8-26-1994

Epidermal growth factor coordinately regulates the expression of prostaglandin G/H synthase and cytosolic phospholipase A2 genes in embryonic mouse cells.

Kenneth P. Chepenik
Thomas Jefferson University

Arturo Diaz
Thomas Jefferson University

Sergio A. Jimenez
Thomas Jefferson University, Sergio.Jimenez@jefferson.edu

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 34, Issue of August 26, pp. 21786–21792, 1994
Printed in U.S.A.

Epidermal Growth Factor Coordinately Regulates the Expression of Prostaglandin G/H Synthase and Cytosolic Phospholipase A_2 Genes in Embryonic Mouse Cells*

(Received for publication, May 3, 1994, and in revised form, June 16, 1994)

Kenneth P. Chepenik‡§, Arturo Diaz¶, and Sergio A. Jimenez¶

From the Department of ‡Anatomy and ¶Rheumatology Division, Department of Medicine, Jefferson Medical College of Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Confluent, primary cultures of mouse embryo palate mesenchyme (MEPM) cells are refractory to activation of phospholipase A_2 (PLA_2) by the calcium ionophore A23187. However, treatment of these cultures with epidermal growth factor (EGF) permits the cells to activate PLA_2 in response to A23187. We have developed this finding by exploring molecular mechanisms by which growth factors modulate mobilization and metabolism of arachidonic acid. We found chronic treatment (>6 h) of confluent MEPM cells with EGF (*a*) increases their ability to metabolize exogenous arachidonic acid to prostaglandin E_2 (PGE_2) and (*b*) stimulated constitutive expression of activities of PLA_2 and cyclooxygenase (CyOx). Immunoprecipitation of [^{35}S]proteins and Western blot analysis revealed EGF treatment stimulated synthesis and accumulation of PLA_{2c}, CyOx-1, and CyOx-2. Northern hybridization analysis revealed EGF increased the steady-state levels of a transcript for the high molecular weight, cytosolic PLA_2 (PLA_{2c}), and both the 2.8- and 4.2-kb transcripts for CyOx-1 and CyOx-2, respectively. *In vitro* nuclear transcription assays showed a parallel increase in the transcription rate of the genes corresponding to CyOx-1 and PLA_{2c}, but not CyOx-2, in response to EGF. Treatment with EGF had no effect on either synthesis of the low molecular weight, group II PLA_2, accumulation of its transcript, or the transcription rate of its gene. Coordinate regulation of activities of PLA_2 and CyOx in response to EGF did not parallel the mitogenic effects of EGF on confluent MEPM cells.

The cellular synthesis of PGs[1] involves mobilization and metabolism of precursor fatty acids (primarily arachidonic acid) from intracellular stores (reviewed in Ref. 1). The primary enzyme involved in mobilization of arachidonic acid appears to be a PLA_2, though some arachidonic acid may be released from phospholipids by sequential hydrolysis via phospholipase C and diglyceride lipase. Unesterified arachidonic acid is metabolized to the PG hydroperoxide PGG_2 and then to the endoperoxide PGH_2 by the same enzyme complex termed PG G/H synthase (E.C. 1.12.99.1; commonly referred to as cyclooxygenase). Therefore, the key enzymes involved in regulating synthesis of PGs are PLA_2 and CyOx (reviewed in Refs. 1, 2).

Mammalian cells appear to contain at least three classes of PLA_2: Group I (PLA_{2I}), low molecular mass (<30 kDa) enzymes which are found in digestive organs, such as pancreas, and are structurally homologous with PLA_2 found in Elapidae venom (3); Group II(PLA_{2II}), low molecular mass (<30 kDa) enzymes which are widely distributed throughout the tissues and cells of the body and have a structure in common with that of Crotalidae venom, where the Cys^{11}-Cys^{77} disulfide bond of PLA_{2I} is missing and replaced by a Cys^{50}-Cys^{132} disulfide bond (3); and the recently purified (4–6) high molecular mass (>60 kDa), cytosolic PLA_2 (PLA_{2c}) which may not be classified as either Group I or II. The extent to which the PLA_{2II} and PLA_{2c} regulate mobilization of arachidonic acid destined for metabolism to PGs is not clear. PLA_{2II} may be secreted from cells and has been implicated as playing a critical role in inflammation (reviewed in Ref. 7). PLA_{2c} is not secreted from cells and, unlike PLA_{2II}, is highly specific for phospholipids which contain arachidonic acid in the *sn*-2 position (5, 8–11), making it a candidate regulatory enzyme in synthesis of eicosanoids (4, 12).

We found recently that activities of PLA_2 may be stimulated readily by treatment of growing cultures of MEPM cells with A23187 (13). In contrast, confluent cultures of MEPM cells were quite refractory to activation of PLA_2 by A23187 unless they were treated briefly with EGF or phorbol ester (13). These findings prompted the hypothesis that regulation of mobilization and metabolism of arachidonic acid might be part of the signaling pathway by which EGF modulated development of the palate. We have explored this hypothesis by determining molecular mechanisms by which EGF (and $TGF_{\beta 1}$) might modulate mobilization and metabolism of arachidonic acid by MEPM cells over an extended period of time. MEPM cells have receptors for both (14–16) of these growth factors, which have have been shown to interact in regulation of metabolism of arachidonic acid in other cellular systems (17).

MATERIALS AND METHODS

Murine Palatal Cell Culture—Mature A/J strain mice (Jackson Laboratories, Bar Harbor, ME) were mated overnight, and the presence of a vaginal plug the following morning (first day of gestation) was considered evidence of mating. Embryos were obtained on the 15th day of gestation and dissected from uteri in sterile phosphate-buffered saline. This developmental stage was chosen as it is a time when it is possible to obtain the maximum number of embryonic palate mesenchyme cells from palates still undergoing organogenesis. Palatal processes dissected from the embryos were minced and then dissociated to produce a suspension of single cells that was seeded into 8-cm^2 culture dishes

* This work was supported by National Institute of Dental Research Grant DE 6553 (to K. P. C.), National Heart, Lung, and Blood Institute Grant HL41214 (to S. A. J.), and the Dean's Overage Research Program of Jefferson Medical College (to K. P. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Anatomy, Jefferson Medical College of Thomas Jefferson University, 1020 Locust St., Philadelphia, PA 19107.

[1] The abbreviations used are: PGs, prostaglandins; CyOx, cyclooxygenase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; GAGs, glycosaminoglycans; HPLC, high pressure liquid chromatography; MEPM, mouse embryo palate mesenchyme; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PLA_2, phospholipase A_2; $TGF_{\beta 1}$, transforming growth factor $beta_1$; cpm, counts/min; kb, kilobase(s).

using routine laboratory procedures (18–20) and grown to confluence in DMEM (high glucose) that contained 10% fetal bovine serum, 2 mM glutamine, 100 μg of streptomycin/ml, 100 units of penicillin/ml, and 0.25 μg of Fungizone/ml (complete DMEM) (18). The cells were then washed with serum-free medium and incubated 24 h in defined medium (19) which consisted of DMEM/Ham's F-12 (50:50, v/v), 1 mg of fetuin/ml, antibiotics and antimycotics as above, and ITS+ (6.25 μg of insulin, 6.25 μg of transferrin, 6.25 ng of selenous acid, 5.35 μg of linoleic acid, and 1.25 mg of bovine serum albumin/ml; Collaborative Research, Lexingham, MA), prior to beginning any treatment regimen. At the end of 24 h, the medium was removed, and the cells incubated in defined medium alone, or defined medium which contained EGF (Collaborative Research) and/or $TGF_{\beta 1}$ (Collaborative Research) at various concentrations. All cell cultures were maintained at 37 °C in a humidified incubator which contained an atmosphere of 5% CO_2, 95% air.

