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Stimulation of Prostaglandin H Synthase mRNA Levels and Prostaglandin Biosynthesis by Phorbol Ester: Mediation by Protein Kinase C

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SUMMARY

We have investigated the mechanisms by which the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulates prostaglandin E_2 (PGE_2) formation in the rat tracheal epithelial cell line EGV-6aigT, which can be grown in serum-free medium. The addition of TPA to cells that were prelabeled with [^{3}H]arachidonic acid did not enhance the release of [^{3}H]arachidonic acid and/or [^{3}H]PGE_2, indicating that TPA does not stimulate phospholipase activity. The addition of exogenous arachidonic acid to cells pretreated with TPA resulted in increased PGE_2 formation, compared with basal levels, indicating an elevation in prostaglandin H synthase (PHS) activity. PHS activity was maximal at 4 hr and was dependent upon the concentration of TPA. Actinomycin D and cycloheximide blocked the TPA response. The recovery of PHS activity of cells in which the existing PHS was inhibited by aspirin was enhanced by TPA treatment. TPA treatment enhanced the expression of PHS mRNA, as measured by Northern analysis. The addition of actinomycin D and cycloheximide reduced the TPA enhancement of PHS mRNA, indicating that the increase in PHS activity required *de novo* RNA and protein synthesis. Furthermore, pretreatment of the cells with protein kinase C inhibitors reduced the TPA-dependent stimulation of PHS activity and the expression of PHS mRNA. The data suggest that TPA-stimulated *de novo* synthesis of PHS is mediated by protein kinase C.

Until recently, it was assumed that the hydrolysis of phospholipids by phospholipases A_2 and C was the rate-limiting step in the biosynthesis of various eicosanoids (1, 2). However, recent data suggest that metabolism of arachidonic acid, by PHS, to the prostaglandin endoperoxides from which the prostaglandins and thromboxane arise is also an important limiting step in eicosanoid biosynthesis. PHS is a membrane-bound glycoprotein, of M_r 67,000–70,000 (3–5), that has two enzymatic activities (3, 6). PHS catalyzes both the bis-oxygenation of arachidonic acid to PGG_2 (cyclooxygenase activity) and a reduction of PGG_2 to PGH_2 (hydroperoxidase activity) (3–5, 7). Both reactions catalyzed by PHS require heme for activity (3, 8), but only the cyclooxygenase activity is blocked by aspirin and other nonsteroidal anti-inflammatory drugs (9–11). However, the regulation and expression of PHS at the cellular level have been studied in a limited number of cells. The cloning of PHS has now provided the tool necessary to investigate the regulation of PHS in cells.

Many growth factors, such as EGF, PDGF, interleukin-1, and the tumor promoter TPA, are known to increase the synthesis of prostaglandins. Many of these agents are known to stimulate the release of arachidonic acid from phospholipids, but more recent data also indicate that some of the agents can enhance *de novo* synthesis of PHS (12–15).

Ohuchi and Levine (16) have shown that TPA has both early and late effects on prostaglandin synthesis in MDCK cells. The early response to TPA involves the release of arachidonic acid from phospholipids, subsequently leading to an increase of prostaglandin synthesis. Later, between 2 and 6 hr, a second effect of TPA occurs, and larger amounts of prostaglandins are produced by the cells. This suggests a stimulation of *de novo* synthesis of PHS by TPA. A recent paper by Wu *et al.* (15) demonstrated that TPA stimulates *de novo* synthesis of PHS in endothelial cells. In a previous study, using RTE cells we observed (17) that TPA enhanced prostaglandin formation and the data suggested that TPA may enhance *de novo* synthesis of PHS. Many of the pleiotropic responses to TPA appear to be mediated via the activation of PKC, a critical point in the signal transduction system (3, 8, 10, 11, 18). We wanted to determine whether TPA stimulates *de novo* synthesis of PHS

ABBREVIATIONS: PHS, prostaglandin H synthase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor; PKC, protein kinase C; PGH_2, prostaglandin H_2; PGG_2, prostaglandin G_2; PDGF, platelet-derived growth factor; TGF, transforming growth factor; FBS, fetal bovine serum; PGE_2, prostaglandin E_2; HPLC, high pressure liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RIA, radioimmunoassay; PGD_2, prostaglandin D_2; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; kb, kilobases; MOPS, 3-(*N*-morpholino)propanesulfonic acid; RTE, rat tracheal epithelial cells; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; DEPC, diethylpyrocarbonate.

in RTE cells and whether the response is mediated by PKC. In the present study, we have examined the mechanisms responsible for the stimulation of prostaglandin production by TPA. For this purpose, we used the rat tracheal epithelial cell line EGV-6aigT, which can be grown in serum-free medium in the absence of other growth factors that may have potential effects on arachidonic acid metabolism. We report here that TPA stimulates *de novo* synthesis of PHS and that this process appears to be mediated by activation of PKC.

