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Growth regulation of primary human keratinocytes by prostaglandin E receptor EP_2 and EP_3 subtypes

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Abstract

We examined the contribution of specific EP receptors in regulating cell growth. By RT–PCR and northern hybridization, adult human keratinocytes express mRNA for three PGE_2 receptor subtypes associated with cAMP signaling (EP_2, EP_3, and small amounts of EP_4). In actively growing, non-confluent primary keratinocyte cultures, the EP_2 and EP_4 selective agonists, 11-deoxy PGE_1 and 1-OH PGE_1, caused complete reversal of indomethacin-induced growth inhibition. The EP_3/EP_2 agonist (misoprostol), and the EP_1/EP_2 agonist (17-phenyl trinor PGE_2), showed less activity. Similar results were obtained with agonist-induced cAMP formation. The ability of exogenous dibutyryl cAMP to completely reverse indomethacin-induced growth inhibition support the conclusion that growth stimulation occurs via an EP_2 and/or EP_4 receptor-adenylyl cyclase coupled response. In contrast, activation of EP_3 receptors by sulprostone, which is virtually devoid of agonist activity at EP_2 or EP_4 receptors, inhibited bromodeoxyuridine uptake in indomethacin-treated cells up to 30%. Although human EP_3 receptor variants have been shown in other cell types to markedly inhibit cAMP formation via a pertussis toxin sensitive mechanism, EP_3 receptor activation and presumably growth inhibition was independent of adenylyl cyclase, suggesting activation of other signaling pathways. © 1998 Elsevier Science B.V.

Keywords: Keratinocyte; Proliferation; Prostaglandin E receptor; Receptor agonist; Cyclic AMP; (Human)

Abbreviations: PGE, prostaglandin E; PGF, prostaglandin F; EP, prostaglandin E receptor; RT–PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction; cAMP, adenosine 3′:5′-cyclic monophosphate; db-cAMP, dibutyryl cAMP (N^6,O^2-dibutyryl adenosine 3′:5′-cyclic monophosphate); NSAIDs, non-steroidal antiinflammatory drugs; GTP, guanylate triphosphate; EFAD, essential fatty acid deficient; SCC, squamous cell carcinoma; EIA, enzyme immunoassay; BrdU, 5-bromo-2′-deoxyuridine; PTX, pertussis toxin; 11-deoxy PGE_1, 11-deoxy prostaglandin E_1 (prosta-13-en-1-ioc acid, 15-hydroxy-9-oxo-, (13E, 15S)-); Misoprostol, prost-13-en-1-oic acid, 11,16-dihydroxy-16-methyl-9-oxo, methyl ester, (11α,13E)-; 17-pt PGE_2, 17-phenyl trinor prostaglandin E_2 (5-heptenoic acid, 7-[3-hydroxy-2-(3-hydroxy-5-phenyl-1-pentenyl)-5-oxocyclopentyl]-, [1R-[1α(Z), 2β(1E,3S*),3α]]-); Sulprostone, 5-heptenamide,7-[3-hydroxy-2-(3-hydroxy-4-phenoxy-1-butenyl)-5-oxocyclopentyl]-*N*-(methylsulfonyl)-, [1R-[1α(Z),2β(1E,3R*),3α]]-; 1-OH PGE_1, prostaglandin E_1 alcohol (prost-13-en-9-one, 1,11,15-trihydroxy-, (11α, 13E, 15S)-); 19-OH PGE_2, 19(R)-hydroxy prostaglandin E_2 (prosta-5,13-dien-1-oic acid, 11,15,19-trihydroxy-9-oxo-, (5Z,11α,13E,15S,19R)-); SSC, saline–sodium citrate buffer; SSPE, saline–sodium phosphate–EDTA buffer; SDS, sodium dodecyl sulfate (lauryl sulfate); DMEM, Dulbecco′s Modified Eagles Medium; PBS, phosphate buffered saline; FBS, fetal bovine serum; IgG, immunoglobulin G; HEPES, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]

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1. Introduction

Substantial evidence suggests that endogenous PGE_2 is important in regulating the growth of both normal and neoplastic epithelial cells. Prostaglandin E_2 (PGE_2) is a major endogenous product of prostanoid metabolism in keratinocytes in intact skin as well as in culture, with increased levels observed during wound healing and in cutaneous neoplasms [1–3]. The importance of PGE_2 in neoplastic development is suggested by the decreased risk for colon cancer formation associated with the use of nonsteroidal antiinflammatory drugs (NSAIDs) [4]. Moreover, NSAID's block tumor formation in tumor promotion models of skin, intestine, and bladder [5–8]. The prostaglandin receptor(s) which are likely to be involved have not been identified. Because skin cancer is the most common epithelial malignancy, the receptor types expressed in human keratinocytes were defined and their effects on growth were characterized in a human tissue culture model.

Recently, four different genes coding for PGE_2 receptors (EP receptors) have been cloned and have been designated as EP_1, EP_2, EP_3, and EP_4 based on the relative agonist or antagonist potencies of a number of different analogues of PGE (For reviews, see [9,10]). The EP receptors are coupled to heterotrimeric GTP binding-proteins (G-proteins), and can also be subgrouped by their respective G-protein interactions [9,10]. Agonist binding to the EP_1 receptor has been shown to mobilize intracellular calcium by a mechanism independent of phospholipase C activation [9]. The EP_2 and EP_4 receptors have been shown to stimulate cyclic AMP production via activation of adenylate cyclase [9]. In humans, eight different splice variants of the EP_3 receptor have been cloned which code for six different functional receptor proteins [11–13]. In all of these splice variants, alternative splicing occurs just 3′ to the seventh transmembrane domain, in a region encoding the carboxy terminal tail of the receptor which is necessary for G-protein interaction. EP_3 receptor variants can mediate multiple signaling pathways. Inhibition of adenylyl cyclase via a pertussis toxin sensitive mechanism, stimulation of adenylyl cyclase, activation of phospholipase C, mobilization of intracellular calcium, and activation of the nitric oxide-guanylyl cyclase pathway by a calcium dependent mechanism have all been described [11,12,14–16].

Prostaglandin receptors are likely to be important in regulating normal epidermal growth as well as the hyperplastic response to wounding and inflammation. Endogenous PGE_2 levels are seen to rise dramatically following wounding of guinea pig epidermis [2] and during inflammation [17]. Exogenous PGE_1 and PGE_2 stimulate proliferative activity in human keratinocytes in vitro [18], in intact skin following topical application or intradermal injection [19,20], and following experimental wounding of mouse skin [21]. Moreover, application of inhibitors of PGE synthesis inhibit the growth of keratinocytes in vitro [22] and in vivo [23], and inhibit the inflammatory hyperplastic response [24]. Growth inhibition in vitro is specifically reversed by PGE_2 [22]. The importance of prostaglandins to reepithelialization following wounding is underscored by experimental animal models of essential fatty acid deficiency (EFAD), where the dietary precursors necessary for PGE synthesis are not present. In EFAD rats, healing of partial thickness burns is delayed up to three times longer [25].

Most importantly, substantial evidence suggests an important role for EP receptors in epithelial neoplasia. Increased production of PGE_2 is commonly observed in carcinomas, and increased levels can be correlated with invasive or metastatic behavior and aggressive growth [3,26–29]. Moreover, administration of PGE_2 and $PGF_{2\alpha}$ to syngeneic mice transplanted with chemically induced squamous cell carcinoma (SCC) markedly enhances the transplantability, growth, and cellular atypia of these tumors [30]. However, different tumors exhibit markedly different responses to PGE_2 or to inhibitors of PGE production. Inhibitors of prostaglandin production inhibit growth of a murine SCC in vivo [31] and a human breast cancer cell line in vitro [32]. However, indomethacin stimulates, and PGE_2 inhibits, growth of the human SCC-25 cell line in vitro [33], and PGE_2 fails to stimulate growth in 75–80% of primary murine mammary tumors, although it stimulates growth in normal mammary epithelium [34]. This heterogeneity of response may be due to altered PGE_2 receptor expression, since scatchard analysis of [^{3}H]-PGE_2 binding to different human and murine

breast cancer cell lines demonstrate different levels of high-affinity and low-affinity binding sites for PGE_2 [35,36]. Loss of specific high-affinity PGE_2 binding sites and increased low-affinity binding sites was associated with an advanced tumorigenic phenotype [35].