Determination of Cellular Proliferation—To measure the effects of EGF on cellular proliferation, incorporation of [*methyl*-^{3}H]thymidine (20 Ci/mmol; DuPont-NEN) into the acid-insoluble fraction of cellular extracts was determined. Essentially, confluent cultures of MEPM cells were incubated for either 24, 48, or 72 h in defined medium alone or in medium which contained 20 ng of EGF/ml. This concentration of EGF was found to be as effective as 10% fetal bovine serum in supporting growth of subconfluent cultures of MEPM cells in defined medium (19). Five μCi of [^{3}H]thymidine were added per milliliter of culture medium for the last 3 h of each 24-h incubation period. The medium was then removed and the cells precipitated over ice for 1 h by addition of 1 ml of fresh, cold (4 °C) 10% trichloroacetic acid to the culture dish. The cells, in 10% trichloroacetic acid, were scraped into Eppendorpf microfuge tubes, stored overnight at 4 °, and then pelleted by centrifugation for 30 s in a microfuge, sonicated to homogeneity in fresh, cold 10% trichloroacetic acid, and washed two times with fresh 10% trichloroacetic acid. The washed precipitates were neutralized with 1 N NaOH and their radioactivities determined by scintillation spectrometry.

Assay for Activities of CyOx and PLA_2—MEPM cells cultured in 8-cm^2 dishes were superfused 15 min with 13 μM [1-^{14}C]arachidonic acid (0.1 μCi/ml; 40–60 mCi/mmol, DuPont-NEN) in defined medium plus 3 mg of defatted bovine serum albumin/ml in order to measure their capacity to metabolize arachidonic acid (total activity of CyOx). The medium was extracted into ethyl acetate according to Flower *et al.* (21) and chromatographed with authentic PGs and arachidonic acid on thin layer chromatography plates in the organic phase of ethyl acetate/isooctane/acetic acid/water (55:25:10:50, v/v). Radiolabeled lipids were located by fluorography, scraped into individual scintillation vials, and their radioactivities determined by scintillation spectrometry. Correction for quench was by the external standards ratio method. In some cases the nature of the radiolabeled product was confirmed by HPLC. In this case, medium which had been purified and concentrated over a C_{18} column was injected, in ethanol, onto an Ultrasphere ODS C_{18} column equipped with a precolumn. Radiolabeled arachidonic acid and its metabolites were separated in a solvent gradient of 0.1% aqueous acetic acid (Solvent A) and acetonitrile (Solvent B) programmed to increase from 32% B to 90% B over a period of 102 min. Radioactivity in the column effluent was detected on-line (model 171 radioisotopic detector, Beckman Instruments, Fullerton, CA). This procedure allows separation of arachidonic acid and 30 of its metabolites one from the other (22).

In order to determine the ability of MEPM cells to mobilize arachidonic acid from endogenous pools and metabolize it to PGs, cells were grown to confluence in complete DMEM and then incubated 24 h in complete DMEM which contained 0.5 μCi/ml of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (60–100 Ci/mmol; DuPont-NEN). This procedure results in incorporation of [^{3}H]arachidonic acid into the *sn*-2 position of all major classes of phospholipids in MEPM cells (23). The medium was then removed and the cells washed three times with warm (37 °C), defined medium which contained 3 mg of defatted bovine serum albumin/ml. The cells were then incubated overnight in defined medium prior to treatment with EGF or $TGF_{\beta 1}$. At the end of the treatment period the medium was collected, extracted into organic solvent, and subjected to thin layer chromatography as described above. The cells were then stimulated with 10 μM A23187 in HEPES-buffered saline (5 mM $CaCl_2$, 0.49 mM $MgSO_4$, 0.373 mM KCl, 0.137 mM NaCl, 3 mg of defatted bovine serum albumin, 20 mM HEPES, pH 7.4). Cellular lipids were extracted into organic solvent according to Bligh and Dyer (24) except the methanol contained 2% acetic acid. Phospholipids were separated from each other by chromatography on Whatman KD6 plates (Fisher) in $CHCl_3/CH_3OH/CH_3COOH/H_2O$, 50:25:8:2 (v/v), along with authentic standards; neutral lipids were separated by chromatography on Whatman KD5 plates in $CHCl_3/CH_3OH/CH_3COOH$, 98:2:1 (v/v). The chromatogram was visualized by staining with I_2 vapors, and the individual lipids were each scraped into a scintillation vial and their radioactivities determined as described above.

Immunoprecipitation of [^{35}S]Proteins—In order to radiolabel proteins synthesized in response to various treatments, cells were incubated in cystine/methionine-free medium for the last 15 min of the treatment period, and then incubated 2 h in cystine/methionine-free medium to which 100 μCi of [^{35}S]methionine/[^{35}S]cystine (>1000 Ci/mmol; Trans ^{35}S-Label™, ICN Biomedicals, Costa Mesa, CA) were added/ml. The cells were then washed and lysed in 1 ml of 50 mM HEPES, pH 7.5, which contained 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 4 μg of leupeptin, and 10 μg of aprotinin. Twenty μl of lysate were precipitated with 10% trichloroacetic acid and the amount of radioactivity determined by scintillation spectrometry. An aliquot of lysate equivalent to 10^6 cpm as trichloroacetic acid-insoluble material was then immunoprecipitated essentially according to Margolis *et al.* (25). Briefly, the sample was incubated at 4 °C for 1 h with normal rabbit serum, then 1 h with Protein A-Sepharose, and then centrifuged 5 min at 10,000 × *g* to produce a clarified supernatant. The clarified supernatant was then incubated 90 min at 4 °C with either rabbit anti-sheep seminal vesicle CyOx ((26) gift of Dr. William Smith, Biochemistry, Michigan State University, East Lansing, MI), rabbit anti-human PLA_{2c} (gift of Dr. James Clark, Genetics Institute, Cambridge, MA), or rabbit anti-human recombinant synovial fluid PLA_{2II} (gift of Dr. Lisa Marshall, Smith Kline, Beecham, King of Prussia, PA), and then 1 h with Protein A-Sepharose (Sigma). This procedure was repeated sequentially with each of the antibodies of interest so as to obtain a measure of the effects of EGF and/or $TGF_{\beta 1}$ on multiple proteins extracted from a single population of cells. The Protein A-Sepharose-antibody-antigen complex was collected by centrifugation, suspended in a small amount of SDS-PAGE sample buffer, and boiled 5 min to solubilize proteins. CyOx and PLA_{2c} were resolved by electrophoresis on 12% gels, and PLA_{2II} was resolved by electrophoresis on 20% gels. The gels were then impregnated with Autoflour (National Diagnostics, Atlanta, GA), dried under vacuum, and visualized by fluorography. Controls consisted of Protein A-Sepharose-protein complexes obtained with normal rabbit serum as well as samples to which microgram quantities of unlabeled, authentic antigen had been added.

Immunoblots (Western Blots)—MEPM cells treated with EGF and/or $TGF_{\beta 1}$ were extracted into SDS-PAGE buffer, separated by electrophoresis on 12% gels, and then blotted onto supported nitrocellulose (MSI, Westboro, MA) according to Towbin *et al.* (27). The blots were then masked with 0.1% Tween 20 and incubated with appropriate dilutions of specific antibodies. Rabbit anti-murine CyOx-2 antibody was obtained from Cayman Chemical Co. (Ann Arbor, MI). Antigen-antibody complexes were visualized using alkaline phosphatase-linked ABC reagents commercially available from Vector Laboratories (Vectastain ABC kit, Vector Laboratories, Burlingame, CA).