Experimental Procedures

Materials. The following radioisotopes were purchased from New England Nuclear (Boston, MA): [5,6,8,9,11,12,14,15^{3}H]arachidonic acid (94.5 Ci/mmol), [^{3}H]PGE_2 (169.5 Ci/mmol), [^{32}P]CTP (800 Ci/mmol), and ^{3}H-labeled standards of arachidonic acid metabolites for HPLC and PGE_2 RIA. Arachidonic acid was purchased from Nu-Check Prep. Inc. (Elysian, MN). EGF and transferrin were obtained from Collaborative Research (Bedford, MA). Insulin, indomethacin, dexamethasone, aspirin, actinomycin D, cycloheximide, tamoxifen, ethidium bromide, and D-sphingosine were purchased from Sigma Chemical Co. (St. Louis, MO). FBS was from Armour Pharmaceutical Co. (Kankakee, IL), calcium ionophore A23187 from Calbiochem Boehringer Corp. (La Jolla, CA), TPA from Chemsyn Science Laboratories (Lenexa, KS), and staurosporine from Boehringer-Mannheim (Indianapolis, IN). TGF-α was from Peninsula Research. HPLC analysis of cell culture media was performed with HPLC-grade methanol, HPLC-grade water, and acetic acid from Fisher Scientific (Fair Lawn, NJ). HPLC-grade hexane was from Mallinckrodt (Paris, KY), 2-propanol and ethyl acetate from Baker Chemical Co. (Phillipsburg, NJ), and 100% ethanol from Grain Processing Corp. (Muscatine, IA). Ecolume from ICN Biomedicals, Inc. (Irvine, CA) was used as a scintillant.

RIA was performed with [^{3}H]PGE_2 from NEN and antiserum to PGE_2 from Advanced Magnetics, Inc. (Cambridge, MA). The assay buffer was prepared using Tris (Bethesda Research Laboratories, Inc., Rockville, MD), sodium azide (Sigma), gelatin (Fisher Scientific), and NaCl (Baker Chemical). Charcoal suspension was prepared using charcoal (Baker Chemical), dextran T70 (Pharmacia Fine Chemicals, Uppsala, Sweden), and bovine serum albumin (Reheis Chemical Co. Phoenix, AZ).

For Western immunoblots, polyclonal anti-PHS antibody raised in goats, by this laboratory, against ram purified seminal vesicle PHS (Oxford Biomedical Research, Oxford, MI) was used. Rabbit polyclonal anti-ram PHS antibody was a gift from Dr. L. Marnett, Vanderbilt University, whereas monoclonal mouse antibody against ram PHS was obtained from Oxford Biomedical Research. Rabbit anti-goat secondary antibodies, goat anti-rabbit IgG conjugated with horseradish peroxidase, goat anti-rabbit IgG, goat anti-mouse IgG, rabbit anti-goat IgG conjugated with horseradish peroxidase, and mouse anti-goat IgG conjugated with horseradish peroxidase were purchased from Cappel (West Chester, PA). Nitrocellulose and Nytran membranes were obtained from Schleicher & Schuell (Keene, NH).

SeaKem GTG agarose (FMS Bioproducts, Rockland, ME) and formaldehyde (Mallinckrodt) were used to make a gel for RNA separation. All solutions were prepared with DEPC-treated water.

Cell culture method. The transformed RTE cell line EGV-6aigT was grown in complete serum-free medium, which contained Ham's F-12 (GIBCO), 5 ng/ml EGF, 5 μg/ml transferrin, 5 μg/ml insulin, 0.1 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin, 50 μM ethanolamine and phosphoethanolamine, 0.8 mM $CaCl_2$, and 0.75 M HEPES, pH 7.3 (all from Sigma), 1.5 mg/ml bovine serum albumin (fraction V) from ICN, and bovine pituitary extract from Clonetics Corp., (San Diego, CA). Medium was changed every 3–4 days except as noted. Cells were maintained at 37° in humidified atmosphere of 5% CO_2 in air. Cells between passages 8 and 15 were used for all experiments.

Analysis of arachidonic acid metabolites by HPLC. Cells were plated in serum-free medium (minus pituitary extract and hydrocortisone) and cultured until they reached about 70–80% confluency. Then media were changed. (a) To study endogenous arachidonic acid metabolism, Ham's F-12 containing 10 μCi of [^{3}H]arachidonic acid/flask was added, and cells were incubated for 24 hr to label endogenous pools of arachidonic acid. The cells were washed to remove unincorporated arachidonic acid and then incubated for an additional 30 min or 3 hr with different agents. (b) To study exogenous arachidonic acid metabolism, cells were nutrient starved for 24 hr, followed by a 3-hr incubation with various agents. Then, 10 μCi of [^{3}H]arachidonic acid (12 nM) plus 10 μM arachidonic acid were added, and cultures were incubated an additional 30 min.