The mechanisms by which PGE acts to regulate growth is poorly understood, although historical data suggest that cyclic AMP is likely to mediate the proliferative response elicited by PGE_2. Substantial evidence exists that cAMP signaling is a positive signal for growth in epidermis [37,38] and normal mammary epithelium [39]. PGE_2 is known to be a major regulator of cAMP production in skin [40]. This suggests that the growth promoting activity of PGE_2 may be mediated via activation of receptors linked to cAMP production. However, it is not known which EP receptor subtype(s) are expressed in the epidermis, although both human and rodent epidermis contain specific binding sites for PGE_2 and PGE_1 [41,42]. Since cutaneous neoplasias are the most common form of human malignancy [43], we sought to determine the EP receptor subtype(s) which are expressed in keratinocytes, and whether these receptor(s) act to regulate cAMP production as well as keratinocyte growth in response to PGE_2 production. These studies provide the foundation for further investigations into the role of PGE_2 receptors in regulating hyperplastic and neoplastic epithelial proliferation.

2. Materials and methods

2.1. Materials

Cyclic adenosine monophosphate EIA kits and the PGE analogues 11-deoxy-PGE_1, 17-phenyl trinor PGE_2, sulprostone, and PGE_1 alcohol (1-OH-PGE_1) were obtained from Cayman Chemical (Ann Arbor, MI). Misoprostol was a generous gift of Searle (St. Louis, MO). Dibutyryl cAMP (db-cAMP) (N^6,O^2-dibutyryl adenosine 3′:5′-cyclic monophosphate), forskolin, PGE_2, pertussis toxin, 5-bromo-2′-deoxyuridine (BrdU), saline–sodium citrate buffer (SSC), saline–sodium phosphate–EDTA buffer (SSPE), 50 × Denhardt's solution, 10% lauryl sulfate (SDS), salmon sperm DNA, and indomethacin were obtained from Sigma (St. Louis, MO). Sterile phosphate buffered saline (PBS), HEPES buffer, and penicillin–streptomycin were obtained from the Washington University School of Medicine tissue culture support center. Dulbecco's Modified Eagle Medium (DMEM) (high glucose, L-glutamine and without sodium pyruvate) was obtained from Gibco-BRL (Gaithersburg, MD). Fetal Bovine Serum (FBS) was obtained from BioWhittaker (Walkersville, MD). Tissue culture plates (Falcon) were obtained from Becton Dickinson (Lincoln Park, NJ). Plasmids containing the EP_4 and EP_{3I} cDNA's (pcDNAI-hEP_4 and pcDNAI-hEP_{3I}) were the generous gift of Dr. K. Metters, Merck-Frost (Quebec, Canada). Mouse monoclonal anti-BrdU (clone Bu20a) and horseradish peroxidase-conjugated rabbit anti-mouse IgG1 were obtained from Dako (Carpinteria, CA). Peroxoblock was obtained from Zymed Laboratories (South San Francisco, CA). Taq DNA polymerase and restriction enzymes were obtained from Boehringer Mannheim (Indianapolis, IN) and Gibco-BRL (Gaithersburg, MD).

2.2. Cell culture and preparation of primary human keratinocytes

Primary adult human keratinocyte cultures were obtained from human epidermis removed during reductive mammoplasty and panniculectomies [22]. Cells were maintained in Dulbecco's Modified Eagle Medium containing 5% fetal bovine serum, penicillin (100 units/ml)/streptomycin (100 μg/ml), and 25 mM HEPES buffer (*N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]) (growth medium) in 95% air, 5% CO_2 at 37°C. All tissue culture plasticware was precoated with collagen (Vitrogen 100, Collagen, Palo Alto, CA).

2.3. Cyclic-AMP assays

Primary adult human keratinocytes were seeded onto 12-well tissue culture plates. Two days after plating (50–70% confluent), indomethacin (1000 × stock dissolved in ethanol) at a final concentration of 3 μg/ml was added to the cultures for 9–16 h to block endogenous PGE_2 production. For experiments using pertussis toxin, pertussis toxin (1000 × stock in water) was added at a final concentration of 20 ng/ml.

Cells were then incubated for 5–9 h at 37°C. At the time of the assay, media was aspirated from the cultures, and serum-free DMEM prewarmed to 37°C was then added containing either vehicle (EtOH), or the desired concentrations of agonist (PGE_2 or analogues) (1000 × stock in EtOH). For agonist dose-response curves, 2 mM isobutyl methyl xanthine (IBMX) was added to inhibit phosphodiesterase activity. After incubation at 37°C for the desired time period (as specified in figure legends), the media was rapidly removed and 0.5 ml of ice cold 10% trichloroacetic acid in phosphate buffered saline was added to the cellular monolayer. The cells were then immediately snap frozen in an ethanol-dry ice bath and stored at −20°C until assayed. Sample preparation and acetylation as well as the cAMP EIA were done following the manufacturers protocol (Cayman Chemical, Ann Arbor, MI). Cyclic AMP levels were normalized to cellular protein quantitated using the bicinchoninic acid method (BCA Protein Assay, Pierce, Rockford, IL).

2.4. Growth Assays: bromodeoxyuridine EIA

Pre-confluent cultures were prepared by seeding keratinocytes at an initial cell density of 100 000 cells/cm^2 on 24 well plates. Cells were allowed to attach and grow for two days at which time the cells were 20–40% confluent. Increasing concentrations of exogenous PGE_2 or selective agonists were added to cells which had been pretreated for 1 h with indomethacin (10 μg/ml) or vehicle (Ethanol). The cells were then incubated in the presence or absence of indomethacin and exogenous prostanoids for 2 days, with daily changes of medium. Ten micromoles of bromodeoxyuridine (BrdU) was added for the last 9 h in culture. At the time of BrdU labeling, the monolayers were between 50–70% confluent.

The BrdU EIA protocol is a modification of a previously reported method [44]. Following ethanol fixation and treatment with peroxoblock (Zymed Laboratories, South San Francisco, CA), the cells were permeabilized with 0.2% Triton X-100 in 25 mM Tris–HCl, 150 mM NaCl, pH 7.6 (TBS). Chromosomal DNA was denatured with 95% formamide in 0.15 mM trisodium citrate buffer for 45 min at 70°C. The cells were blocked at room temperature with TBS/0.1% Tween 20 containing 0.1 mg/ml bovine serum albumin (blocking buffer), then incubated sequentially with primary anti-BrdU (diluted 1:50 in blocking buffer) for 30 min, secondary horseraddish peroxidase (HRP)-conjugated rabbit anti-mouse-IgG1 (1:1000 in blocking buffer) for 30 min, and finally substrate (0.2 mg/ml *o*-phenylenediamine in TBS/0.1% Tween 20 containing 0.03% H_2O_2). The reaction was stopped with 2 N HCl, and the optical density measured at 490 nm. Negative control wells (background wells) were treated identically except that no BrdU was added. Specific BrdU uptake was determined by subtracting background wells (no BrdU added) from wells receiving BrdU. Results were normalized to cellular protein quantitated by the bicinchoninic acid method (BCA Protein Assay, Pierce, Rockford, IL).

2.5. Reverse-transcription polymerase chain reaction (RT–PCR)

Total RNA was extracted from adult human primary keratinocytes by the acid guanidinium thiocyanate–phenol–chloroform method [45]. Primers and reaction temperature for first strand DNA synthesis are as described in the figure legend (Fig. 1). First strand DNA synthesis was otherwise performed using Superscript II reverse transcriptase and the supplied buffer per the manufacturers instructions (Gibco BRL, Gaithersburg, MD).

For EP_4 receptor PCR, amplification was performed on a Thermolyne Temptronic thermocycler (Barnstead/Thermolyne, Dubuque, IA) for 30 cycles. Denaturation, annealing, and polymerization temperatures were 95°C × 1 min, 56°C × 1 min, and 72°C × 30 s, respectively. Reaction components were 1.0 unit Taq DNA polymerase, 20 mM Tris–Hcl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 200 μM dNTP's, and 10 pmol each of forward primer [5′-ATCTTACTCATTGCCACC-3′] and reverse primer [5′-TCTATTGCTTTACTGAGCAC-3′] in a 50 microliter reaction volume. The 212 bp product encodes nucleotides 1201–1412 in the cDNA sequence described in Bastien et al. [46]. The positive control PCR reaction contained approximately 10 ng of hEP_4-pcDNAI.