Northern Blot Analysis—Cells were grown to confluence in T-75 flasks and treated 24 h with or without growth factors. They were then lysed in 4 M guanidinium isothiocyanate, 5 mM sodium citrate, pH 7.0, 0.5% Sarkosyl, and 0.1 M β-mercaptoethanol. Total RNA was isolated by the discontinuous $CsCl_2$ gradient as described in Sambrook *et al.* (28). Ten μg of total RNA/sample was denatured in formaldehyde and subjected to electrophoresis in 0.8% agarose, 6% formaldehyde gels in 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0. Equal loading of RNA was verified by visual inspection of ethidium bromide-stained ribosomal RNA. RNA was transferred in a vacuum blotting system (LKB 2016 Vacugene, Piscataway, NJ) to a supported nitrocellulose membrane in 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) and cross-linked to the membrane by UV irradiation (UV Stratalinker, Stratagene, La Jolla, CA). Membranes were prehybridized overnight at 42 °C in 50% formamide, 5 × concentrated Denhardt's solution (0.5 g of Ficoll 400, 0.5 g of polyvinylpyrrolidone, 0.5 g of bovine serum albumin, in 500 ml of H_2O), 5 × SSPE (1.8 M NaCl, 0.1 M $NaHPO_4$, 0.01 M EDTA), 0.1% SDS, and 200 μg of salmon sperm DNA/ml of solution. The membranes were then hybridized overnight in the same solution to which was added the appropriate cDNA radiolabeled with [α-^{32}P]dCTP by nick translation (29) to specific activities higher than 10^8 cpm/μg DNA. The cDNAs employed were: an 800-base pair *Eco*RI/*Pst*I cDNA fragment of rat type II PLA_2 (PLA_{2m}; gift of J. Ishizaki, Shionogi & Co., Ltd., Osaka, Japan; (30)), a 1.8-kb *Eco*RI cDNA fragment of mouse CyOx I (gift of Dr. David DeWitt, Biochemistry, Michigan State University, East Lansing, MI; (31)), a 4.2-kb *Eco*RI cDNA for mouse CyOx-2 (gift of Dr. Dean Kujubu (32)), a 2.4-kb *Eco*RI cDNA fragment of mouse PLA_{2c} (gift of Dr. James Clark, Genetics Institute, Cambridge, MA (4)), and a 600-base pair *Eco*RI/*Bam*HI cDNA fragment of human actin. After hybridizations the blots were washed at 55 °C in decreasing concentrations of SSC,

with a final wash in 0.25 × SSC, 0.1% SDS. Autoradiographs prepared by exposing the blots to X-omat-AR film were quantified by scanning laser densitometry.

In Vitro Nuclear Transcription Assay—The transcription rates of PLA_2 and CyOx genes were determined by the *in vitro* nuclear run-off assay (33). For this purpose, MEPM cells obtained as described above were cultured in T-175 flasks and incubated in the presence or absence of EGF (20 ng/ml) for 24 h. At the end of the incubation period, cell layers were trypsinized, nuclei prepared as described previously (34), and stored at −70 °C until used. The transcription reactions were carried out in a volume of 200 µl of 10 mM Tris, pH 8.0, 90 mM KCl_2, 3 mM $MgCl_2$, 2 mM dithiothreitol, 1 unit of RNasin Ribonuclease Inhibitor (Promega, Madison, WI)/ml, 0.4 mM each of ATP, UTP, and GTP, and 0.5 mCi [α-^{32}P]CTP (DuPont-NEN, 800 Ci/mmol). Incubations were for 25 min at 25 °C, and incorporation of [α-^{32}P]CTP was followed by trichloroacetic acid precipitation of 1-µl aliquots. Transcription was terminated by the addition of 900 µl of a buffer containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM KCl_2, 1 mM EDTA, and 0.5% SDS. To each sample 100 µg of yeast tRNA and 100 µg of proteinase K were added per ml, digested for 60 min at 42 °C, extracted with phenol/chloroform, and precipitated in 10% trichloroacetic acid and 10% (v/v) saturated sodium pyrophosphate. The pellets were washed with 70% ethanol, dried, and dissolved in 100 µl of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1% SDS. An additional 100 µg of yeast tRNA was added, the solution made 2.5 M in ammonium acetate, and the nucleic acids precipitated by addition of 100% ethanol. The pellets were dissolved in 100 µl of a buffer containing 20 mM Tris-HCl, 10 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.5, and incubated for 30 min at 37 °C with 100 µg of RNase-free DNase/ml and 1 unit RNasin/µl. Samples were extracted with phenol/chloroform and precipitated with ethanol in 0.3 M sodium acetate. Labeled transcripts were resuspended in prehybridization buffer, and duplicate 5-µl aliquots were precipitated in 10% trichloroacetic acid. The precipitates were trapped on filter paper and their radioactivities determined by scintillation spectrometry. 17 × 10^6 cpm from each sample were adjusted to 500 µl in the same buffer and were hybridized to filters containing linearized, denatured, and immobilized Bluescript plasmid or plasmids containing cDNAs for murine CyOx-1, murine CyOx-2, rat PLA_{2m}, or murine PLA_{2c}. The dots were previously cut out and prehybridized in 50% formamide, 5 × Denhardt's solution, 4 × SSC, 0.1% SDS, 0.1 mg of salmon sperm DNA/ml for 4 h. Hybridizations were carried with continuous shaking at 42 °C for 48 h. After hybridization, the filters were washed for 15 min in 2 × SSC at room temperature, then for 15 min in 0.2 × SSC at 55 °C, and treated with RNase A (10 µg/ml in 2 × SSC) for 5 min at 37 °C. The filters were then washed in 2 × SSC, 0.1% SDS for 15 min at room temperature and dried. Autoradiographs were obtained and scanned in a laser densitometer. The amount of ^{32}P hybridized to

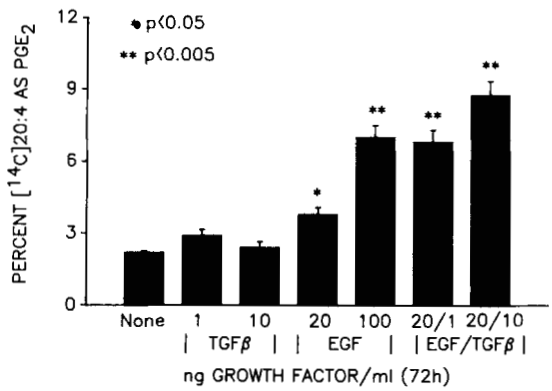

FIG. 1. **Effects of EGF and $TGF_{\beta 1}$ on metabolism of exogenous arachidonic acid to PGE_2 by MEPM cells.** Confluent, primary cultures of MEPM cells were incubated 72 h with growth factors, as indicated, or with medium alone (*None*) and then superfused with 13 µM [^{14}C]arachidonic acid for 15 min. The medium was extracted and analyzed for radiolabeled PGs by thin layer chromatography and scintillation spectrometry. *, $p < 0.05$ when compared with controls (*None*); **, $p < 0.005$ when compared with controls, $TGF\beta_1$ alone, or EGF alone, as determined by *t* test (35). Each data point represents the mean (± S.E.) of three separate experiments.