Arachidonic acid and its metabolites were extracted from incubation media by acidification to pH 3.0 with glacial acetic acid and application to a PrepSep-C_{18} (octadecyl) column. Methanol (100%) used to elute eicosanoids was evaporated in silated glassware, and residues were reconstituted in 30% methanol for HPLC. Recovery of the internal standard [^{14}C]PGE_2 during sample preparation was about 90%.

Arachidonic acid metabolites were separated by reverse phase HPLC, with a C_{18} Ultrasphere ODS column (Altex Scientific Inc., Beckman Instruments Inc., Berkeley, CA), using methanol and acetate buffered water (pH 5.05) at a flow rate of 1.1 ml/min, as described previously (19). PGE_2 and PGD_2 were subsequently separated using a normal phase system consisting of a μ-Porasil (10-μm) column (Waters Associates, Milford, MA) eluted with hexane/ethanol/acetic acid (994:6:1) for 25 min, followed by a linear gradient to 80% of hexane/ethanol/acetic acid (90:10:1) for an additional 60 min, at a flow rate of 3.0 ml/min (20).

RIA for PGE_2. EGV-6aigT cells grown in 24-well plates were subjected to 24-hr starvation (Ham's F-12 with no supplements). Then, various inhibitors or vehicle were added, followed by incubation with TPA. Subsequently, arachidonic acid was added for an additional 30 min. Media were collected, and the concentration of PGE_2 in the media was determined by RIA using specific antibody against PGE_2, as described previously (17).

Immunoblotting of PHS protein. EGV-6aigT cells grown in 150-cm^2 Integrid dishes until they reached about 70–80% confluency were nutrient starved for 24 hr, and then various compounds or vehicle were added, followed by incubation with TPA. Media were discarded, the cell monolayers on each dish were washed twice with PBS, and cells were scraped into 5 ml of 100 mM Tris-HCl, pH 8.0, containing 10 mM sodium diethyldithiocarbamate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 250 μM leupeptin (all from Sigma). Subsequently, cells were sonicated three times for 20 sec each time (on ice), and centrifuged for 20 min at 10,000 × *g*. The supernatant was then centrifuged for 1 hr at 100,000 × *g*. The microsomal pellet was resuspended in 1 ml of 100 mM phosphate buffer, pH 8.0, and the protein concentration was measured by the method of Lowry *et al.* (21). The microsomal protein (25–100 μg from each sample) was applied to a 7.5% polyacrylamide-SDS gel and electrophoretically separated, as described by Laemmli (22). Proteins were then electrophoretically transferred overnight to a nitrocellulose membrane, using a current of 300 mA, in Seprabuff buffer (Integrated Separation System, Hyde Park, MA). Subsequently, the blot was saturated with 3% bovine serum albumin for 15 min at 60°, and then the nitrocellulose was incubated either with the anti-PHS antibody (goat, rabbit, or mouse) at various dilutions or with nonimmune goat serum, at room temperature for 1 hr. After washing, the blots were incubated with appropriate secondary antibody diluted 1:100. After washing, the membrane preparation was treated with IgG conjugated with horseradish peroxidase (1:3000), at room temperature for 15 min, followed by blot treatment with diaminobenzidine solution for color development and visualization (23). In these experiments, ram seminal vesicle microsomes or purified PHS obtained from Oxford Biomedical (Oxford, MI) were used as a positive control.

Analysis of PHS gene expression. Cell cultures grown in serum-free medium in 150-cm^2 Integrid dishes were nutrient starved for 24 hr, followed by pretreatment with inhibitors or vehicle and subsequent

treatment with TPA. Media were then discarded, and cells were washed three times with ice-cold PBS, scraped into 10 ml/dish ice-cold PBS, and transferred to 50-ml polystyrene conical tubes, for centrifugation at 110 × *g* for 5 min at 4°. Total cytoplasmic RNA from treated or control cells was extracted by the method of Gilman (24). Briefly, the cell pellet was lysed with ice-cold lysis buffer for 5 min and centrifuged; the supernatant was transferred to microcentrifuge tubes containing 20% SDS, mixed, extracted twice with a phenol/chloroform/isoamyl alcohol (25:24:1) mixture and once with a chloroform/isoamyl alcohol mixture (24:1), and centrifuged. The aqueous phase, which was removed to a clean tube, was precipitated with sodium acetate and ethanol and incubated overnight in −20°. Finally, RNA was washed and resuspended in sterile DEPC-treated water. The yields of total cellular RNA were about 40–135 $\mu g/10^7$ cells, as quantified spectrophotometrically.