EP_3 receptor PCR was performed using identical conditions except that denaturation and annealing temperatures were set at 30 s rather than 1 min. The forward [5′-GGCACGTGGTGCTTCATC-3′] and re-

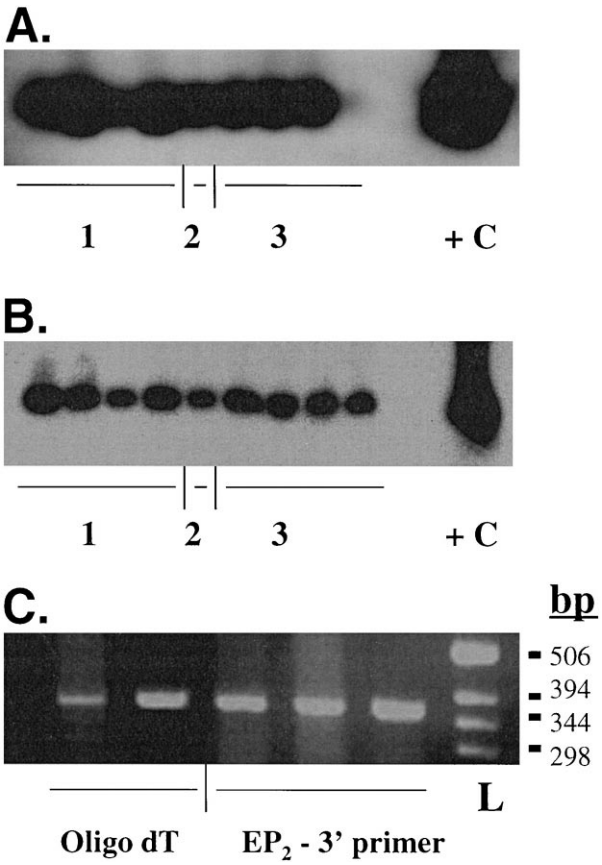

Fig. 1. Primary adult human keratinocytes express mRNA for Prostaglandin E receptor subytes (EP_4, EP_3, and EP_2) by RT–PCR. (A) RT–PCR and southern hybridization with EP_4 receptor specific primers from 4 different RNA preparations from individual 1, a single RNA preparation from individual 2, and 4 separate RNA preparations from individual 3. Primary adult human keratinocytes were grown on 100 mm dishes coated with collagen and RNA extracted as detailed in Section 2. Reverse transcription was performed using the EP_4 PCR reverse primer (see Section 2) at 37°C for 10 min, then at 50°C for 50 min. PCR and southern hybridization was performed as described. (B) RT–PCR and southern hybidization with primers and conditions specific for the EP_3 receptor. Samples and reverse transcription conditions are as stated for Fig. 1(A), except the EP_3 reverse primer was used for first strand synthesis. (C) Ethidium-bromide stained 2% agarose gel of EP_2 RT–PCR products from a RNA isolated from keratinocytes from a single individual. Lanes 1 and 2 (from left to right) represent duplicate reactions using oligo dT primers for first strand synthesis. Reverse transcription was done at 37°C for 1 h. Lanes 3–5 represent three seperate RNA preparations from the same individual, with the reverse transcription carried out using the EP_2 PCR reverse primer (see Section 2) for first strand synthesis as above (Fig. 1(A) and (B)). Lane L represents the DNA size ladder.

verse [5′-GGGTCCAGGATCTGGTTC-3′] primers were designed from sites of considerable DNA sequence homology between the EP_1, EP_3, and EP_4 receptors. However, the primers specifically amplified only EP_3 under the conditions utilized. The forward primer corresponds to amino acids GTWCFI in the second extracellular loop of EP_3 receptors. The reverse primer corresponds to amino acids LNQILDP in the seventh transmembrane domain. The 416 bp product encodes a fragment which is upstream of the known alternative splice sites for EP_3 splice variants. The positive control PCR reaction contained approximately 10 ng of hEP_{3I}-pcDNAI.

Following electrophoresis in a 2% agarose gel and staining with ethidium bromide, bands were poorly visualized in the EP_4 and EP_3 reactions. Therefore, southern hybridization was performed following transfer to nylon membranes (GeneScreen, NEN Research Products, Boston, MA) using full-length [^{32}P]-cDNA probes prepared by random primed labeling with [^{32}P]-dCTP (3000 Ci/mmol, Amersham, Arlington Heights, IL) under stringent conditions. Hybridization signals were visualized by autoradiography using Kodak Biomax film (Eastman Kodak, Rochester, NY) at −80°C overnight with a single enhancing screen.

EP_2 receptor PCR was performed using a forward primer [5′-CTTACCTGCAGCTGTACG-3′] and reverse primer [5′-GATGGCAAAGACCCAAAGG-3′] which amplify a 368 basepair fragment encoding nucleotides 740 to 1107 of the human EP_2 receptor using PCR conditions as described by Regan et al. [47]. The PCR product was band purified from a 2% agarose gel, and the identity confirmed by restriction digest using 4 separate restriction enzymes (Data not shown).

2.6. Northern Hybridization

Poly(A)$^+$ RNA was prepared using the oligotex spin column chromotagraphy procedure (Qiagen, Chatsworth, CA). Poly(A)$^+$ RNA was electrophoresed in 1% agarose gels containing 0.22 M formaldehyde and transferred to nylon membranes (GeneScreen, NEN Research Products, Boston, MA).

Probes used for hybridization of EP_2 receptor mRNA were prepared by random priming (Ready-to-Go, Pharmacia Biotech, Piscataway, NJ) using

[α^{32}P]dCTP (3000 Ci/mmol, Amersham, Arlington Heights, IL). The EP_2 receptor probe was prepared by labeling a 368 bp RT–PCR fragment obtained from human keratinocyte total RNA as described above. An EP_4 receptor antisense riboprobe was prepared from pcDNAI-hEP_4 by digestion with Asp700, followed by in vitro transcription with SP6 RNA polymerase using the Riboprobe Gemini System II (Promega, Madison, WI). An EP_3 receptor antisense riboprobe was prepared using the SP6 transcriptional promoter as above from a KspI restriction digest of pcDNAI-hEP_{3I}. Riboprobes were labeled with [α^{32}P]CTP (800 Ci/mmol, Amersham, Arlington Heights, IL).

In all cases, the membranes were prehybridized in 6 × SSPE, 50% formamide, 1% SDS, and 5 × Denhardt's reagent at 42°C for 1–2 h. Hybridization was done in the same buffer for 18–24 h at 42°C. Membranes were washed as described in the figure legends (Fig. 2) and exposed for 1–3 days in a Molecular Dynamics phosphoimager (PhosphoImager, model 425B; Sunnyvale, CA).

3. Results

3.1. Primary non-confluent human keratinocytes express transcripts for the EP_2, EP_4, and EP_3 subtypes of prostaglandin E receptors: EP_2 transcripts are expressed at higher levels than EP_4 transcripts

To determine which receptors are expressed transcriptionally in non-confluent human keratinocytes in vitro, RT–PCR was performed which demonstrated products of the correct size for EP_2, EP_4, and EP_3 receptors (Fig. 1). No bands were visible in paired negative control reactions in which reverse transcriptase was omitted (Data not shown).