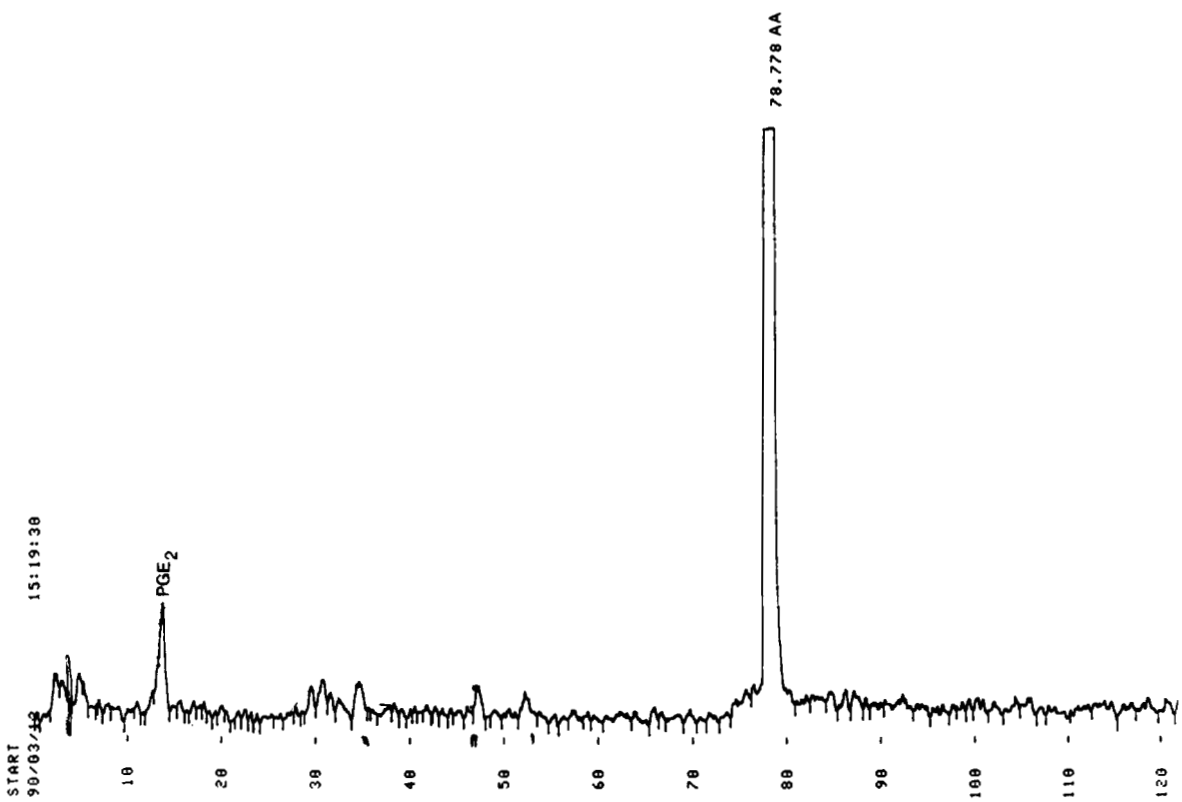

FIG. 2. **High pressure liquid chromatographic separation of metabolites of [^{14}C]arachidonic acid.** MEPM cells were incubated with 20 ng EGF/ml medium and superfused with 13 µM [^{14}C]arachidonic as in Fig. 1. Purified and concentrated medium was injected onto an Ultrasphere ODS column, with precolumn. Arachidonic acid and its metabolites were separated in a solvent gradient of 0.1% aqueous acetic acid (Solvent A) and acetonitrile (Solvent B) programmed to increase from 32% B to 90% B over a period of 102 min. Radioactivity in the column effluent was detected online. 137,000 disintegrations/min as ^{14}C were injected onto the column. Untreated cells did not synthesize sufficient [^{14}C]PGs to detect by HPLC (data not shown). *PGE_2*, prostaglandin E_2; *AA*, arachidonic acid.

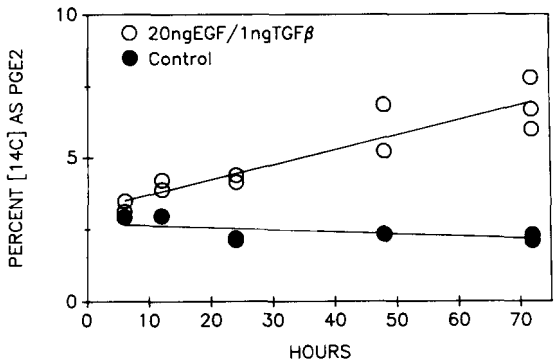

FIG. 3. **Effects of EGF and $TGF\beta_1$ on metabolism of exogenous arachidonic acid by MEPM cells treated for various lengths of time.** Confluent, primary cultures of MEPM cells were incubated 6–72 h in defined medium alone (●) or with EGF and $TGF_{\beta1}$ (○). At various intervals the cells were superfused with [^{14}C]arachidonic acid and the medium analyzed for production of [^{14}C]PGE_2, as described in Fig. 1. Each data point represents a separate experiment. Analysis of variance revealed significant ($p < 0.001$) interaction between time and growth factor treatment in stimulating release of [^{14}C]PGE_2.

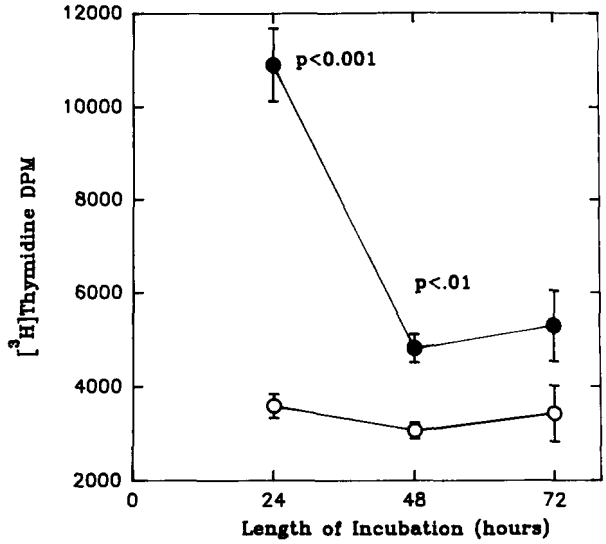

FIG. 4. **Effects of EGF on synthesis of DNA by MEPM cells.** Confluent MEPM cells were incubated 24 h in defined medium, and then for 24, 48, or 72 h in defined medium which contained 20 ng of EGF/ml (●), or in defined medium alone (○). Five μCi of [^{3}H]thymidine/ml of medium was added to each culture for the last 3 h of each 24-h period. Radiolabeled cells were collected in cold (4 °C) 10% trichloroacetic acid, precipitated overnight at 4 °C, and the radioactivity in the precipitates determined as described under "Materials and Methods." Each data point represents the mean (± S.E.) of three separate determinations. Values of p for comparisons of treated to controls at each time point were obtained by t test (35).

each blot was determined by scintillation counting.

Laser Densitometry—Autoradiographs and blots were scanned with a Molecular Dynamics Personal Densitometer (Molecular Dynamics, Sunnydale, CA). Volume integrations with subtraction of appropriate backgrounds were performed with software provided by Molecular Dynamics.

Statistical Analysis—Analysis of variance was performed using software from Human Systems Dynamics (Northbridge, CA). Data were analyzed also using a modified t test according to formulas outlined by Wallenstein *et al.* (35).

