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Increase in Epidermal Growth Factor Receptor and Its mRNA Levels by Parathyroid Hormone (1-34) and Parathyroid Hormone-Related Protein (1-34) During Differentiation of Human Trophoblast Cells in Culture

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Abstract Human cytotrophoblasts in culture aggregate and fuse to form syncytiotrophoblasts. This process is associated with an increase in epidermal growth factor receptor (EGFR) expression [Alsat et al.: J Cell Physiol 154:122–128, 1993]. Recent studies have demonstrated the presence of parathyroid hormone-related protein (PTHrP) in the human uterus and placenta. This led us to study the effect of PTH (1-34) and PTHrP (1-34) on the expression of EGFR during this differentiation process. Both peptides induced a concentration-dependent increase in EGF binding, with a maximal effect at the physiological concentration of 1 nM. EGFR protein level assessed by cross-linking and immunoblotting and EGFR biological activity assessed by measuring its EGF-induced autophosphorylation were increased 2- and 2.5-fold, respectively, when cells were treated for 24 h with 0.1 μM PTHrP or PTH compared to control cells. This effect was time-dependent with a maximum at 3 h of treatment. This treatment also increased trophoblast cell EGFR mRNA levels, suggesting transcriptional regulation of the EGFR. To ascertain whether activation of protein kinase C (PKC) or protein kinase A (PKA) is involved in this PTH effect, we determined EGFR protein level and EGFR autophosphorylation after exposure of cells to PKA inhibitor and PKC inhibitor, alone or together with the peptide. The presence of a PKC inhibitor blocked a further increase in EGFR number by PTH, while PKA inhibitor had no effect. These results show that PTH and PTHrP increase the synthesis of EGF receptors which are strongly expressed in syncytiotrophoblasts and suggested that these peptides might be involved in human placental development.

Key words: trophoblast differentiation, EGFR autophosphorylation, EGFR mRNA, PKA inhibitor, PKC inhibitor

The membrane receptor for epidermal growth factor (EGF) is a well-characterized 170 kDa tyrosine kinase [Cohen et al., 1982] comprising several regulatory domains with multiple functions. EGF binding to its receptor activates the protein kinase, which phosphorylates various cellular proteins, as well as the EGF receptor (EGFR) itself. This autophosphorylation process of EGFR is required to mediate physiological actions of EGF and reflects the presence of biologically active receptors in the cell [Schlessinger, 1986].

EGF has been shown to play a fundamental role in regulating the differentiation and endocrine functions of cultured trophoblast cells [Truman and Ford, 1986; Maruo et al., 1987; Morrish et al., 1987] and the human placenta contains large amounts of EGFR predominantly expressed in the syncytiotrophoblast [Magid et al., 1985; Rao et al., 1985]. In vitro, isolated human cytotrophoblasts aggregate and fuse to form syncytial cells [Kliman et al., 1986]. This morphological differentiation is associated with an increase in the levels of both EGFR and its mRNA levels [Alsat et al., 1993]. In addition, we have shown in a preliminary study that parathyroid hormone (PTH) is able to increase EGF binding

Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; FCS, fetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hCG, human chorionic gonadotropin; kb, kilobase; PAGE, polyacrylamide gel electrophoresis; PKA, protein kinase A; PKC, protein kinase C; PTH, parathyroid hormone; PTHrP, PTH-related protein; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

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capacity during the differentiation of human trophoblast cells in culture [Alsat et al., 1991].