In order to establish the relative quantities of the different receptors and to establish the size and/or number of EP_3 splice variants which are expressed in keratinocytes, northern hybridization was performed using poly(A)$^+$ RNA from cultured non-confluent human keratinocytes (Fig. 2). A band of approximately 3.1 kb was observed for the EP_2 receptor (Fig. 2(A)) and a faint band of approximatley 3.8 kb was

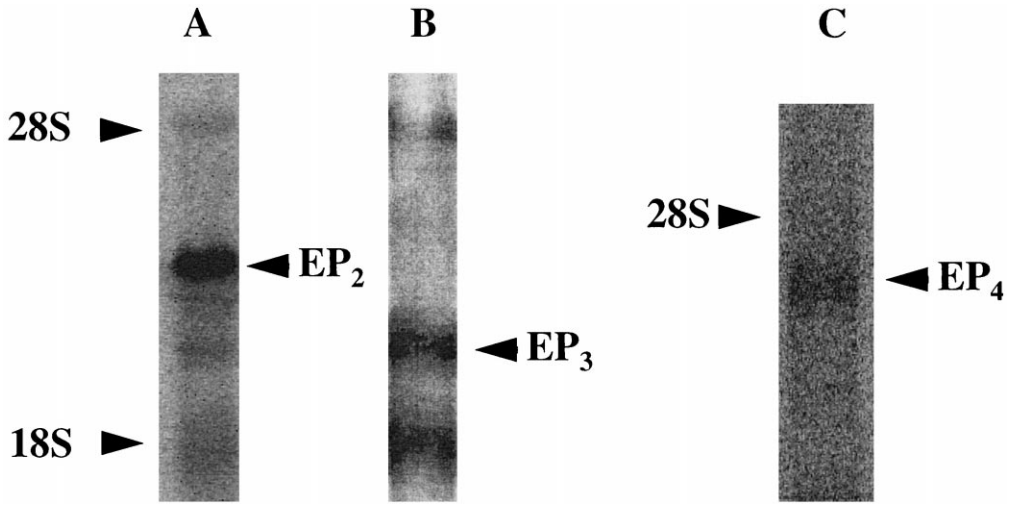

Fig. 2. Northern hybridization of poly(A)$^+$ enriched RNA with [^{32}P]-labeled probes specific for EP_2 (Fig. 2(A)), EP_{3I} (Fig. 2(B)), and EP_4 (Fig. 2(C)). (A) 3 μg poly (A)$^+$ RNA hybridized with EP_2 probe as described in experimental procedures. The membrane was washed twice at room temperature and once at 42°C with 2 × SSPE with 0.1% lauryl sulfate. This was followed by a final wash with 1 × SSPE with 0.1% lauryl sulfate at 50°C. (B) and (C) 7 μg of poly (A)$^+$ RNA hybridized with EP_3 and EP_4 probes as described in experimental procedures. The membranes were washed sequentially, first at room temperature, then at 42°C with 2 × SSPE with 0.1% lauryl sulfate followed by 1 × SSPE with 0.1% lauryl sulfate.

observed for the EP_4 receptor (Fig. 2(C)). We also performed northern hybridization for EP_2 receptors on poly(A)$^+$ RNA isolated from HaCat cells, a spontaneously immortalized keratinocyte cell line, and SCC-25 cells, a squamous cell carcinoma cell line. Both cell lines exhibited expression of the 3.1 kb EP_2 transcript (Data not shown). A faint band was also observed by northern hybridization of poly(A)$^+$ RNA from HaCat cells with the EP_4 receptor probe (Data not shown).

We next attempted to demonstrate the presence of EP_3 transcripts. Using an EP_{3I} riboprobe, an intense band at approximately 2.4 kb was apparent (Fig. 2(B)). This 2.4 kb band was also observed by northern hybridization using poly(A)$^+$ RNA prepared from SCC-25 cells (data not shown). In addition, the probe also hybridized strongly with regions corresponding with residual 28S and 18S ribosomal RNA (approximately 1.9 and 5 kb). However, the signal intensity at these sites was considerably less than that observed with 10 μg of total RNA which was run in a parallel lane, suggesting that these bands represented cross-hybridization with ribosomal RNA and not specific hybridization with the EP_3 receptor.

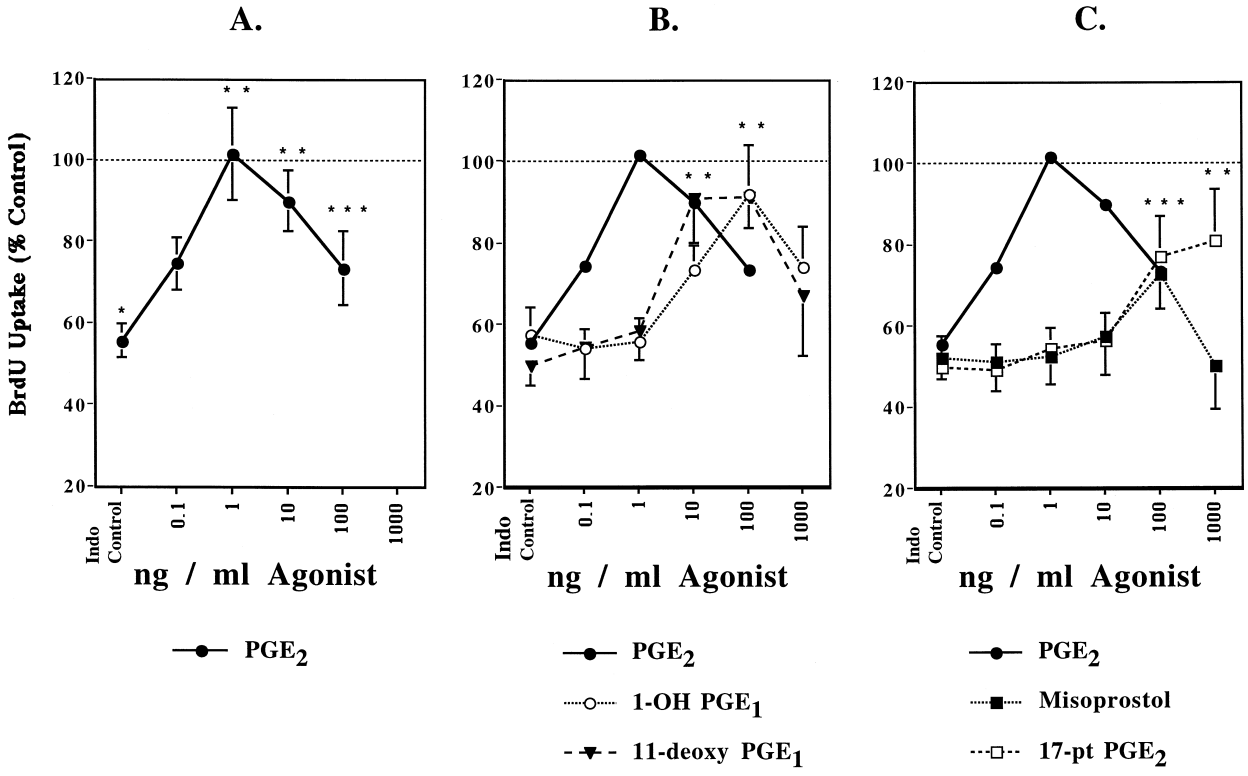

Fig. 3. Agonists specific for EP_2 and EP_4 receptors reverse indomethacin-induced growth inhibition in primary adult human keratinocytes. Receptor-specific agonist stimulation of bromodeoxyuridine uptake in indomethacin-treated non-confluent primary adult keratinocytes (approximately 50–70% confluent at the end of experiment). Expressed as percent of control cells which are grown in the absence of indomethacin and agonists. Mean and s.e.m. of 5–7 separate experiments. (A) Indomethacin inhibits growth to approximately 55% of control cells ($* = P < 0.01$, paired *t*-test). Exogenous PGE_2 addition restores growth to basal growth levels ($** = P < 0.05$ compared with indomethacin control; ANOVA) and demonstrates a bell-shaped dose-response pattern, with 100 ng/ml PGE_2 less effective than 1 ng/ml PGE_2 ($*** = P < 0.1$, paired *t*-test). (B) Growth restoration by EP_2 and EP_4 specific agonists. 1-OH PGE_1 and 11-deoxy PGE_1 compared with PGE_2. ($**$ = not significantly different than the response observed for PGE_2 at 1 ng/ml, but significantly different than the indomethacin control, $P < 0.05$, ANOVA). (C) Growth restoration by $EP_3 > EP_2$, EP_4 agonist misoprostol and the $EP_1 > EP_2$, EP_4 agonist 17-pt-PGE_2. ($**$ = not significantly different than the response observed for PGE_2 at 1 ng/ml, but significantly different from the indomethacin control, $P < 0.05$, ANOVA). ($***$ = less than the response observed with 1 ng/ml PGE_2, $P = 0.095$, paired *t*-test).