RESULTS

Treatment of confluent cultures of MEPM cells for 72 h with as much as 10 ng $TGF_{\beta1}$/ml had no effect on the ability of these cells to metabolize exogenous arachidonic acid to PGs (Fig. 1). However, treatment with EGF promoted metabolism of exogenous arachidonic acid to PGE_2 in a dose-dependent fashion (Fig. 1). Though $TGF_{\beta1}$ by itself had no effect, it potentiated the ability of EGF to increase metabolism of exogenous arachidonic acid to PGE_2 (Fig. 1). PGE_2 was the predominant PG synthesized, as determined by thin layer chromatography and HPLC (Fig. 2).

The growth factors stimulated metabolism of exogenous arachidonic acid to PGE_2 in a linear fashion for at least 72 h (Fig. 3). In contrast, EGF stimulated cellular synthesis of DNA only in the first 24 h of treatment; thereafter synthesis of DNA returned to that typical of the nonproliferative state (Fig. 4). Initiation of a single round of DNA synthesis in response to addition of a growth factor to confluent, quiescent cultures of these embryonic cells is a response typical of cultures of nontransformed cells (36).

Once it was determined that the growth factors could indeed alter the capability of MEPM cells to metabolize exogenous arachidonic acid, cells prelabeled with [^{3}H]arachidonic acid were used to determine whether the growth factors similarly altered the ability of the cells to mobilize and/or metabolize endogenous stores of arachidonic acid. In this case, confluent MEPM cells prelabeled with [^{3}H]arachidonic acid and treated with EGF released relatively more free [^{3}H]fatty acid and [^{3}H]PGE_2 from endogenous stores than did controls (Fig. 5*A*). Forty-eight ± 0.4% of the total ^{3}H released as free fatty acid and PGE_2 from cells treated with EGF was found as PGE_2, whereas only 15 ± 0.5% of the total ^{3}H released as free fatty acid and PGE_2 from control cells was found as PGE_2 (Fig. 5*A*). $TGF_{\beta1}$ did not appear to potentiate significantly the effects of EGF on release of [^{3}H]arachidonic acid from endogenous stores or conversion of endogenous [^{3}H]arachidonic to [^{3}H]PGE_2 (Fig. 5*A*).

Confluent cultures prelabeled with [^{3}H]arachidonic acid and then treated with EGF mobilized twice as much endogenous [^{3}H]free fatty acid and [^{3}H]PGE_2 in response to the calcium ionophore A23187 than did controls (Fig. 5*B*). Thirty % of the radiolabel mobilized by EGF-treated, ionophore-stimulated cells was converted to [^{3}H]PGE_2, whereas ionophore-stimulated controls (no EGF treatment) converted only 10% of the mobilized radiolabel to [^{3}H]PGE_2 (Fig. 5*B*). Again, $TGF_{\beta1}$ did not appear to potentiate the effects of EGF on mobilization and metabolism of endogenous stores of [^{3}H]fatty acid (Fig. 5*B*). Consistent with previous findings (13), [^{3}H]fatty acid was released from all major classes of intracellular phospholipids in response to treatment with EGF and ionophore (Fig. 5*C*). No radiolabel was released from triglycerides since the amount of label found in the triglyceride fraction of EGF-treated cells (4.1 ± 0.3%, $n = 3$) was not significantly different from that found in controls (5.4 ± 0.6%, $n = 3$) subsequent to stimulation with the ionophore.

Increased mobilization and metabolism of arachidonic acid in response to EGF was accompanied by increased synthesis of CyOx and PLA_{2c} protein (Fig. 6). Scans of the autoradiographs presented in Fig. 6 revealed a 5—6-fold increase in CyOx and 2.5–3-fold increase in PLA_{2c} within 24 h of treatment. Increased synthesis of these proteins continued for at least 72 h (Fig. 6), at which time synthesis of CyOx was as much as 33 times that of controls and synthesis of PLA_{2c} was as much as six times that of controls. $TGF_{\beta1}$ itself did not have a noticeable effect (less than 2-fold increase relative to controls) on synthesis of CyOx or PLA_{2c} after 24 or 72 h of treatment (Fig. 6). However, a potentiating affect of $TGF_{\beta1}$ on EGF-induced synthesis of CyOx was readily detected after 72 h of treatment of cells with both growth factors (Fig. 6; 20 ng EGF/ml induced a 15-fold increase in CyOx whereas 20 ng of EGF and 1 ng of $TGF_{\beta1}$/ml induced a 33-fold increase in CyOx). There was no detectable effect of the growth factors on synthesis of proteins immunoprecipitated with antibody to PLA_{2II} (data not shown).

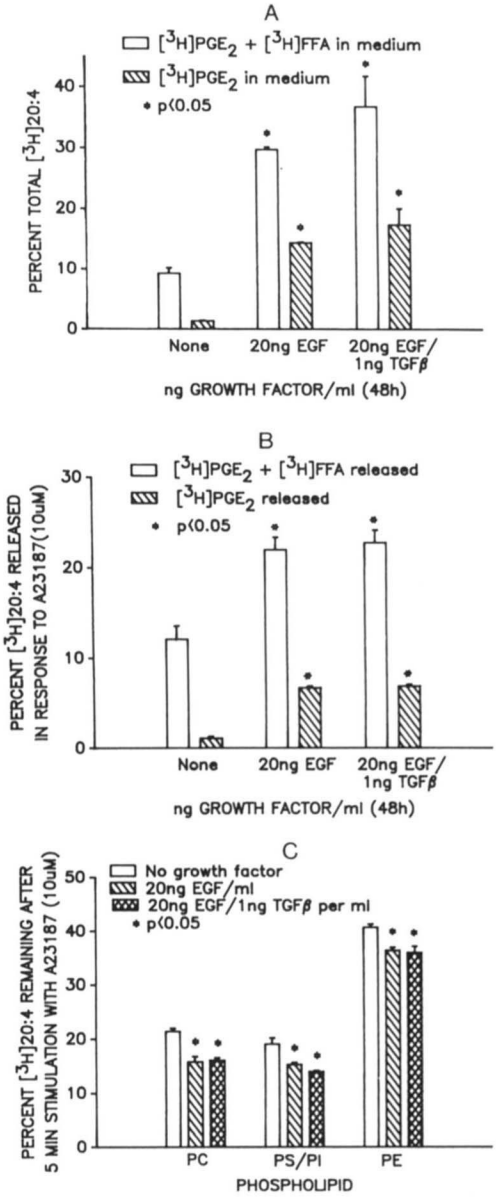

FIG. 5. **Effects of growth factors on mobilization and metabolism of endogenous arachidonic acid by MEPM cells.** Confluent, primary cultures of MEPM cells were radiolabeled with [^{3}H]arachidonic acid, washed, and then incubated 48 h with the indicated growth factors. The medium was then collected and extracted. The cells were then washed and stimulated 5 min with 10 μM A23187 in HEPES-buffered saline containing 5 mM $CaCl_2$. Cells and HEPES-buffered saline were collected together and extracted. The various extracts were analyzed for radiolabeled fatty acids, PGs, and phospholipids by thin layer chromatography. *A*, release of radiolabeled fatty acid ([MDSU3H]*FFA*) and PGE_2 ([^{3}H]*PGE_2*) into the medium during the initial 48-h incubation period. *B*, radiolabeled fatty acid and PGE_2 released in response to A23187; *C*, depletion of radiolabeled fatty acid from cellular phospholipids. *None*: no addition of growth factors; *PC*, *PS/PI*, and *PE*, 1,2-radyl-*sn*-glycero-3-phosphocholine, -serine/-inositol fraction, -ethanolamine. Each *bar* represents the mean (± S.E.) of three separate experiments. Values of *p*, indicated by *, on comparing the response of EGF-treated cells to their appropriate controls were determined by *t* test (35).