RNA (8 μg) was fractionated by electrophoresis in a denaturating formaldehyde/1.2% agarose gel. For each experiment, parallel gels were run, one of which was stained with ethidium bromide, for examination for any errors in loading or possible degradation of the RNA, and the other of which was transferred overnight to nitrocellulose or Nytran membranes, via capillary action in 10× SSC buffer. Filters were then baked for 2 hr at 80° and prehybridized for 2–4 hr at 42°. Subsequently, filters were hybridized (24) overnight to a 1.6-kb ram PHS RNA probe (Oxford Biomedical Research), which was labeled with [^{32}P]CTP by an *in vitro* transcription random priming kit obtained from Boehringer-Mannheim (Indianapolis, IN). The latter probe was a 1.6-kb *Eco*RI fragment of ovine PHS cDNA obtained from Oxford Biomedical Research.

In other experiments, poly(A$^+$) RNA was isolated, by the batch method, from cells that had been treated with F-12 medium and TPA as described above (25). Poly(A$^+$) RNA was fractionated in a denaturing formaldehyde/0.8% agarose/1× MOPS gel. The total volume of the gel was 100 ml and included 2 μl of ethidium bromide for visualization of the RNA. The samples were denatured by addition of formamide, formaldehyde, and MOPS and heating for 15 min at 55°. The gel electrophoresis was run at 80 V for 5 hr. The gel was transferred overnight, via capillary action in 10× SSC buffer, to a Nytran membrane. The filters were then baked for 2 hr at 80° and prehybridized for 2–4 hr at 42°. Subsequently, the filters were hybridized overnight to a PHS cDNA probe, which was labeled with [^{32}P]CTP (New England Nuclear-Dupont, Wilmington, DE) using the random priming label kit obtained from Boehringer-Mannheim. The probe contains a 1.6-kb *Eco*RI fragment of ovine PHS obtained from Oxford Biomedical Research. Then, the blot was prehybridized again and probed with β-actin from Oncor (Gaithersburg, MD), using the same ^{32}P and the same random priming labeling kit.

Results

Stimulation of arachidonic acid metabolism by TPA and other agonists. To characterize the major arachidonic acid metabolites produced by the EGV-6aigT cell line grown in serum-free medium, cells were first prelabeled for 24 hr by incubation with 10 μCi of [^{3}H]arachidonic acid. The cells were then washed and incubated with medium (control) or medium containing different agonists. Fig. 1 shows the reverse phase HPLC profile of arachidonic acid metabolites produced by TPA-stimulated and nonstimulated cultures. The major metabolite co-eluted with standards of PGE_2 and PGD_2. To further characterize this metabolite, the peak was analyzed by normal phase HPLC, which separates PGE_2 and PGD_2 (data not shown). In all cases, only one peak was detected, which coeluted with the PGE_2 standard. In addition to PGE_2, control cells also produced small but detectable amounts of a metabolite that coeluted with $PGF_{2\alpha}$.

Stimulation of the cultures with the calcium ionophore A23187 increased PGE_2 production about 50%. In addition,

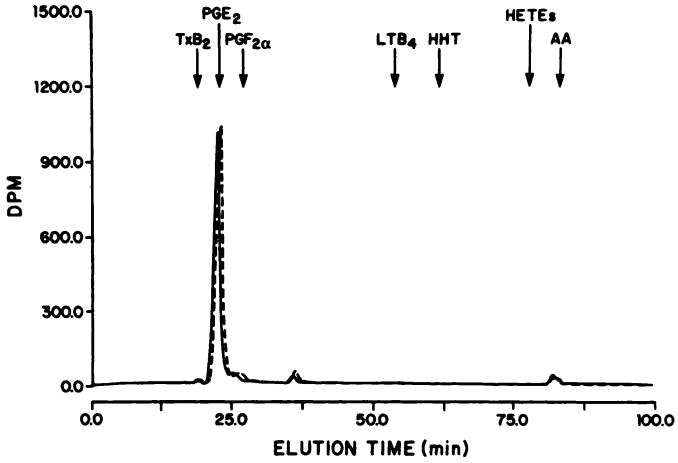

Fig. 1. Reverse phase HPLC analysis of the [^{3}H]arachidonic acid metabolites produced by EGV-6aigT cells treated with TPA. Cells at 70–80% confluence that were nutrient starved were incubated with [^{3}H]arachidonic acid (10 μCi/flask) for 24 hr. After the cells were washed, the cultures were incubated with medium containing 50 nM TPA or medium plus vehicle, for 30 min. The media were collected and analyzed. *Solid line*, control; *dashed line*, TPA-treated cells. *Arrows*, elution times of the standards. *TxB_2*, thromboxane B_2; *LTB_4*, leukotriene B_4; *AA*, arachidonic acid; HHT, 12-hydroxyheptadecatrienoic acid; HETE, hydroxy eicosatetraenoic acid.

small amounts of metabolites co-eluting with thromboxane B_2, HHT, and HETE(s) were detected in A23187-stimulated cultures (data not shown). Neither TPA (Fig. 1) nor other agents, such as EGF, TGFα, and FBS (data not shown), stimulated release and metabolism of arachidonic acid. This experiment shows clearly that TPA does not stimulate the release of arachidonic acid from membrane phospholipids in EGV-6aigT cells grown in serum-free medium.

