for their ability to inhibit the TPA stimulation of phospholipid metabolism and comitogenesis in bovine lymphocytes. As shown in Figure 1, β-ionone exhibits a broad dose-effectiveness relationship similar to that reported previously for retinoic acid inhibition of TPA-mediated comitogenesis (3). If epoxidation of the 5,6-double bond of β-ionone is in fact a necessary step in producing the active inhibitory species, one might expect the exogenously added epoxide to inhibit at lower doses and to yield a sharper dose-response curve than the parent compound. As can be seen in Figure 1, the epoxide is active over a narrow

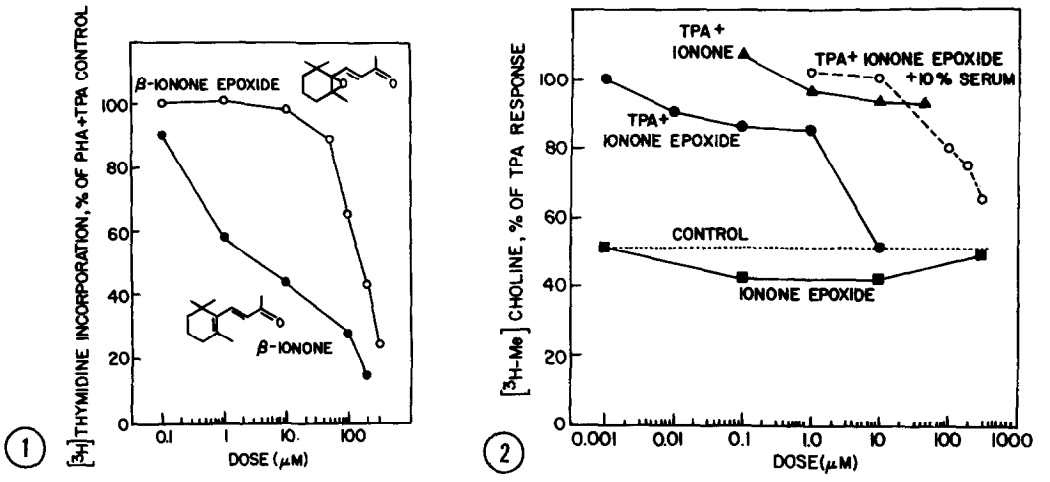

Figure 1. Dose-response curves for the inhibition of lymphocyte mitogenesis by β-ionone and 5,6-epoxy-β-ionone. PHA, TPA and β-ionone were all added to cell cultures at time zero and cultures were pulsed with [^{3}H]thymidine 48 to 50 h later. Points represent the means of triplicates. Standard errors were < 10%.

Figure 2. Dose-response curves for the inhibition of [^{3}H-Me]choline incorporation into phospholipids by β-ionone and 5,6-epoxy-β-ionone. β-Ionone or its epoxide were added to cultures 0.5 h before [^{3}H-Me]choline and TPA. After a 1 h incorporation, cells were harvested and the label incorporated into the phospholipid fraction was determined. Points are the averages of duplicates. The level of incorporation in untreated control cultures is indicated by the dotted line. (■—■) 5,6-epoxy-β-ionone; (●—●) TPA plus 5,6-epoxy-β-ionone; (▲—▲) TPA plus β-ionone; (O--O) TPA plus 5,6-epoxy-β-ionone with medium containing 10% bovine serum.

range of concentration as expected. The observation that the effective range is shifted to higher concentrations, however, deviates from the expected result. This situation, as discussed below, appears to reflect a problem in delivering the chemically reactive epoxide to the appropriate cellular sites rather than to an inherently lesser activity.

That the inhibitory action of 5,6-epoxy-β-ionone toward TPA-induced comitogenesis does not merely represent toxicity is supported by two observations. First, inhibitory doses of this compound do not affect the number of cells excluding trypan blue within the 48 h test; and secondly, if the addition of the epoxide to lymphocyte cultures is delayed 10 h or more after

the addition of PHA and TPA, then the inhibition of TPA action is significantly diminished (less than 15% inhibition with 300 μM epoxide). This latter point is in accord with the previously demonstrated sensitive interval for retinoic acid effects, suggesting that the target sites for the active species are only transiently available during the early stage of lymphocyte activation (3).

To gain further insights into the early action of phorbol esters and their retinoid antagonists in the lymphocyte system, we have also examined the effects of these agents on lipid metabolism. As previously demonstrated TPA elicits a two-fold increase in the incorporation of [^{3}H-Me]choline into phospholipids within 1 h (1). This response is also antagonized by retinoic acid with a broad dose-response curve similar to that found in the retinoid inhibition of TPA-mediated comitogenesis (3). However, in this metabolic area β-ionone is essentially without effect (Figure 2). This lack of activity in the early assay system could reflect less efficient metabolism of this agent as contrasted with the physiologically more significant retinoic acid. Were this the case, then supplying an ionone metabolite more proximate to the active inhibitory species could be expected to result in significant inhibition of this response to TPA. It is clear from the results presented in Figure 2 that 5,6-epoxy-β-ionone fulfills this expectation.

Furthermore, when this experiment was repeated with the inclusion of 10% bovine serum in the incubation medium the narrow range of inhibitory doses of the epoxide was found to be shifted more than one order of magnitude toward higher concentrations. This finding suggests that the epoxide is inactivated or sequestered by serum. Although the mechanism of this inactivation is uncertain, it could involve binding to serum components, nonspecific chemical reactions or possibly the action of an epoxide cleaving enzyme. In any case, this observation suggests that the failure of low levels of 5,6-epoxy-β-ionone to inhibit TPA-enhanced DNA synthesis may result from competing side reactions which reduce the concentration of the chemically

reactive epoxide.

Also shown in Figure 2 is the fact that 5,6-epoxy-β-ionone does not affect the [^{3}H-Me]choline incorporation into the phospholipids of control cultures. This finding is accepted as further evidence that this agent is not simply toxic, but interacts selectively with some cellular component which is essential to the phorbol ester response.

Taken together, these observations on β-ionone and its epoxide support the concept that formation of an analogous epoxide may be involved in the antagonism of TPA action by retinoic acid. Preliminary studies on the anti-TPA effects of the retinoic acid epoxide are consistent with this concept and will be reported elsewhere. These results further attest to the usefulness of the bovine lymphocyte system for exploring the molecular mechanism of action of both the tumor promoting phorbol esters and their retinoid antagonists.

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