PTH, an 84-amino acid protein, is a well-known hypercalcemic hormone [Habener et al., 1984]. PTH-related protein (PTHrP), a 141-amino acid molecule, has been recently isolated, sequenced, and cloned from a number of human and animal tumors associated with the paraneoplastic syndrome of humoral hypercalcemia [reviewed by Orloff et al., 1989; Malette, 1991]. These two peptides, which show a high degree of homology in their amino-terminal domains, have biological activities which can vary according to the tissue or the cell line used. Interestingly, they are able to interact at the cellular level with growth factors to modulate the expression of differentiated functions. For example, PTH opposes the effect of transforming growth factor (TGF) β on DNA synthesis and collagen production in osteoblast-enriched cultures [Centrella et al., 1988]. PTH increases insulin-like growth factor I (IGFI) concentrations in bone cultures thus modulating IGFI-stimulated collagen synthesis [Canalis et al., 1989]. Similarly, PTHrP modulates the effect of TGF β on DNA and collagen synthesis in fetal rat bone cells [Centrella et al., 1989]. Lastly, both peptides act synergistically with EGF to increase immortalized human keratinocyte growth [Henderson et al., 1992].

Specific receptors for PTH have been described in the human placenta [Lafond et al., 1988]. PTH-like bioactivity inhibited by synthetic PTHrP (1-34) has been reported in ovine placenta [Rodda et al., 1988] and PTHrP has recently been detected in human placenta by means of an immunohistochemical technique [Asa et al., 1990; Kramer et al., 1991]. In addition, human smooth muscle cells and endometrial stromal cells in culture express PTHrP mRNA and secrete bioactive PTHrP [Casey et al., 1992]. It is therefore of interest to study the role of these peptides in the human placenta. In the present study, we used human trophoblast cells in culture to determine whether both PTH (1-34) and PTHrP (1-34) could increase the expression of biologically active EGFR; the signal transduction pathway involved in this process is also discussed.

MATERIALS AND METHODS

Materials

Human recombinant ^{125}I-EGF (914–1,295 Ci/mmol), (γ-^{32}P) ATP (3,000 Ci/mmol), and (α-^{32}P) dCTP (3,000 Ci/mmol) were purchased from Amersham International (Buckinghamshire, UK). Human PTH (1-34) and an EGFR-cDNA probe (64-2 fragment) were kindly provided by Dr. F. Caulin and Dr. F. Bellot from Rorer Biotechnology (King of Prussia, PA). Human PTHrP (1-34) was purchased from Bachem (Hannover, Germany). A polyclonal antibody against the C-terminal part of EGFR was a generous gift from J. Schlessinger (New York University Medical Center, New York, NY). Protein kinase A (PKA) inhibitor was a competitive cAMP antagonist (Rp-cAMPs) from Biolog Life Science Institute (Bremen, Germany). The bisindolylmaleimide GF 109203X, a potent and selective protein kinase C (PKC) inhibitor, was a generous gift from Glaxo Laboratories Research Group (Les Ulis, France) [Toullec et al., 1991].

Cell Isolation and Culture

Term placentas were obtained after caesarean section from mothers with uncomplicated pregnancies. The study was approved by the local ethics committee. Placental tissues were aseptically processed within 30 min; isolated cytotrophoblasts were obtained from villous tissue after trypsin-DNase digestions and Percoll gradient by the method of Kliman et al. [1986] with minor changes as previously described [Alsat et al., 1991].

For primary culture, the purified cytotrophoblasts were diluted to a density of 1×10^6 cells/ml with Dulbecco's modified Eagle's medium (DMEM) containing 25 mM HEPES and supplemented with 2 mM glutamine, 20% heat-inactivated fetal calf serum (FCS), and antibiotics (100 μg/ml streptomycin, 100 IU/ml penicillin, 100 μg/ml gentamycin, and 2.5 μg/ml amphotericin B). The cells were plated and allowed to spread and adhere for at least 15 h in humidified 5% CO_2/95% air at 37°C. The characteristics of these cultured cells have been described elsewhere: syncytiotrophoblasts were characterized after 48 h of culture by means of scanning electron microscopy, immunohistochemistry, progesterone and β human chorionic gonadotropin (hCG) secretion [Malassiné et al., 1990], and human placental growth hormone (GH-V) production [Evain-Brion et al., 1990].