TPA-dependent stimulation of PGE_2 production from exogenous arachidonic acid. The effect of TPA and other agents on the conversion of exogenous arachidonic acid was examined. Reverse phase HPLC analysis (data not shown) indicated that cells produced PGE_2 as the major eicosanoid from exogenous arachidonate. Thus, in all subsequent experiments, PGE_2 production was measured by specific RIA. The influence of different growth factors and TPA on PGE_2 production from exogenous arachidonic acid was also examined. TPA was the only agent that stimulated PGE_2 production from exogenous arachidonic acid. Serum and TGFα only slightly stimulated PGE_2 synthesis, whereas EGF, insulin, and transferrin did not alter PGE_2 formation (data not shown).

As shown in Fig. 2, the increase in PGE_2 formation from exogenous arachidonic acid was dependent upon the duration of TPA exposure. TPA-stimulated PGE_2 synthesis occurred after an apparent lag period of 2 hr. Maximum stimulation occurred after a 4-hr incubation with TPA. Subsequently, a slow decrease occurred, and PGE_2 synthesis reached control levels after a 12–24-hr incubation with TPA. As shown in Fig. 3, TPA stimulated PGE_2 production in a concentration-dependent manner. Nonstimulated cells produced 1.28 ± 0.11 ng of $PGE_2/10^5$ cells. The minimum effective concentration was 3 nM, which caused a 53% increase. Maximum stimulation, a 233% increase (up to 4.27 ± 0.39 ng of $PGE_2/10^5$ cells), was obtained with a 1 μM concentration of TPA. Higher doses had less pronounced effects, probably due to TPA toxicity.

PGE_2 production was abolished in control as well as in TPA-

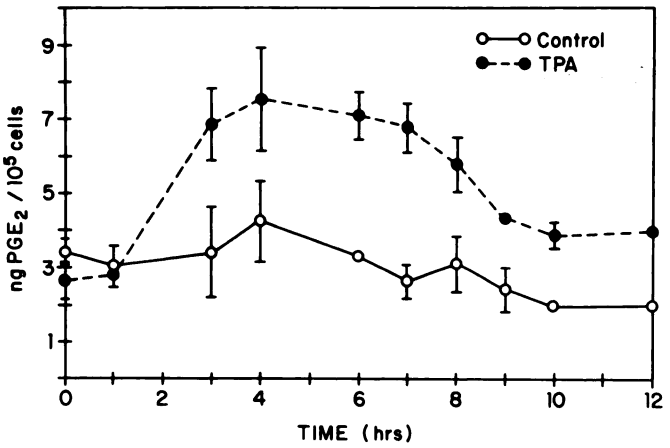

Fig. 2. Time-dependent stimulation of PHS activity by TPA. To nutrient-starved cells, 50 nM TPA was added, and the cells were incubated for the indicated period of time. Then, 10 μM arachidonic acid was added and the cells were incubated for an additional 30 min. The media were collected and analyzed for PGE_2 by RIA. Values are the mean ± standard deviation of three determinations.

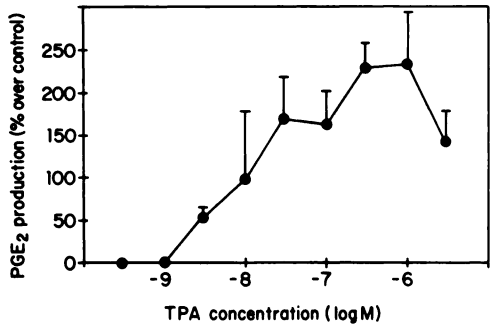

Fig. 3. Dependence of PHS stimulation on the concentration of TPA. Nutrient-starved cells were incubated for 4 hr with TPA at various concentrations, followed by an additional 30-min incubation with 10 μM arachidonic acid. The media were collected and analyzed for PGE_2 by RIA. Values are the mean ± standard deviation of three determinations.

stimulated cultures by indomethacin, an inhibitor of PHS (data not shown). Dexamethasone also blocked the TPA-induced increase in PGE_2 synthesis, in a concentration-dependent manner (data not shown).