3.2. Reversal of indomethacin-induced growth inhibition: EP receptor agonist dose-response profiles

This set of experiments was designed to determine which PGE_2 receptor(s) are involved in the ability of exogenous PGE_2 to reverse indomethacin-induced growth inhibition in primary cultures of non-confluent adult human keratinocytes [22]. Growth was measured by the incorporation of bromodeoxyuridine into cellular DNA during S phase of DNA replication by a specific enzyme immunoassay (See Section 2). Indomethacin (10 μg/ml) significantly ($P < 0.01$) inhibited growth of non-confluent primary keratinocytes to approximately 50% of control levels (cells grown in the absence of indomethacin) (see Fig. 3). Addition of exogenous PGE_2 reversed the indomethacin-induced growth arrest in a dose-dependent manner, with a maximal effect observed at 1 ng/ml which completely reversed indomethacin-induced growth inhibition. Higher concentrations of PGE_2 did not result in any further increase in growth rates above control levels, but resulted in a paradoxical decrease in bromodeoxyuridine incorporation. The highest concentration of PGE_2 tested (100 ng/ml), showed significantly less activity ($P < 0.1$) than the maximal concentration of 1 ng/ml at reversing indomethacin-induced growth inhibition.

To determine which receptor(s) mediate this effect, the ability of different receptor-specific agonists (See Table 1) to restore indomethacin-induced growth arrest was determined. In Fig. 3, exogenous addition of 11-deoxy PGE_1, 1-OH PGE_1, misoprostol, and 17-phenyl trinor PGE_2 to indomethacin-treated, non-confluent keratinocytes all resulted in a significant reversal of growth inhibition in a dose-dependent manner. Agonists with greater specificity for EP_2 and EP_4 receptors, 11-deoxy PGE_1 and 1-OH PGE_1, caused nearly complete reversal of indomethacin-induced growth inhibition at a concentration of 100 ng/ml. At this concentration, growth reversal was not significantly different from the maximal response for PGE_2 (1 ng/ml). In contrast, the maximal response for misoprostol ($EP_3 > EP_2$), observed at an agonist concentration of 100 ng/ml, was less than that for PGE_2 ($P = 0.095$; paired t-test) (Fig. 3(C)). All agonists, with the exception of 17-phenyl trinor PGE_2, showed a bell-shaped dose-response profile as was seen with PGE_2, with bromodeoxyuridine uptake reaching a maximal level, followed by decreased uptake at higher concentrations. The rank order of activity of all agonists was PGE_2 > 11-deoxy PGE_1 > 1-OH PGE_1 > 17-phenyl trinor PGE_2 = misoprostol.

Table 1
Reported specificities of EP receptor agonists references [10,16,47,60,61]

Receptor Agonist	Relative Specificity
PGE_2	$EP_2 = EP_4 = EP_3 = EP_1$
11-deoxy PGE_1	EP_2,$EP_4 > EP_3 > EP_1$
1-OH PGE_1	EP_2,EP_4
19-OH PGE_2	EP_2
Misoprostol	$EP_3 > EP_2 = EP_4 \gg EP_1$
17-Phenyl trinor PGE_2	$EP_1 > EP_3 > EP_2$,EP_4
Sulprostone	$EP_3 > EP_1 \gg EP_2 > EP_4$

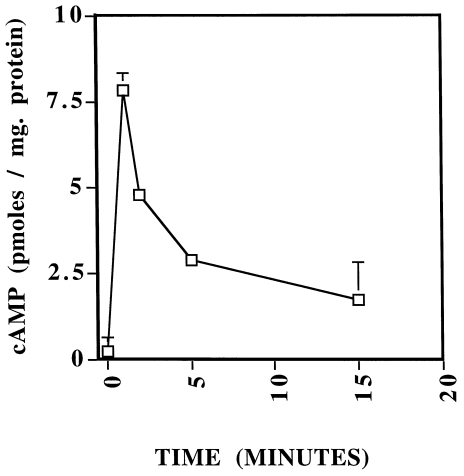

Fig. 4. Stimulation of cAMP by 100 nM PGE_2 over time in the absence of phosphodiesterase inhibitors. Non-confluent primary adult human keratinocytes were pretreated overnight with indomethacin (3 μg/ml) and then stimulated for the indicated times with 100 nM PGE_2 in serum free DMEM. Mean and s.e.m. of two separate experiments done in triplicate. Cyclic AMP was normalized to cellular protein.

3.3. Agonist dose-response profiles for activation of adenylyl cyclase

In the above experiments, EP_2 and EP_4 selective agonists showed the greatest ability to reverse indomethacin-induced growth inhibition. Since EP_2 and

EP_4 receptors are known to stimulate cAMP production, and cAMP has been suggested to be a positive growth signal in keratinocytes, we examined the ability of the different receptor agonists to stimulate cAMP production. Fig. 4 illustrates a typical time course experiment measuring cyclic AMP levels in pre-confluent keratinocytes in response to 100 nM PGE_2 in the absence of phosphodiesterase inhibitors.

To determine which receptor mediates this increase in cAMP levels, cAMP dose-response curves elicited by a panel of selective agonists for the various EP receptor types were generated (Fig. 5(A)–(C)). A common phenomenon associated with G-protein coupled receptors is the desensitization and down-regulation of receptor-mediated second messenger response pathways [10]. This phenomenon has been observed in PGE_2 receptors [48,49]. In preliminary experiments, down-regulation or desensitization of the cAMP response to PGE_2 was observed. Cells which had been pretreated with indomethacin to block receptor down-regulation or desensitization by endogenously produced PGE_2 showed approximately a 4-fold increased cAMP response (in the presence of IBMX) to exogenous PGE_2 than cells not treated with indomethacin (Data not shown). Therefore, to maximize the observed difference in cAMP responses elicited by the different receptor agonists, dose-response profiles were done in cells pretreated for 9 h in the presence of 3 μg/ml indomethacin.

In Fig. 5(A), dose-response profiles for the EP_2 and EP_4 receptor agonists 11-deoxy PGE_1 and 1-OH PGE_1, and the EP_2 receptor agonist 19-OH PGE_2, are compared with the dose-response profile for PGE_2. PGE_2 stimulates a dose-dependent increase in