Increased synthesis of PLA_{2c} and CyOx was accompanied by accumulation of PLA_{2c}, CyOx-1, and CyOx-2 proteins (Fig. 7*A*). The anti-sheep seminal vesicle CyOx antibody would immunoprecipitate both isoforms of CyOx as it cross-reacts with CyOx-2 (Fig. 7*B*); the anti-CyOx-2 antibody did not detectably cross-react with sheep seminal vesicle cyclooxygenase (Fig. 7*B*).

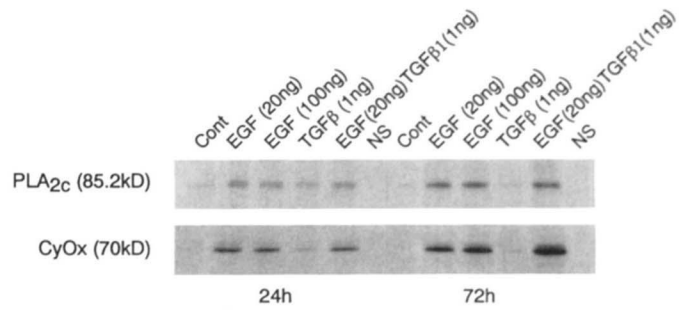

FIG. 6. **EGF stimulates synthesis of CyOx and PLA_{2c} by MEPM cells.** Confluent cultures were incubated for 24 or 72 h in defined medium alone (*Cont*) or medium which contained growth factors as indicated in the figure. The cells were then washed, incubated 15 min in defined medium which lacked methionine and cystine, and then incubated 2 h in methionine(−)/cystine(−) medium which contained 100 μCi of [^{35}S]methionine/[MDSU35S]cystine/ml. The cells were then lysed to yield a supernatant which was precipitated with the appropriate antiserum and protein A-Sepharose. Radiolabeled proteins in the immunoprecipitates were separated by SDS-PAGE and visualized by fluorography. *NS*, radioactivity immunoprecipiated by normal rabbit serum. Data are representative of two separate experiments.

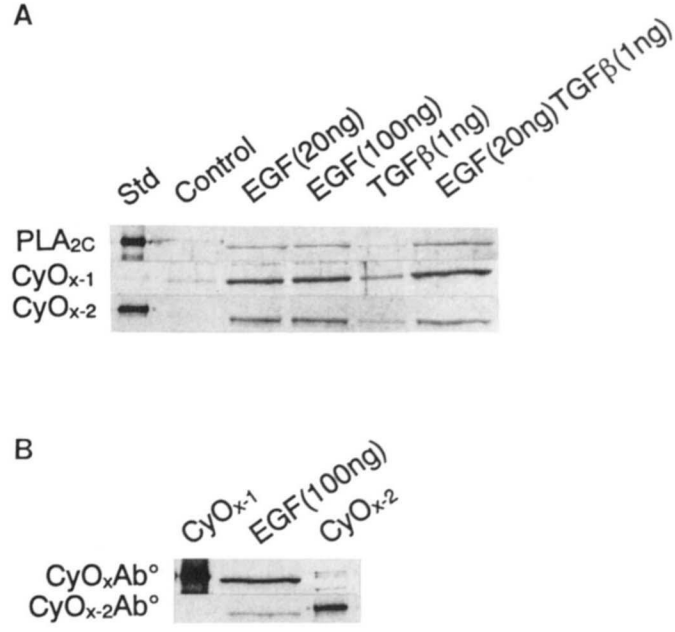

FIG. 7. **Western blot analysis of the effects of EGF and $TGF_{\beta 1}$ on accumulation of CyOx and PLA_{2c} in MEPM cells.** *A*, duplicate samples of cells extracts obtained as described in Fig. 6 were resolved by SDS-PAGE, blotted, and stained with antibody to either sheep seminal vesicle CyOx, mouse CyOx-2, or human PLA_{2c}. Data are representative of two separate experiments. *B*, authentic sheep seminal vesicle CyOx (CyOx-1), mouse CyOx-2, and MEPM cell extract were resolved on the same gel by SDS-PAGE and stained with antibody to either sheep seminal vesicle CyOx (CyOx-1) or mouse CyOx-2 to determine whether there was cross-reaction between the antibodies.

Laser scanning quantitation of autoradiographs prepared from Northern blots revealed a 4-fold increase in steady-state levels of the 2.8- and 4.2-kb transcripts for CyOx-1 and CyOx-2, respectively, and a 2.5-fold increase in steady-state levels of a 3.0-kb mRNA for PLA_{2c} within 24 h of treatment with EGF (Fig. 8). In contrast, there was no apparent effect (less than 1.6 times control values) of any of the growth factor treatments on steady-state levels of the 2.2-kb mRNA for the low molecular weight, Group II PLA_2 (*PLA_{2m}*; Fig. 8). The steady-state level of mRNA for PLA_{2c} in TGF_{B1}-treated cells was 73 ± 12% (n = 3) of controls.

In vitro nuclear run-off assays revealed the increased steady-state mRNA levels for CyOx-1 and PLA_{2c} in response to EGF

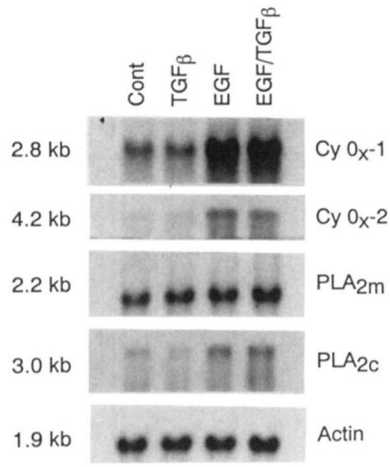

FIG. 8. **Effects of EGF and $TGF_{\beta 1}$ on steady-state levels of mRNA for CyOx, PLA_{2m}, PLA_{2c}, and actin in MEPM cells.** Confluent, primary cultures of MEPM cells were cultured 24 h with defined medium alone (*Cont*) or with medium which contained either 1 ng of $TGF_{\beta 1}$ (TGF_{β}), 20 ng of EGF (*EGF*), or 1 ng of $TGF_{\beta 1}$ and 20 ng of EGF (EGF/TGF_{β}) per ml. Total RNA was extracted as described under "Materials and Methods." Ten μg of RNA/sample were electrophoresed on a 0.8% agarose gel, blotted onto nitrocellulose filters, and hybridized to ^{32}P-labeled cDNAs for CyOx-1, CyOx-2, PLA_{2m}, PLA_{2c}, or actin. Autoradiographs prepared from the filters were scanned with a laser densitometer. The size of the mRNAs detected by the various cDNAs is indicated on the *left* side of the autoradiographs. Northern blots are representative of three separate experiments.