TPA stimulation of PGE_2 production by increased *de novo* PHS synthesis. To determine whether the TPA enhancement of PGE_2 formation in EGV-6aigT cells was associated with *de novo* synthesis of PHS, the effects of inhibitors of transcription (actinomycin D) and of protein synthesis (cycloheximide) were tested. As shown in Table 1, both inhibitors blocked TPA-induced PGE_2 production, indicating that PGE_2 production was dependent on *de novo* synthesis of PHS. To obtain additional evidence for TPA induction of PHS synthesis, cells were pretreated with aspirin to inhibit any preexisting PHS and were then incubated with TPA, FBS, EGF, or combinations of the above. PGE_2 production was measured 2 and 4 hr after the addition of the agents listed above. Fig. 4 shows that PHS activity only partially recovered after a 4-hr incubation in the aspirin-treated cells in media containing EGF or FBS. However, cells incubated with TPA showed enhanced recovery of PHS activity after 2-hr treatment. A marked enhancement of the PHS activity (about 3-fold) occurred following a 4-hr incubation with TPA. Combinations of TPA and

TABLE 1

Influence of transcriptional and translational inhibitors on PGE_2 production stimulated by TPA

Nutrient-starved cells were pretreated for 30 min with the inhibitor, followed by an incubation with TPA for 4 hr. Then, 10 μM arachidonic acid was added and the cells were incubated for an additional 30 min. Media were removed and analyzed by RIA. Numbers in parentheses are numbers of determinations.

	Treatment	PGE_2 production
		ng/10⁵ cells
A.	None	1.78 ± 0.15 (11)
	Actinomycin D (10 μg/ml)	1.96 ± 0.22 (3)
	TPA (50 nM)	3.17 ± 0.36 (12)
	Actinomycin + TPA	1.70 ± 0.04 (3)
B.	None	1.70 (2)
	Cycloheximide (10 μg/ml)	1.47 ± 0.10 (3)
	TPA (50 nM)	3.83 ± 0.29 (3)
	Cycloheximide + TPA	1.53 ± 0.12 (3)

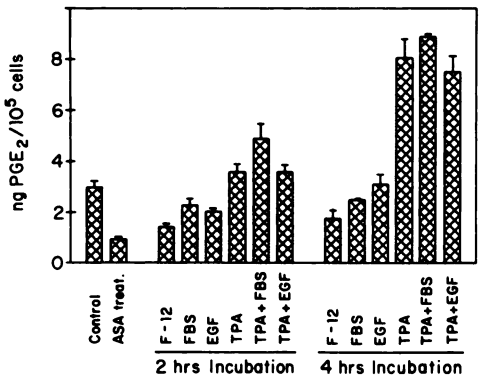

Fig. 4. Recovery of PHS activity in aspirin-treated cells: effect of TPA, EGF, and FBS. Nutrient-starved cells were pretreated with 300 μM aspirin for 30 min, to inhibit preexisting PHS. The cells were then treated with the agents listed, for 2 or 4 hr, followed by the addition of 10 μM arachidonic acid and a 30-min additional incubation. *F-12*, medium alone; *FBS*, treatment with medium containing 5% FBS; *ASA treat.*, aspirin treatment. The media were removed and analyzed for PGE_2 by RIA. Values are the mean ± standard deviation of three determinations.

TABLE 2

Influence of PKC inhibitors on PGE_2 production stimulated by TPA

Nutrient-starved cells were pretreated for 30 min with the PKC inhibitor, followed by a 4-hr incubation with TPA or vehicle. Then, 10 μM arachidonic acid was added for an additional 30 min. Media were removed and analyzed by RIA. Values are the mean ± standard deviation of three determinations.

	Treatment	PGE_2 production
		ng/10⁵ cells
A.	None	1.64 ± 0.06
	Staurosporine (100 nM)	1.45 ± 0.09
	TPA (50 nM)	3.17 ± 0.36
	Staurosporine + TPA	1.68 ± 0.12
B.	None	1.23 ± 0.12
	Sphingosine (20 μM)	0.84 ± 0.04
	TPA (50 nM)	3.16 ± 0.62
	Sphingosine + TPA	1.31 ± 0.08

EGF or of TPA and FBS did not further increase stimulation of PHS activity.

It is well established (3, 8, 10, 11) that many TPA responses are mediated by PKC. To test whether PKC is involved in TPA stimulation of *de novo* synthesis of PHS, cell cultures were preincubated with staurosporine or sphingosine (PKC inhibitors) and then treated with TPA. As shown in Table 2, both PKC inhibitors had no effect on basal levels of PHS activity but abolished the TPA-stimulated increase in PHS activity.

These data indicate that PKC is involved in the stimulation of *de novo* synthesis of PHS by TPA.

Western analysis of PHS. To obtain direct evidence that TPA induces *de novo* synthesis of PHS, attempts were made to show an increase in PHS protein levels, by using immunochemical techniques. Isolated microsomal proteins were fractionated by SDS-polyacrylamide gel electrophoresis, electroblotted on nitrocellulose filters, and treated with antibodies against ram PHS. Three different antibodies were used, commercially available mouse anti-PHS, a rabbit anti-PHS, and a goat anti-PHS. Our attempts, however, were unsuccessful, because none of the antibodies cross-reacted with the enzyme isolated from EGV-6aigT cells. Enzyme isolated from Syrian hamster embryo fibroblasts, which also have PHS activity, did react with the antibodies (data not shown). Thus, it was not possible to directly show an increase in PHS protein levels.