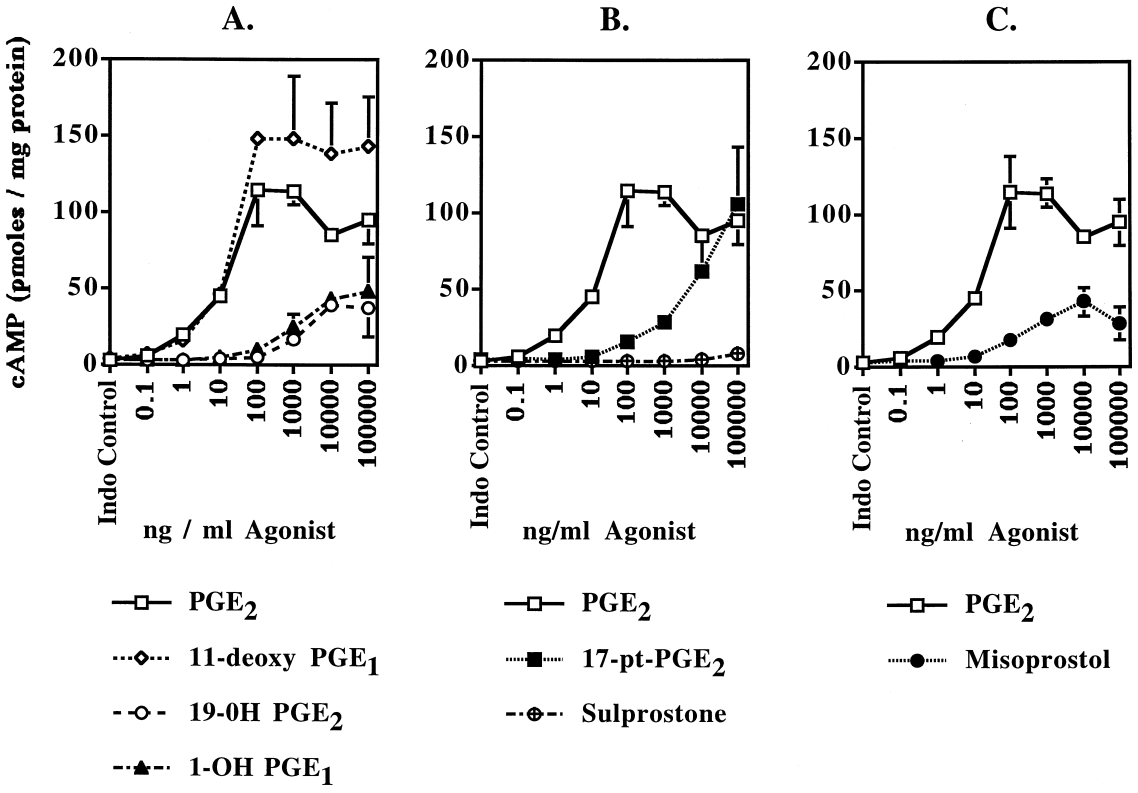

Fig. 5. Agonists specific for EP_2 and EP_4 receptors stimulate cAMP production in primary adult human keratinocytes. Agonist dose-response curves for cAMP stimulation in the presence of 2 mM isobutyl methyl xanthine (IBMX), a phosphodiesterase inhibitor. Non-confluent primary adult human keratinocytes pretreated overnight with indomethacin (3 μg/ml) were stimulated for 15 min with increasing concentrations of agonist with indomethacin (3 μg/ml) in serum free DMEM. Control cells received indomethacin alone. Results are expressed as the mean and standard deviation of two separate experiments using pooled triplicate wells for each cAMP determination.

cAMP levels with an EC_{50} of 7.8 ng/ml (27.3 nM) which is in general agreement with the reported EC_{50} for EP_2 receptors in other cell types [47,49]. The curve for 11 deoxy-PGE_1 was similar to that for PGE_2. However, while 19-OH PGE_2 and 1-OH PGE_1 exhibited similar dose-response curves, both showed decreased potency for cAMP stimulation when compared with PGE_2.

Fig. 5(B) and (C) demonstrates the cAMP dose-response curves generated by the $EP_1 > EP_2$ specific agonist 17-phenyl trinor PGE_2, the $EP_3 > EP_2/EP_4$ agonist misoprostol, and the EP_3/EP_1 agonist sulprostone. Compared with the dose-response profile for PGE_2, 17 phenyl trinor PGE_2 was less active. However, 17 phenyl trinor PGE_2 exhibited greater potency than misoprostol, 19-OH PGE_2, or 1-OH PGE_1. Misoprostol elicited a cAMP response similar to that for 19-OH PGE_2 and 1-OH PGE_1. Sulprostone did not elicit a cAMP response except at the highest dose (100 μg/ml). The relative potency for the different agonists were in the order 11d-PGE_1 = PGE_2 > 17 phenyl trinor PGE_2 > 19-OH-PGE_2 = 1-OH-PGE_1 = misoprostol > > sulprostone.

Activation of EP_3 receptors has been shown to dramatically inhibit forskolin-induced cAMP production with IC_{50}'s of 0.1 to 3 nM [11]. This activity is blocked by pertussis toxin, suggesting interaction with heterotrimeric G-proteins containing the $G_{i\alpha}$ subunit. To examine this, we stimulated non-confluent keratinocytes with forskolin, a direct activator of adenylyl cyclase, both in the presence and absence of increasing concentrations of sulprostone. Sulprostone was unable to decrease the cAMP production in response to forskolin up to concentrations of 100 ng/ml (approx. 350 μM) (data not shown). Moreover, pretreatment with pertussis toxin had no effect on the dose-response curves for cAMP production using the EP_3 agonists misoprostol and sulprostone (data not shown).

3.4. Indomethacin-induced growth inhibition is reversed by exogenous addition of dibutyryl cAMP

To demonstrate whether the increase in cAMP levels obtained by activation of EP_2 and/or EP_4 receptors is the signaling mechanism involved in stimulating BrdU incorporation, we next sought to determine whether BrdU uptake could be restored to control levels in indomethacin-treated keratinocytes by treatment with a cell permeable analogue of cAMP (dibutyryl cAMP). If cAMP is required for PGE_2 stimulated growth, then addition of exogenous cAMP should reverse indomethacin-induced growth inhibition. Dibutyryl cAMP was shown to dose-dependently restore growth to control levels in indomethacin treated keratinocytes (Fig. 6).

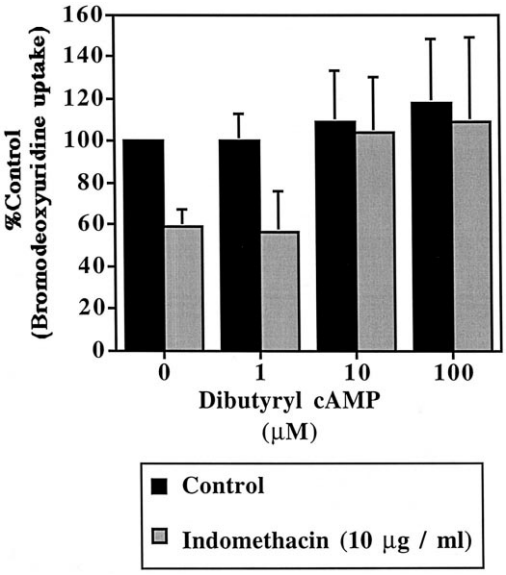

Fig. 6. Dibutyryl cAMP dose-dependently restores growth in indomethacin (10 μg/ml) treated non-confluent keratinocytes. Cells were treated with dibutyryl cAMP in the presence and absence of indomethacin for two days with daily changes of media and reagents. Growth was measured by bromodeoxyuridine uptake by EIA as described in experimental procedures. Expressed as percent of control cells (no indomethacin and no dibutyryl cAMP). Mean and standard deviation of two experiments done in quadruplicate.

3.5. Sulprostone inhibits bromodeoxyuridine uptake in indomethacin-treated non-confluent keratinocytes

Sulprostone, which is virtually devoid of EP_2 or EP_4 binding activity, is a potent agonist for EP_3 and EP_1 receptors. In Fig. 7, sulprostone induced a dose-dependent decrease in BrdU uptake up to 30% greater at 0.1 ng/ml than cells treated with indomethacin alone ($P = 0.118$; ANOVA). At concentrations higher than 10 ng/ml, the decrease in BrdU uptake began to

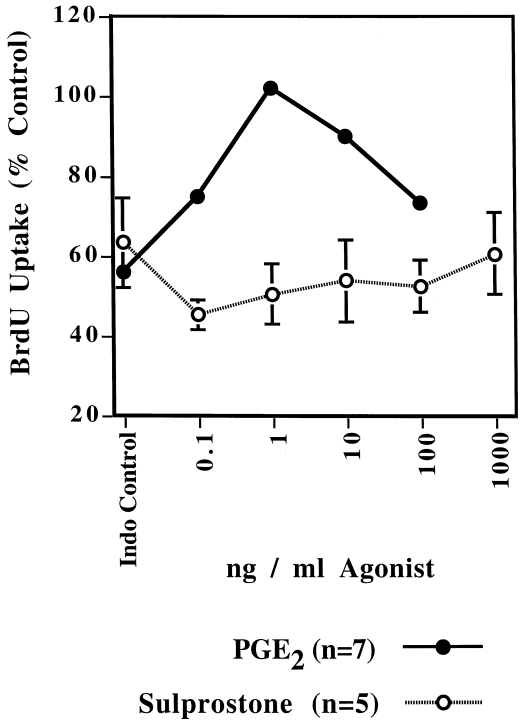

Fig. 7. Sulprostone inhibits growth of primary adult human keratinocytes in the absence of endogenous PGE_2. Dose-response growth assay by bromodeoxyuridine uptake EIA using increasing concentrations of the $EP_3 > EP_1$ agonist sulprostone compared with PGE_2. Results and experimental protocol are as described in Fig. 3 and experimental procedures. Results are expressed as the mean and s.e.m. of 5 separate experiments for sulprostone and 7 experiments for PGE_2 (see Fig. 3(A)).

normalize to levels obtained in the indomethacin-treated control cells.