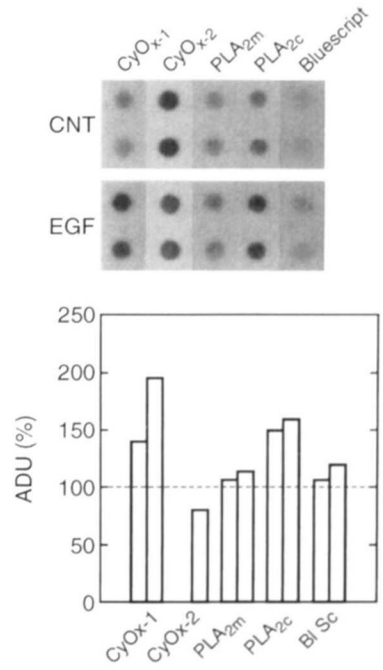

FIG. 9. **Effects of EGF on the transcription rate of CyOx-1, CyOx-2, PLA_{2m}, and PLA_{2c} in MEPM cells.** Confluent, primary cultures of MEPM cells were incubated 24 h in the presence or absence of EGF (20 ng/ml). Nuclei were isolated and *in vitro* transcription assays were conducted as described under "Materials and Methods." Labeled transcripts from each sample (1.7×10^7 cpm) were hybridized to the filter-bound cDNAs for CyOx-1, CyOx-2, PLA_{2m}, PLA_{2c}, or Bluescript plasmid (*Bl Sc*). After washing and digestion with RNase A, the filters were processed by autoradiography. Autoradiographs were scanned in a laser densitometer and the resulting values obtained from samples incubated with EGF (*open bars*) expressed in arbitrary densitometric units (*ADU*) as a percentage relative to values from samples incubated with media alone (value of 100%; *dashed line*). Each *bar* represents the average of duplicate hybridizations obtained in a single experiment.

were at least partially the result of an increased transcription rate (Fig. 9) of the genes for CyOx-1 and PLA_{2c}. The transcription rate of the genes for PLA_{2m} (group II PLA_2) and CyOx-2 were not enhanced by treatment of MEPM cells with EGF (Fig. 9).

DISCUSSION

Chronic treatment of MEPM cells with EGF clearly enhanced their capacity to metabolize arachidonic acid to PGE_2. In parallel, EGF induced accumulation of the mRNA and protein for both CyOx-1 and CyOx-2. In contrast, induction of cyclooxygenase by treatment of some other types of cells with mitogens was not accompanied by induction of the mRNA for CyOx-1, but was accompanied by induction of the mRNA for CyOx-2 (37–39). EGF-induced accumulation of CyOx-1 mRNA in these embryonic cells reflects to some extent enhanced gene transcription, whereas accumulation of CyOx-2 mRNA reflects primarily regulation of message stability. Evett *et al.* (40) reported dexamethasone inhibition of mitogen-induced accumulation of CyOx-2 mRNA in NIH3T3 cells was at the level of regulation of message stability since dexamethasone did not alter the rate of gene transcription. These various findings lead to the conclusion that there is both cell and agonist specificity as to which isoform(s) of CyOx is(are) induced and the molecular mechanism by which that induction takes place.

Although $TGF_{\beta 1}$ stimulates synthesis of PGs by various types of cells (*i.e.* human lung fibroblasts (41) and microvessel endothelial cells (42)), it had no detectable effect on synthesis of PGs by MEPM cells. However, $TGF_{\beta 1}$ potentiated the stimulatory effects of EGF. This is similar to the situation found with vascular smooth muscle cells (17), wherein $TGF_{\beta 1}$ alone is a moderate inducer of activity of CyOx but significantly potentiates the inducing effects of EGF. These findings most likely reflect $TGF_{\beta 1}$ modulation (up-regulation) of receptor levels for EGF (43). Such receptor up-regulation would result in an enhanced response to EGF. It should be noted that interaction of growth factors in modulating receptor levels is quite complex since treatment of MEPM cells with $TGF_{\beta 1}$ in the presence of serum factors down-regulates receptors for EGF (44).

The finding that $TGF_{\beta 1}$ potentiated the effects of EGF on metabolism of exogenous arachidonic acid without potentiating metabolism of endogenous arachidonic acid may at first seem paradoxical. However, it may be that only those molecules of arachidonic acid mobilized from endogenous stores in juxtaposition to molecules of CyOx are metabolized to eicosanoids and that not all molecules of CyOx are in juxtaposition to intracellular sites of mobilization of arachidonic acid. Exogenous arachidonic acid would be metabolized by any available molecule of CyOx, whereas endogenous arachidonic acid would be metabolized by only those molecules of CyOx coupled to the intracellular site of acyl mobilization. In such a case it would be possible to observe a level of metabolism of exogenous arachidonic acid that may not be matched by levels of metabolism of endogenous arachidonic acid. Indeed, although the amount of arachidonic acid released from endogenous stores was increased by EGF, less than half was metabolized to PGE_2. Smith *et al.* (1) have suggested tight coupling exists between the endogenous pool of arachidonic acid which is mobilized and destined for conversion to PGs and the pool of CyOx through which it is metabolized.

EGF induced constitutive expression of activities of PLA_2 and CyOx in MEPM cells, as measured by mobilization and metabolism of endogenous arachidonic acid. This effect of EGF on activity of these enzymes did not simply reflect the mitogenic properties of EGF in that they did not parallel the effects of EGF on synthesis of DNA. Furthermore, constitutive expression of activities of PLA_2 was coordinated with increased steady-state mRNA levels for the high molecular weight, cytosolic PLA_{2c}, but not PLA_{2m} (a PLA_{2II}). Several investigators

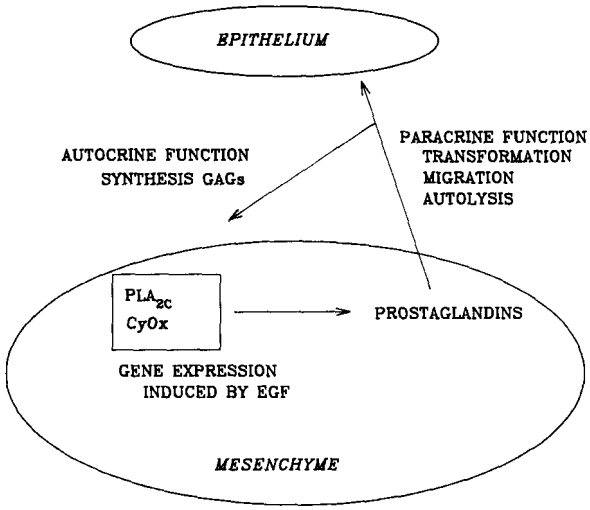

FIG. 10. **A model depicting EGF regulation of gene expression of enzymes requisite to the synthesis of lipid signaling molecules as a mechanism for regulating mesenchymal-mesenchymal and mesenchymal-epithelial interactions during development of the palate.** In this model, EGF induces palate mesenchymal cells to synthesize prostaglandins by inducing expression of PLA_{2c} and CyOx. These prostaglandins may then serve as molecular signals by which the mesenchyme specifies terminal differentiation of the palate (reviewed in Ref. 48), and by which mesenchymal cells signal each other to synthesize GAGs (49). This model is consistent with the evidence that EGF plays some sort of role in modulating differentiation of the palate (reviewed in Ref. 50), that agents which inhibit synthesis of prostaglandins will inhibit terminal differentiation of the palatal epithelium *in vivo* and *in vitro* (51, 52) and that synthesis of GAGs in palatal mesenchyme is important in reorientation of the secondary palatal shelves from the vertical to the horizontal position during embryogenesis (53).

reported very recently that either EGF (45) or $interleukin_{1\beta}$ (46) will increase mRNA levels and protein synthesis of PLA_{2c} in rat mesangial cells, and Hoeck *et al.* (47) found a similar affect of TNF_{α} on HeLa cells. However, to the best of our knowledge the findings herein are the first to document EGF-induced coordinate expression of the genes for PLA_{2c} and CyOx at the level of transcription, accumulation of mRNA, and synthesis of protein. Such coordinate regulation of PLA_{2c} and CyOx makes it likely the high molecular weight, cytosolic PLA_2 is regulatory for mobilization of arachidonic acid destined for metabolism to prostaglandins in this embryonic cell system. Furthermore, such chronic (long term) regulation of gene expression of PLA_{2c} and CyOx provides a mechanism by which EGF may induce a signaling pathway thought to be involved in regulating development of the palate (Fig. 10). These findings support the hypothesis that induction of synthesis of lipid signaling molecules is a major mechanism by which EGF regulates development of the palate (Fig. 10).