Analysis of PHS gene expression. Treatment of the cells with TPA enhanced the expression of PHS mRNA, as measured by Northern analysis. As seen in Fig. 5, the level of PHS mRNA (2.8 kb) was higher in the RNA obtained from cells treated for 4 hr with TPA than the RNA from control cells. In contrast, the level of actin probe hybridization was essentially the same for TPA and control cells. Thus, TPA enhanced the expression of the 2.8-kb PHS mRNA. A mRNA of >2.8 kb hybridized with the PHS cDNA probe and was apparent in this sample prepared from the TPA-treated cells, as shown in Fig. 5. The appearance of the larger form of PHS mRNA was unpredictable; it was a random event that occurred with both control and TPA-treated samples. A >2.8 kb mRNA for PHS has been observed by other workers.[1]

Northern analysis of PHS mRNA levels obtained from cells pretreated with various inhibitors, followed by TPA stimulation, was performed. Fig. 6 summarizes these results. TPA treatment enhanced the levels of PHS mRNA by approximately 4-fold. Actinomycin D and cycloheximide blocked the induction

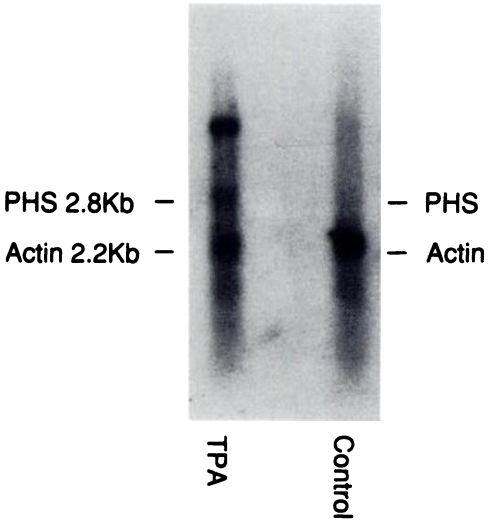

Fig. 5. Stimulation of PHS mRNA by TPA. Nutrient-starved cells were incubated with either medium or medium containing 50 nM TPA, for 4 hr. The poly(A$^+$) RNA was isolated and purified as described in Experimental Procedures. The RNA was separated on a formaldehyde/agarose gel, transferred to Nytran, and hybridized first with a 1.6-kb PHS cDNA probe labeled with [^{32}P]CTP. The Nytran was later rehybridized with an actin probe labeled with [^{32}P]CTP.

[1] K. Seibert-Boyd, personal communication.

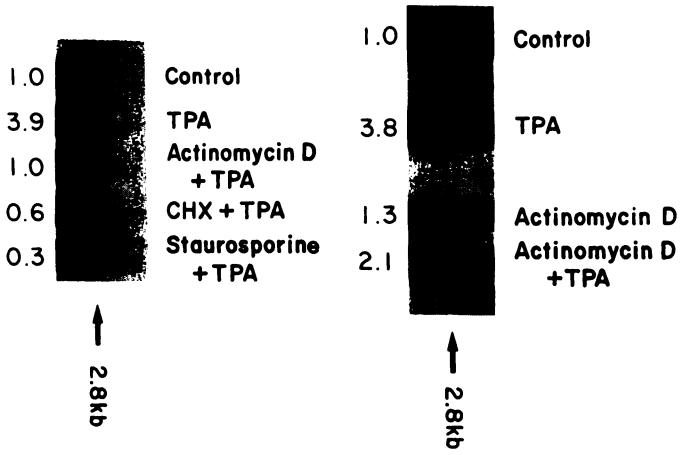

Fig. 6. Northern analysis of PHS mRNA in cells treated with TPA: the effect of inhibitors on expression. Nutrient-starved cells were pretreated for 30 min with inhibitors of protein synthesis, RNA synthesis, or PKC and were then incubated with TPA for 4 hr. The RNA was isolated and purified as described in Experimental Procedures. The RNA (8 μg) was separated on a formaldehyde/agarose gel, transferred to Nytran, and hybridized with a 1.6-kb PHS RNA probe labeled with [^{32}P]CTP. Northern blots were quantitated by densitometry, with controls taken as 1.0. The *numbers to the left* of the blots are the results of these measurements and are expressed as fold increases over the respective control. Concentrations used: TPA, 50 nM; actinomycin D, 10 μg/ml; cycloheximide (*CHX*), 10 μg/ml; staurosporine, 100 nM.

of PHS mRNA, indicating that both transcription and translation are required. The mRNA levels were reduced to control levels or below. The addition of the PKC inhibitors staurosporine (Fig. 6) and sphingosine (data not shown) also reduced the TPA-induced increase in PHS mRNA levels. These data support the conclusions drawn from the biochemical measurements described above, namely that the enhancement of *de novo* synthesis of PHS by TPA involves activation of PKC.