4. Discussion

Mechanistic studies of the role of PGE_2 in vivo are complicated by the difficulty in dissecting direct effects of PGE_2 on cellular proliferation from indirect contributions from increased blood flow and inflammatory infiltrates. Previously, it was shown that endogenous PGE_2 stimulates growth of non-confluent human keratinocytes in vitro [22]. Keratinocytes growing in non-confluent culture phenotypically and biochemically resemble the ''activated'' basal cell population responsible for reepithelialization following cutaneous wounds [50,51], and after application of non-mutagenic tumor promoters [7]. Thus, non-confluent cultures offer an attractive model for studying the receptor-mediated growth promoting effects of PGE_2 on activated keratinocytes. In this report, we demonstrate by RT–PCR and northern hybridization that non-confluent primary adult human keratinocytes express message for EP_2, EP_4, and EP_3 receptors. Previously, PGE_2 was shown to increase cAMP levels in human and rodent skin [40–42]. However, demonstration of specific PGE_2 receptor subtype expression in skin has not been reported. In addition, the receptor subtype(s) and signaling mechanism which mediate the proliferative response is unknown. We demonstrate that EP_2, and possibly EP_4 receptors, mediate the growth promoting effects of endogenous PGE_2 production, and that this effect is dependent on cAMP signaling. Moreover, the concentration of PGE_2 (1 ng/ml) which gives a maximal growth response in indomethacin-treated keratinocytes (Fig. 3) is in general agreement with both the observed concentrations of endogenous PGE_2 produced by non-confluent cultures (5.09 ± 3.29 ng/ml/24 h; mean and standard deviation, $n = 11$) and the EC_{50} for stimulation of adenylyl cyclase (7.8 ng/ml). These observations, plus the observed bell-shaped growth response curve suggest that PGE_2 levels are tightly regulated to maximize growth and prevent receptor down-regulation. In addition, keratinocytes also express at least one splice variant of the EP_3 receptor subtype, activation of which causes growth inhibition in a cAMP-independent manner.

By northern analysis (Fig. 2), the EP_2 receptor subtype, as described by Regan et al. [47], appears to be present at much higher levels than the levels observed for the EP_4 receptor subtype, suggesting that the EP_2 receptor is the predominant receptor responsible for both the observed growth effects (Fig. 3) and the stimulation of cAMP production (Fig. 5) by agonists specific for EP_2 and EP_4 receptor subtypes. However, little is known about post-transcriptional regulation of these receptors. Since mRNA expression does not always correlate with protein levels, a significant contribution by EP_4 receptors cannot be excluded. In addition, a band of approximately 2.4 kb was observed by northern hybridization using an EP_{3I} riboprobe. The size of this band is in agreement with a 2.4–2.5 kb band observed by northern blotting in previous studies of other tissue types [11–13].

To determine which receptor subtype(s) mediate proliferation, and whether these same subtype(s) also stimulate adenylyl cyclase, agonist dose-response profiles were generated using the agonists listed in Table 1. Agonists reported to have higher degrees of selectivity for EP_2 and/or EP_4 receptor activation stimulated cAMP production as well as reversed indomethacin-induced growth inhibition. The increase in cAMP observed for these agonists appears to be a necessary signal for PGE_2 stimulated growth, since addition of exogenous dibutyryl cAMP was also able to completely reverse indomethacin-induced growth arrest (Fig. 6). Taken together, this data indicates that proliferation is stimulated by EP_2 and/or EP_4 receptors coupled to adenylyl cyclase activation, although the northern data suggest that EP_2 receptors are the predominant receptor type mediating this effect. However, it is unclear whether the observed growth effects are mediated by direct stimulation of cells to enter the cell cycle, or by blocking entry into terminal differentiation or apoptosis. The ability of cAMP-elevating agents to inhibit epidermal growth factor (EGF)-induced differentiation has been suggested in the epidermoid carcinoma cell line A431 [52,53]. In addition, keratinocytes undergo apoptosis [54], and a role for PGE_2 and cAMP in negative regulation of apoptosis is demonstrated in other cell types [55,56].

Contrary to the growth promoting effects of EP_2/EP_4 receptors, sulprostone, which is virtually devoid of activity at either EP_2 or EP_4 receptors, caused a decrease in bromodeoxyuridine uptake when compared with indomethacin-treated cells alone. This suggests that isolated activation of EP_3 receptors constitutes a negative growth signal. However, this effect does not appear to result from direct inhibition of adenylyl cyclase. Sulprostone had no effect on basal cAMP production (Fig. 5), nor did it inhibit the cAMP response elicited by forskolin, a direct activator of adenylyl cyclase, both in the presence and absence of pertussis toxin (data not shown). It is possible that the growth inhibitory effects observed with sulprostone are in part mediated by EP_1 receptors. Using RT–PCR, preliminary data suggests that the EP_1 receptor is expressed in normal adult confluent keratinocytes, HaCat cells, and in epidermoid carcinoma cells (A431) (data not shown). However, the rather potent growth stimulating activity of 17-phenyl trinor PGE_2, with rank agonist potency of $EP_1 > EP_2 > EP_3$, and the relatively poor growth stimulatory activity of misoprostol ($EP_3 > EP_2 > EP_4 > EP_1$), suggests that the EP_3 receptor subtype is primarily responsible for the observed growth inhibition.

The absence of any observable effect of EP_3 receptor activation on cAMP production suggests that the growth inhibitory activity of sulprostone could be the result of activation of alternative second messenger cascades, possibly activation of phospholipase C and/or intracellular calcium mobilization [11,12,16]. Negative growth regulation by EP_3 receptors could result from direct mitogenic inhibition, increased apoptosis, or increased rate of entry into terminal differentiation. PGE_2 receptors acting through phospholipase C and calcium signaling are likely to be important regulators of keratinocyte differentiation since calcium and protein kinase C are potent inducers of keratinocyte differentiation [57–59].

In conclusion, these observations are relevant to our understanding of the role of PGE_2 in regulating normal, hyperplastic, and neoplastic cell growth. In parallel with the observations reported here, both PGE_2 and cAMP stimulate proliferation in normal mammary epithelial cells [34,39], but PGE_2 has variable activity in stimulating growth of different breast cancer cell lines [32,34,35]. This heterogeneity of response may be due to altered PGE_2 receptor expression. Planchon et al. [35], using scatchard analysis of specific [^{3}H]-PGE_2 binding, demonstrated that Ha-ras transfected MCF-7 (MCF-7ras) human ductal breast carcinoma cells exhibited more than 80% loss of high-affinity PGE_2 binding sites ($K_d = 0.1$ nM) and a two-fold increase in low-affinity binding sites ($K_d = 35$ nM) compared with non-transfected MCF-7 cells. Moreover, the MCF-7ras cells exhibit an advanced tumorigenic phenotype. The presence of high-affinity binding sites (K_d of 0.1 nM) in the human breast cancer cell line (MCF-7) is consistent with the K_d observed for EP_3 receptor subtypes [9–11]. The low-affinity binding sites may represent EP_2 receptors, since PGE_2 was shown to stimulate cAMP production [35]. Moreover, the loss of these high-affinity receptors and increase in low-affinity receptors was associated with increased tumorigenicity and a more aggressive histologic phenotype. In this report, we have shown that selective stimulation

of PGE_2 receptors modulate epithelial cell growth, thus raising the possibility that alterations of normal receptor number or function could be associated with increased tumorigenicity, tumor proliferation, or invasive behavior in epithelial malignancies.

