Requirements for Protein Synthesis and Calcium for Stimulation of Prostaglandin Synthesis in Cultured Rat Liver Cells by Tumor Promoters

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ABSTRACT

The tumor promoters, 12-O-tetradecanoylphorbol-13-acetate (TPA), phorbol-12,13-didecanoate, teleocidin, and dihydroteleocidin, at nM levels, but not the non-tumor-promoting 4a-phorbol-12,13-didecanoate even at μM concentrations, stimulated arachidonic acid metabolism in cultured rat liver cells. These liver cells synthesize primarily prostaglandin I₂ [measured as its nonenzymatic hydrolytic product, 6-keto-prostaglandin F₁α (PGF₁α)]. The production of 6-keto-PGF₁α increased with time of incubation with TPA and was essentially complete in 4 hr. Cycloheximide, at nM levels, blocked the TPA-stimulated 6-keto-PGF₁α production in a dose-dependent manner; this inhibition was related to inhibition of protein synthesis. Chelation of Ca²⁺ by ethyleneglycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid, treatment of the cells with the Ca²⁺ channel blocker, nifedipine, or inhibition of intracellular Ca²⁺ mobilization by 8-(diethylamine)octyl-3,4,5-trimethoxybenzoate hydrochloride also inhibited TPA-stimulated 6-keto-PGF₁α production. The steroid antiinflammatory drug, dexamethasone, a potent in vivo inhibitor of tumor promotion, was an inhibitor of 6-keto-PGF₁α stimulation by TPA.

INTRODUCTION

One of the pleiotropic biochemical responses to TPA treatment in mouse skin and cultured cells is increased prostaglandin synthesis (5). TPA is known to be a potent tumor promoter in the 2-stage model of carcinogenesis. This model was originally demonstrated in skin (3), but it also has been shown to be operative in liver (1, 19, 20), bladder (9), mammary gland (2), stomach (8), and cultured cells (18).

Although prostaglandin production has been associated with tumor formation, a causal relationship has never been demonstrated (11). The most compelling evidence that prostaglandin production is involved in the process of tumor formation has come from inhibition of tumor formation in vivo. Several inhibitors of prostaglandin synthesis are capable of inhibiting tumor promotion by TPA, and this inhibition can be overcome by addition of specific prostaglandins (15, 17). The possible relationship between prostaglandin synthesis and tumor formation has increased interest in mechanisms involved in regulation of prostaglandin synthesis and especially in mechanisms leading to stimulation of arachidonic acid metabolism by tumor promoters.

Arachidonic acid, the precursor of prostaglandins, is metabolized by a complex series of reactions. Inhibitors that act at different steps of the overall reaction have been described. For example, nonsteroidal antiinflammatory drugs, such as aspirin and indomethacin, inhibit the enzyme cyclooxygenase and block prostaglandin synthesis; the antiinflammatory steroids, such as dexamethasone, inhibit expression of acylhydrolase activity, possibly by inducing the synthesis of an inhibitor (6, 10). Antioxidants, such as butylated hydroxyanisole, inhibit both cyclooxygenase and lipoxigenase activities and block production of prostaglandins, hydroxy-6,8,11,14-eicosatetraenoic acids and leukotrienes (14). The retinoids and protease inhibitors block TPA-stimulated prostaglandin production by some but not all cells (12). Most of the above inhibitors of arachidonic acid metabolism have been shown to block tumor production in vivo (12).

In this paper, we will describe the stimulation of prostaglandin biosynthesis in a normal rat liver cell line by several tumor promoters [TPA, PDD, teleocidin, and dihydroteleocidin (21)]. This TPA stimulation is inhibited by cycloheximide and dexamethasone. We also will show that EGTA, which decreases the extracellular Ca²⁺ concentration by chelation, nifedipine, which blocks the membrane Ca²⁺ channels in the membrane (4), and TMB-8, which inhibits the mobilization of intracellular bound Ca²⁺ (16), inhibit prostaglandin synthesis stimulated by TPA.

MATERIALS AND METHODS

An established cell line for normal rat liver, C-9 (23), was grown as monolayers using MEM containing 2 mM L-glutamine and supplemented with 10% (v/v) fetal bovine serum. Cells treated with trypsin-EDTA (0.25%) were seeded at 0.8 × 10⁶ cells/80-mm tissue culture dishes (P60; Falcon Plastics, Oxnard, Calif.) in 4 ml of the serum-supplemented medium. After 1 day of growth, the medium was removed, and each dish was washed twice with 2 ml of MEM and then incubated for 4 hr in 2 ml of MEM containing the indicated substances (unless otherwise indicated) at 37° in an atmosphere of 95% air-5% CO₂. The culture media were collected and analyzed by radioimmunoassay for PGF₂α (measured as 6-keto-PGF₁α) (7).