Acknowledgment—We thank Dr. John Flynn, Jefferson Medical College, for performing analysis by HPLC.

REFERENCES

1. Smith, W. L., Marnett, L. J., and DeWitt, D. L. (1991) *Pharmacol. Ther.* **49,** 153–179
2. DeWitt, D. L. (1991) *Biochim. Biophys. Acta* **1083,** 121–134
3. Waite, B. M. (1987) in *The Phospholipases: Handbook of Lipid Research*, Vol. 5, pp. 169–171, Plenum Press, New York
4. Clark, J. D., Lih, L-L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) *Cell* **65,** 1043–1051
5. Leslie, C. C. (1991) *J. Biol. Chem.* **266,** 11366–11371
6. Sharp, J. D., White, D. L., Chiou, X. G., Goodson, T., Gamboa, G. C., M°Clure, D., Burgett, S., Hoskins, J., Skatrud, P. L., Sportsman, J. R., Becker, G. W., Kang, L. H., Roberts, E. F., and Kramer, R. M. (1991) *J. Biol. Chem.* **266,** 14850–14853
7. (1991) *Immunol. Today* **12,** 143–146
8. Clark, J. D., Milona, N., and Knopf, J. L. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 7708–7712
9. Diez, E., and Mong, S. (1990) *J. Biol. Chem.* **265,** 14654–14661
10. Kim, D. K., Kudo, I., Fujimori, Y., Mizushima, H., Masuda, M., Kikuchi, R., Ikizawa, K., and Inoue, K. (1990) *J. Biochem. (Tokyo)* **108,** 903–906
11. Kim, D. K., Kudo, I., and Inoue, K. (1991) *Biochim. Biophys. Acta* **1083,** 80–88
12. Kramer, R. M., Roberts, E. F., Manetta, J., and Putman, J. E. (1991) *J. Biol. Chem.* **266,** 5268–5272
13. Chepenik, K. P. (1989) *Lipids* **24,** 829–832
14. Nexo, E., Hollenberg, M. D., Figureroa, A., and Pratt, R.M. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 2782–2785
15. Yoneda, T., and Pratt, R. M. (1981) *Science* **213,** 563–565
16. Linask, K. K., D'Angelo, M., Gehris, A. L., and Greene, R. M. (1991) *Exp. Cell Res.* **192,** 1–9
17. Pash, J. M., and Bailey, J. M. (1988) *FASEB J.* **2,** 2613–2618
18. Chabot, M. C., and Chepenik, K. P. (1986) *J. Craniofacial. Genet. Dev. Biol.* **6,** 223–234
19. Chepenik, K. P., and Grunwald, G. B. (1988) *J. Craniofacial. Genet. Dev. Biol.* **8,** 147–153
20. Chepenik, K. P., and Haystead, T. A. J. (1989) *J. Craniofacial. Genet. Dev. Biol.* **9,** 285–301
21. Flower, R. J., Cheung, H. S., and Cushman, D. W. (1973) *Prostaglandins* **4,** 325–341
22. Renzi, P. M., and Flynn, J. T. (1992) *Am. J. Physiol.* **263,** H1213-H1221
23. George, M., and Chepenik, K. P. (1985) *Biochim. Biophys. Acta* **836,** 45–55
24. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
25. Margolis, B., Rhee, S. G., Felder, S., Mervic, M., Lyall, R., Levitzke, A., Ullrich, A., Zilberstein, A., and Schlsessinger, J. (1989) *Cell* **57,** 1101–1107
26. Smith, W. L., and Rollins, T. E. (1982) *Methods Enzymol.* **86,** 213–222
27. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning a Laboratory Manual*, 2nd Ed., pp. 7.7–7.83, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
29. Rigby, P. W. J., Dieckmann, M., Rhodes, C., and Berg, P. (1977) *J. Mol. Med.* **113,** 237–251
30. Ishizaki, J., Ohara, O., Nakamura, E., Tamaki, M., Ono, T., Kanda, A., Yoshida, N., Teraoka, H., Tojo, H., and Okamoto, M. (1989) *Biochem. Biophys. Res. Commun.* **162,** 1030–1036
31. DeWitt, D. L., and Smith, W. L. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 1412–1416
32. Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W., and Herschman, H. R. (1991) *J. Biol. Chem.* **266,** 12866–12872
33. Greenberg, M. E., and Ziff, E. B. (1984) *Nature* **311,** 433–438
34. Olsen, A. S., and Prockop, D. J. (1987) *Methods Enzymol.* **144,** 74–83
35. Wallenstein, S., Zuker, C. L., and Fleiss, J. L. (1981) *Circ. Shock* **8,** 503–517
36. Todaro, G. J., Lazar, G. K., and Green, H. (1965) *J. Cell. Comp. Physiol.* **66,** 325–334
37. Hla, T., and Neilson, K. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7384–7388
38. Xie, W., Chipman, J. G., Robertson, D. L., Erikson, R. L., and Simmons, D. L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2692–2696
39. Simmons, D. L., Xie, W., Chipman, J. G., and Evett, G. E. (1991) in *Prostaglandins, Leukotrienes, Lipoxins, and PAF* (Bailey, J. M., ed) pp. 67–78, Plenum Press, New York
40. Evett, G. E., Xie, W., Chipman, J. G., Robertson, D. L., and Simmons, D. L. (1993) *Arch. Biochem. Biophys.* **306,** 169–77
41. Diaz, A., Varga, J., and Jimenez, S. (1989) *J. Biol. Chem.* **264,** 11554–11557
42. Moatter, T., and Gerritsen, M. E. (1992) *J. Cell. Phys.* **151,** 571–578
43. Assoian, R. K., Frolik, C. A., Roberts, A. B., Miller, D. M., and Sporn, M. B. (1984) *Cell* **36,** 35–41
44. Sharpe, P. M., Brunet, C. L., and Ferguson, M. W. J. (1992) *Int. J. Dev. Biol.* **36,** 275–282
45. Maxwell, A. P., Goldberg, H. J., Tay, A. H., Li, Z. G., Arbus, G. S., and Skorecki, K. L. (1993) *Biochem. J.* **295,** 763–766
46. Schalkwijk, C. G., Vervoordeldonk, M., Pfeislschifter, J., van den Bosch, H. (1993) *FEBS Lett.* **333,** 339–343
47. Hoeck, W. G., Ramesha, C. S., Chang, D. J., Fan, N., and Heller, R. A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4475–4479
48. Ferguson, M. W. J. (1988) *Development* **103,** 41–60
49. Greene, R. M., MacAndrew, V. I., and Lloyd, M. R. (1982) *Biochem. Biophys. Res. Commun.* **107,** 232–238
50. Pratt, R. M. (1987) *Curr. Topics Dev. Biol.* **22,** 175–193
51. Montenegro, M. A., and Pas de la Vega, Y. (1982) *Arch. Oral Biol.* **27,** 771–775
52. Montenegro, M. A., and Palomino, H. (1990) *J. Craniofacial Genet. Dev. Biol.* **10,** 83–94
53. Brinkley, L. L., and Morris-Wiman, J. (1984) *Curr. Topics Dev. Biol.* **19,** 17–36