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References

[1] A.P. Pentland, J. George, C. Moran, P. Needleman, Biochim. Biophys. Acta 919 (1987) 71–78.
[2] J.T. Lord, V.A. Ziboh, W.D. Cagle, S. Kursunoglu, G. Redmond, Adv. Prostaglan. Thrombox. Res. 7 (1980) 865–869.
[3] E.E. Vanderveen, R.C. Grekin, N.A. Swanson, K. Kragballe, Arch. Dermatol. 122 (1986) 407–412.
[4] F.M. Giardiello, G.J. Offerhaus, R.N. DuBois, Eur. J. Cancer 31A (1995) 1071–1076.
[5] V.E. Reeve, M.J. Metheson, M. Bosnic, C. Boehm-Wilcox, Cancer Lett. 95 (1995) 213–219.
[6] M.A. Shibata, R. Hasegawa, T. Shirai, Y. Takesada, S. Fukushima, Int. J. Cancer 55 (1993) 1011–1017.
[7] F. Marks, G. Furstenberger, T. Heinzelmann, K. Muller-Decker, Toxicol. Lett. 82 (1995) 907–917.
[8] M. Pollard, P.H. Luckert, Science 214 (1981) 558–559.
[9] M. Negishi, Y. Sugimoto, A. Ichikawa, J. Lipid Mediators Cell Signal 12 (1995) 379–391.
[10] F. Ushikubi, M. Hirata, S. Narumiya, J. Lipid Mediators Cell Signal 12 (1995) 343–359.
[11] A. Schmid, K.H. Thierauch, W.D. Schleuning, H. Dinter, Eur. J. Biochem. 228 (1995) 23–30.
[12] M. Kotani, I. Tanaka, Y. Ogawa, T. Usui, K. Mori, A. Ichikawa, S. Narumiya, T. Yoshimi, K. Nakao, Mol. Pharmacol. 48 (1995) 869–879.
[13] J.W. Regan, T.J. Bailey, J.E. Donello, K.L. Pierce, D.J. Pepperl, D. Zhang, K.M. Kedzie, C.E. Fairbairn, A.M. Bogardus, D.F. Woodward, D.W. Gil, Br. J. Pharmacol. 112 (1994) 377–385.
[14] H. Sakai, E. Kumano, A. Ikari, N. Takeguchi, J. Biol. Chem. 270 (1995) 18781–18785.
[15] S. An, J. Yang, S.W. So, L. Zeng, E.J. Goetzl, Biochemistry 33 (1994) 14496–14502.
[16] G. Asboth, S. Phaneuf, G.N. Europe-Finner, M. Toth, A.L. Bernal, Endocrinology 137 (1996) 2572–2579.
[17] M.E. Goldyne, J. Invest. Dermatol. 64 (1975) 377–385.
[18] J. Zhang, K. Maruyama, K. Iwatsuki, I. Ono, F. Kaneko, Exp. Dermatol. 3 (1994) 164–170.
[19] C.B. Bentley-Phillips, H. Paulli-Jorgensen, R. Marks, Arch. Dermatol. Res. 257 (1977) 233–237.
[20] N.J. Lowe, R.B. Stoughton, J. Invest. Dermatol. 68 (1977) 134–137.
[21] A. Lupulescu, Prostaglandins 10 (1975) 573–579.
[22] A.P. Pentland, P. Needleman, J. Clin. Invest. 77 (1986) 246–251.
[23] A.J. Francis, R. Marks, Br. J. Dermatol. 97 (1977) 395–399.
[24] R. Marks, A.J. Francis, Br. J. Dermatol. 97 (1977) 395–399.
[25] T.K. Hulsey, J.A. O'Neill, W.R. Neblett, H.C. Meng, J. Pediatr. Surg. 15 (1980) 505–508.
[26] P.H. Rolland, P.M. Martin, J. Jacquemier, A.M. Rolland, M. Toga, J. Natl. Cancer Inst. 64 (1980) 1061–1070.
[27] J.H. Botha, K.M. Robinson, N. Ramchurren, K. Reddi, R.J. Norman, J. Natl. Cancer Inst. 76 (1986) 1053–1056.
[28] I. Klapan, V. Katic, F. Culo, V. Cuk, J. Cancer Res. Clin. Oncol. 118 (1992) 308–313.
[29] F. Culo, I. Klapan, V. Katic, T. Kolak, B. Bakula, Med. Oncol. Tumor Pharmacother. 9 (1992) 35–39.
[30] A. Lupulescu, Oncology 37 (1980) 418–423.
[31] C.H. Snyderman, M.M. Abbas, R. Wagner, F. D'Amico, Arch. Otolaryngol. Head Neck Surg. 121 (1995) 1017–1020.
[32] M. Noguchi, M. Earashi, I. Miyazaki, M. Tanaka, T. Sasaki, Prostaglandins Leukotrienes Essent. Fatty Acids 52 (1995) 381–386.
[33] T.M. ElAttar, H.S. Lin, J. Oral Pathol. Med. 22 (1993) 425–427.
[34] K.E. McKenzie, G.K. Bandyopadhyay, W. Imagawa, K. Sun, S. Nandi, Prostaglandins Leukotrienes Essent. Fatty Acids 51 (1994) 37–43.
[35] P. Planchon, N. Veber, V. Magnien, L. Israel, A.B. Starzec, Mol. Cell. Endocrinol. 111 (1995) 219–223.
[36] A.M. Fulton, J.J. Laterra, C.M. Hanchin, J. Cell. Physiol. 139 (1989) 93–99.
[37] H. Green, Cell 15 (1978) 801–811.
[38] K. Takahashi, S. Nakanishi, S. Imamura, J. Invest. Dermatol. 101 (1993) 646–651.
[39] S.P. Ethier, R.M. van de Velde, K.C. Cundiff, Exp. Cell Res. 182 (1989) 653–658.
[40] K. Adachi, K. Yoshikawa, K.M. Halprin, V. Levine, Br. J. Dermatol. 92 (1975) 381–388.
[41] J.T. Lord, V.A. Ziboh, S.K. Warren, Endocrinology 102 (1978) 1300–1309.
[42] J.T. Lord, V.A. Ziboh, J. Invest. Dermatol. 73 (1979) 373–377.
[43] R. Marks, Cancer 75 (1995) 607–612.
[44] J.P. Magaud, I. Sargent, D.Y. Mason, J. Immunol. Methods 106 (1988) 95–100.

[45] P. Chomczynski, N. Sacchi, Anal. Biochemistry 162 (1987) 156–159.
[46] L. Bastien, N. Sawyer, R. Grygorczyk, K.M. Metters, M. Adam, J. Biol. Chem. 269 (1994) 11873–11877.
[47] J.W. Regan, T.J. Bailey, D.J. Pepperl, K.L. Pierce, A.M. Bogardus, J.E. Donello, C.E. Fairbairn, K.M. Kedzie, D.F. Woodward, D.W. Gil, Mol. Pharmacol. 46 (1994) 213–220.
[48] M. Negishi, Y. Sugimoto, I. Atsushi, S. Narumiya, A. Ichikawa, J. Biol. Chem. 268 (1993) 9517–9521.
[49] L. Zeng, S. An, E.J. Goetzl, Immunology 86 (1995) 620–628.
[50] Z. Bata-Csorgo, C. Hammerberg, J.J. Voorhees, K.D. Cooper, J. Clin. Invest. 95 (1995) 317–327.
[51] J.N. Mansbridge, A.M. Knapp, J. Invest. Dermatol. 89 (1987) 253–263.
[52] R.L. Konger, T.C.K. Chan, J. Cell. Physiol. 156 (1993) 515–521.
[53] S. Iwashita, K. Mitsui, Y. Shoji-Kasai, M. Senshu-Miyaike, J. Biol. Chem. 265 (1990) 10702–10708.
[54] C.A. McCall, J.J. Cohen, J. Invest. Dermatol. 97 (1991) 111–114.
[55] E.J. Goetzl, S. An, L. Zeng, J. Immunology 154 (1995) 1041–1047.
[56] M. Tsujii, R.N. DuBois, Cell 83 (1995) 493–501.
[57] J.J. Reiners, T.J. Slaga, Cell 32 (1983) 247–255.
[58] R. Le Panse, B. Coulomb, V. Mitev, B. Bouchard, C. Lebreton, L. Dubertret, Mol. Pharmacol. 46 (1994) 445–451.
[59] C.B. Evans, S. Pillai, M.E. Goldyne, Prostaglandins Leukotrienes Essent. Fatty Acids 49 (1993) 777–781.
[60] R.A. Coleman, S.P. Grix, S.A. Head, J.B. Louttit, A. Mallett, R.L. Sheldrick, Prostaglandins 47 (1994) 151–168.
[61] R.A. Lawrence, R.L. Jones, Br. J. Pharmacol. 105 (1992) 817–824.