Protein synthesis in the cells was determined by incubating the cells with 1 μCi [³H]leucine for 1 hr and measuring the incorporation of [³H]leucine in trichloroacetic acid-precipitable material. Stock solutions of TPA (1 mg/ml), nifedipine (10 mg/ml), TMB-8 (10 mg/ml), PDD (1 mg/ml), Ac₄-PDD (10 mg/ml), teleocidin (10 mg/ml), dihydroteleocidin (10 mg/ml), and dexamethasone (1 mg/ml) were made in dimethyl sulfoxide and kept at −20°. Stock solutions of cycloheximide (1 mg/ml) and EGTA (0.1 mM) were made in water and kept at −20°.
Materials. Phorbol esters (TPA, 4α-PDD, PDD) were purchased from CMC Cancer Research Chemicals, Inc. (Brewster, N. Y.). TMB-8 was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Nifedipine was obtained from Charles Pfizer and Co. (New York, N. Y.). Teleocidin and dihydroteleocidin were gifts from Dr. T. Sugimura, National Cancer Center, Research Institute, Tokyo, Japan. The rat liver cells (the C-9 cell line) were obtained from the American Type Culture Collection (Rockville, Md.). [3H]leucine (specific activity, 120 Ci/mmole) was purchased from ICN (Irvine, Calif.). All other reagents were from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

The tumor promoters TPA, PDD, teleocidin, and dihydroteleocidin stimulated PGE2 (measured as 6-keto-PGF1α) production by C-9 rat liver cells in culture in a dose-dependent manner. The non-tumor-promoting phorbol ester 4α-PDD, even at concentrations 3 orders of magnitude higher than those of the tumor promoters, did not (Chart 1). Teleocidin was the most effective stimulator of prostaglandin production, about 2 times more effective than TPA. As expected, indomethacin (3.4 x 10⁻⁶ M) and aspirin (6.0 x 10⁻⁵ M) inhibited TPA-stimulated production of 6-keto-PGF1α. The C-9 liver cells synthesize PGE2 and PGF2α as well as PGI2, but only 4% and 3% as much, respectively. Stimulation of arachidonic acid metabolism by TPA increased the synthesis of PGE2 and PGF2α to the same extent as that of 6-keto-PGF1α. Increased levels of 6-keto-PGF1α after TPA treatment were found also when measured after resolution by reverse-phase high-performance liquid chromatography. Stimulation of 6-keto-PGF1α production in the C-9 cells after addition of 30 nM TPA took 4 hr for completion (Chart 2). Thus, in most of our experiments, a TPA concentration of 0.3 x 10⁻⁷ M and a 4 hr incubation time were used; in most experiments, only the synthesis of 6-keto-PGF1α was measured.

Inhibition of protein synthesis by cycloheximide blocked the stimulation of 6-keto-PGF1α synthesis by TPA (Chart 3) as well as PGF2α production (not shown); these cycloheximide concentrations did not affect the cell viability as measured by trypan blue exclusion. The dose-dependent inhibition of TPA-stimulated 6-keto-PGF1α production and protein synthesis was parallel (Chart 3).

The C-9 cells, treated with the Ca²⁺ ionophore A-23187 (2 µM) for 1 hr, produced 20 to 50 times more 6-keto-PGF1α, showing that intracellular Ca²⁺ plays a role in 6-keto-PGF1α production. The requirement for Ca²⁺ for TPA-stimulated prostaglandin synthesis in C-9 rat liver cells was demonstrated with the use of reagents that inhibit Ca²⁺ function by different mechanisms. EGTA, which chelates Ca²⁺ ions in the extracellular medium, inhibited the TPA stimulation of 6-keto-PGF1α in a dose-dependent manner (Chart 4). The concentration of Ca²⁺ in the medium is 1.8 mM; 2 mM EGTA completely inhibited 6-keto-PGF1α production in our experimental conditions. At 4 hr, 2 mM EGTA did not affect 6-keto-PGF1α in the nonstimulated cells. Thus, the Ca²⁺ ion concentration is critical for TPA-induced prostaglandin synthesis.

The effects of 2 Ca²⁺ antagonists, nifedipine and TMB-8, are shown in Table 1. These 2 reagents interfere with the Ca²⁺ influx and mobilization of intracellular Ca²⁺, respectively (nifedipine blocks Ca²⁺ channels in the cell membrane, and TMB-8 inhibits the translocation of intracellular Ca²⁺ ions). Stimulation of 6-keto-PGF1α synthesis by TPA was inhibited by both nifedipine and TMB-8 in a dose-dependent way. When both Ca²⁺ antagonists were added at low concentrations, which by themselves had no
Table 1

Inhibition of TPA-stimulated 6-keto-PGF_{1\alpha} production by Ca^{2+} antagonists

<table>
<thead>
<tr>
<th>Nifedipine (m)</th>
<th>0</th>
<th>0.5 x 10^{-6} M</th>
<th>2.5 x 10^{-6} M</th>
<th>1.3 x 10^{-5} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of inhibition</td>
<td>TMB-8</td>
<td>TMB-8</td>
<td>TMB-8</td>
<td>TMB-8</td>
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<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.6 x 10^{-6}</td>
<td>0</td>
<td>27</td>
<td>34</td>
<td>48</td>
</tr>
<tr>
<td>2.9 x 10^{-6}</td>
<td>28</td>
<td>37</td>
<td>42</td>
<td>54</td>
</tr>
<tr>
<td>1.5 x 10^{-5}</td>
<td>62</td>
<td>70</td>
<td>69</td>
<td>72</td>
</tr>
</tbody>
</table>