Cell Treatment

After plating, cells were treated with 0.1 μM PTH or 0.1 μM PTHrP in buffer (DMEM supplemented with 20% FCS) or with buffer alone

(control) for the times stated. When described, the effects of specific PKA and PKC inhibitors were tested on PTH-stimulated EGFR number and phosphorylation: 24 h cultured cells were treated for 3 h with 0.1 mM PKA inhibitor (a competitive cAMP inhibitor not hydrolyzed by common cyclic nucleotide-dependent phosphodiesterases) or with 5 μM PKC inhibitor [Toullec et al., 1991] in the presence and absence of 0.1 μM PTH.

EGF Binding Assay

After the appropriate time of culture the medium was aspirated and the cells were rinsed twice with DMEM containing 20 mM HEPES and 0.1% bovine serum albumin (BSA), pH 7.5 (binding medium). Unless otherwise stated, binding studies were performed at room temperature for 90 min with 38 pM ($7.3–10.6 \times 10^4$ cpm) ^{125}I-EGF in 1 ml of binding buffer. At the end of the incubation period, the cells were chilled on ice and rinsed rapidly with ice-cold phosphate-buffered saline (PBS), pH 7.4. Cell-associated radioactivity was then measured using a gamma-spectrometer (LKB, Pharmacia, St. Quentin-Yvelines, France) after cell lysis with 0.5 M NaOH [Mirlesse et al., 1990]. Specific binding was calculated from triplicate determinations by subtracting from total binding the nonspecific binding determined in the presence of an excess of unlabeled EGF (0.5 μM). Nonspecific binding was always less than 1% of total added radioactivity. Protein content was measured in cellular lysates in 0.5 M NaOH by means of the standard Bio-Rad assay.

Affinity Labeling of EGFR

Covalent cross-linking of ^{125}I-EGF to EGFR of trophoblast cells cultured in the absence or presence of PTH or PTHrP was performed using disuccinimidyl suberate (DSS) as the cross-linking reagent [Massagué, 1987]. Cultured cells were washed 3 times with buffer A (DMEM containing 25 mM HEPES and 0.2% BSA, pH 7.7). After 30 min at 4°C, the medium was aspirated and replaced with 1 ml of buffer A containing 1 nM ^{125}I-EGF. Incubation was performed for 3 h at 4°C. The cells were then washed 3 times with ice-cold buffer A and cross-linking was initiated by the addition of 10 μl of 25 mM DSS to 2 ml of BSA-free buffer A. After 15 min at 4°C with agitation, the cells were rapidly washed twice with buffer B (0.25 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4, 0.3 mM PMSF), scraped off into 1 ml of this buffer, and pelleted by centrifugation for 2 min at 12,000*g*. The cell pellets were solubilized with 60 μl of buffer C containing 125 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.0, 1% Triton X-100, and 10 μg/ml of the following protease inhibitors: leupeptin, antipain, aprotinin, soybean trypsin inhibitor, and benzamidine. After 40 min at 4°C, insoluble material was removed by centrifugation for 15 min at 12,000*g* and the solubilized proteins were subjected to sodium dodecyl sulfate (SDS) gel electrophoresis and autoradiography.

Cell Membrane Protein Preparation

Cytotrophoblast cells from term placentas were cultured as described above in the presence and absence of 0.1 μM PTH or PTHrP. The cells were washed twice with ice-cold PBS (pH 7.4) and once with homogenization buffer (0.25 M sucrose, 10 mM Tris HCl, pH 7.8, 1 mM $MgCl_2$, and 10 μg/ml of leupeptin, antipain, aprotinin, soybean trypsin inhibitor, and benzamidine). The cells were scraped off into 1 ml of buffer and homogenized in a Dounce homogenizer. The homogenate was centrifuged at 800*g* for 10 min and the supernatant was further centrifuged at 12,000*g* for 15 min. The resulting pellet (crude membranes) was resuspended either in lysis buffer (pH 7.4) containing 50 mM Tris, 125 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, and 10 μg/ml leupeptin and aprotinin for immunoblotting assays, or in buffer containing 20 mM Tris HCl, 50 mM NaCl, 1 mM $CaCl_2$, and 2 mM $MgCl_2$ (pH 7.4) for phosphorylation assays.