Discussion

The main purpose of the investigations presented here was to determine the mechanisms responsible for the stimulation of PGE_2 production by TPA. For that purpose, we used the EGV-6aigT cell line, because it can grow in serum-free medium in the absence of other growth factors, which have the potential to affect arachidonic acid metabolism. The initial studies showed that PGE_2 was the major eicosanoid produced by this cell line from endogenous as well as exogenous arachidonic acid. Neither TPA nor other agents used stimulated the release and subsequent metabolism of endogenous arachidonic acid. However, when the metabolism of exogenous arachidonic acid was explored, thus bypassing the phospholipase step, TPA was the only agent that stimulated PGE_2 production. The use of exogenous arachidonic acid permits an estimation of the PHS activity in cells and, thus, the data suggest that TPA is acting either by activating PHS or by increasing *de novo* synthesis of PHS in cells.

Our experimental observations indicate induction of *de novo* synthesis of PHS by TPA in EGV-6aigT cells. First, the time course of PGE_2 synthesis from exogenous arachidonic acid showed a 2-hr lag period, which is in agreement with *de novo* synthesis of PHS. Second, inhibitors of transcription and protein synthesis blocked PHS activity stimulated by TPA. Third, in cells pretreated with aspirin, an irreversible inhibitor of PHS

that inhibits any preexisting PHS, the PHS activity recovered completely after a 2-hr incubation with TPA and showed enhanced PGE_2 production after a 4-hr incubation with TPA, when compared with the control. Fourth, Northern analysis of PHS mRNA showed an increase in PHS transcripts after incubation with TPA, which was blocked by actinomycin D and cycloheximide.

The most direct evidence that TPA induces *de novo* synthesis of PHS would have been estimation of the TPA-dependent increase in PHS protein. However, this was not possible to obtain, because the three antibodies available to us, namely a mouse monoclonal and two polyclonal antibodies raised against ram PHS in goats or rabbits, did not cross-react with PHS obtained from the RTE cells. At low dilutions of the antibodies, cross-reaction was observed with a microsomal protein of M_r 68,000, but nonimmune sera also cross-reacted (data not shown). Recently, Lin *et al.* (26) reported that PDGF increased PHS mRNA levels in NIH 3T3 cells but did not stimulate *de novo* synthesis of PHS. This difference between the data of Lin *et al.* and our work either is due to differences in the mechanism of action of PDGF and TPA or is indicative of the differences in the biochemical mechanisms of arachidonic acid metabolism in fibroblasts and epithelial cells.

Several recent studies demonstrated that prostaglandin production is dependent on protein synthesis (13, 16, 27–30), and it has been suggested that in some tissues the principal mechanism for the regulation of prostaglandin formation may depend upon resynthesis of PHS after autoinactivation, which occurs rapidly following conversion of PGG_2 to PGH_2 (31–33). Induction of PHS by various bioactive compounds has been described in several different cell types, by EGF in human amnion cells (12), TGF-β in mouse osteoblastic cells (34), interleukin-2 in human endothelial cells (35), interleukin-1 in human dermal fibroblasts (14), PDGF in Swiss 3T3 cells (13), and TPA in human endothelial cells (15). TPA directly activates PKC, a Ca^{+2}- and phospholipid-dependent protein serine/threonine kinase that is an enzyme of central importance in signal transduction (36). Subsequent studies have shown that PKC is also the major cellular receptor for TPA (37–40). TPA might exert its stimulatory effects on PGE_2 production by the PKC pathway. The PKC inhibitors sphingosine and staurosporine abolished the TPA stimulatory effect on PGE_2 formation as well as on PHS mRNA accumulation. The fact that cycloheximide inhibits TPA stimulation of PHS mRNA suggests a requirement for protein synthesis, which directly or indirectly increases PHS gene transcription. Similar inhibition by cycloheximide of the TPA-enhanced mRNA level for TGF-α was reported by Raymond *et al.* (41). On the other hand, Ben-Ze'ev and Raz (42) have shown that cycloheximide may disrupt the intermediate filament protein vimentin, thus disrupting the organization of the cell cytoskeleton, including endoplasmic reticulum and the Golgi apparatus, which would result in inhibition of protein synthesis. As a further result, vimentin RNA level decreases. It is possible that vimentin plays a regulatory role at the macromolecular level. Its inhibition could affect the production of other proteins, including the one required for PHS gene expression. Our findings may be related to the recently reported observation that dexamethasone inhibits EGF-dependent *de novo* synthesis of PHS, a process that also was found to require protein synthesis (43). Our results suggest that TPA stimulates *de novo* synthesis of PHS in the EGV-6aigT cell line grown in serum-free medium and that this process is mediated by PKC activation.

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