* Data are expressed as the mean percentage of inhibition of 6-keto-PGF_{1\alpha} levels in culture fluids of triplicate dishes. The individual values agreed within 20% of the mean.

effect, they were found to act synergistically. At the concentrations used, these 3 Ca^{2+} antagonists did not affect basal 6-keto-PGF_{1\alpha} production, viability as measured by trypan blue exclusion, or protein synthesis when measured at 4 hr.

Antiinflammatory steroids are effective inhibitors of tumor promotion in vivo (12). They are effective inhibitors also of arachidonic acid metabolism in vivo and in cell culture (12). Dexamethasone inhibited stimulation of 6-keto-PGF_{1\alpha} synthesis by TPA in C-9 cells (Chart 5), even at nM levels (2.55 x 10^{-8} M dexamethasone inhibited the TPA-stimulated 6-keto-PGF_{1\alpha} synthesis by 50%). Inhibition by dexamethasone did not occur immediately; there was a lag of 45 to 60 min before inhibition was seen, and maximum inhibition was seen only after 6 hr (Chart 5).

**DISCUSSION**

Rat liver cells (C-9), like many other cells (12, 22), were stimulated by tumor promoters to metabolize arachidonic acid. These cells metabolize arachidonic acid by way of the cyclooxygenase, but not by the lipooxygenase, pathway; they produce 6-keto-PGF_{1\alpha}, PGF_{2\alpha}, and PGE_{2}. The synthesis of all 3 products was stimulated by TPA.

In this study, we have shown that inhibitors of protein synthesis, blockers of Ca^{2+} transport, and the antiinflammatory steroid dexamethasone inhibited the TPA-stimulated 6-keto-PGF_{1\alpha} production of C-9 cells. Inhibition of any of several enzymes could account for the effects of these drugs: (a) acylhydrolases, either a phospholipase A_{2} or a combined phospholipase C and diacylglycerol lipase attack; (b) cyclooxygenase; (c) prostaglandin hydrolase; and (d) prostacyclin synthetase, endoperoxide E isomerase, or endoperoxide F reductase. Since PGF_{2\alpha} and 6-keto-PGF_{1\alpha} both were inhibited by cycloheximide, EGTA, and dexamethasone, the block appears to be at or before the prostaglandin hydrolase reaction. Because the antiinflammatory steroids have been shown to block phospholipase activity in some cells (11, 12) and because TPA has been shown to stimulate acylhydrolase activity in a dog kidney cell line (5, 11, 12), it is reasonable to conclude that it is the acylhydrolase reaction that is being affected. The demonstration that Ca^{2+} is required for some phospholipase activities is consistent with this conclusion.

The identity of the protein the synthesis of which is being inhibited by cycloheximide and which is required for complete expression of TPA-stimulated PGI_{2} production is unknown. TPA-stimulated PGI_{2} production is particularly sensitive to regulation by this short-lived protein, since stimulation of PGI_{2} production by combined EGF and vasopressin treatment is not inhibited by a similar treatment with cycloheximide. Several proteins must be considered as possible short-lived protein(s).

**Acylhydrolase.** In dog kidney cells, as in C-9 cells, arachidonic acid metabolism was stimulated by TPA, and the TPA stimulation was inhibited by cycloheximide. TPA stimulated acylhydrolase activity in the dog kidney cells and cycloheximide inhibited this activity (13). Thus, the essential short-lived protein could be the acylhydrolase. If so, mechanisms of deacylation in TPA and EGF-vasopressin stimulation are different (only TPA stimulation was inhibited by cycloheximide).

**Growth Factor.** TPA could be inducing the synthesis of a growth factor which then stimulates prostaglandin production (13). The C-9 cells are stimulated to produce prostaglandins by EGF, platelet-derived growth factor, sarcoma growth factor, and interleukin 1.

**Regulator of Acylhydrolase.** Just as the antiinflammatory steroids induce the synthesis and/or activation of a protein that inhibits phospholipase A_{2} (6, 10), TPA could be inducing the synthesis and/or activation of a protein that stimulates an acylhydrolase.

**TPA Receptor.** The short-lived protein could also be the cell receptor for TPA, which, as a result of binding to TPA, is no

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[Diagram and tables are not directly transcribed due to the complexity of the content, but related figures and tables are referenced throughout the text.]
longer available; e.g., it is internalized. More receptor must be synthesized before TPA stimulation can continue.

Decaylation of cellular lipids may be causally related to tumor production, and the metabolism of arachidonic acid either by the lipoxygenase or cyclooxygenase pathway could secondarily amplify or inhibit the tumor-promoting processes.

REFERENCES

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