Western Immunoblotting

Samples (5 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA) at 20 V for 1 h using a semidry transfer cell (Bio-Rad, Richmond, CA). Immunoblotting was performed with a rabbit polyclonal anti-EGFR antibody against the C-terminal domain diluted 1:200 and revealed with donkey anti-rabbit IgG horseradish peroxidase (HRP)-linked antibodies diluted 1:10,000 and chemiluminescence (ECL, Amersham).

Phosphorylation of Membrane Protein

Cell membrane proteins (5 μg) were incubated in the presence or absence of 0.15 μM EGF with 5 μM (γ-^{32}P) ATP in a final volume of 50 μl in 16 mM HEPES buffer (pH 7.4) contain-

ing 0.1 mM AMP-PNP, 1 mM $MnCl_2$, and 0.1% BSA. After 10 min at 4°C, the reaction was terminated by adding 10 μl of Laemmli sample buffer and heating at 100°C for 3 min. The samples were then processed for SDS-PAGE and autoradiography. In some experiments, the radioactive EGFR band was excised from dried gels and counted in 5 ml of liquid scintillation cocktail (Beckman, Palo Alto, CA) to determine the relative ^{32}P incorporation.

Immunoprecipitation of EGFR

Membrane proteins from trophoblast cells cultured for 24 or 48 h were isolated and phosphorylated as described above. Labeled EGFR was precipitated using a polyclonal antibody against the C-terminal fragment diluted 1:50 in 500 μl of 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM PMSF, 1 mM sodium orthovanadate, and 10 μg/ml of the following protease inhibitors—leupeptin, antipain, aprotinin, soybean trypsin inhibitor, and benzamidine—for 2 h at 4°C. Immune complexes were conjugated to 1% protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) for 1 h at 4°C. The beads were then washed 4 times with 1 ml of the same buffer and the samples were subjected to 7.5% SDS-PAGE followed by autoradiography of the dried gel. Relative ^{32}P incorporation into the 170 kDa EGFR protein was evaluated as indicated above.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated from cultured cells by acid guanidinium thiocyanate phenol-chloroform extraction according to Chomczynski and Sacchi [1987] and quantitated by measuring absorbance at 260 nm. For Northern blotting, 15 μg of total RNA was denatured in 0.01 M phosphate buffer (pH 7.0) with 1 M glyoxal, fractionated by means of 1% agarose gel electrophoresis, and transferred to a nitrocellulose membrane [Thomas, 1980]. A 64-2 fragment of EGFR cDNA was used as the probe. The probe was labeled with (α-^{32}P) dCTP using a random primer DNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Prehybridization was carried out for 3 h at 42°C in 50% formamide containing 5× SSC, 5× Denhardt's solution, 0.1% SDS, and 0.1 μg/ml herring sperm DNA. The hybridization was performed overnight at 42°C in the buffer used for prehybridization but containing 10% dextran sulfate and the ^{32}P-labeled probe. The blots were washed 4 times in 2× SSC-0.1% SDS at 52°C for 30 min. After washing the blots were exposed to AR X-ray film (Amersham) and autoradiographed for 2 days at −80°C.

Data Analysis

All data are representative of at least two experiments performed with different placental culture preparations. The binding data are the means of triplicate determinations in a representative experiment, unless otherwise stated. The characteristics of EGF binding were assessed by making Scatchard plots of bound (B) and free (F) EGF at equilibrium from the EGF displacement data. The binding parameters were calculated by graphic analysis according to Rosenthal [1967]. Statistical significance was defined using Student's t-test, with a threshold of $P < 0.05$.

RESULTS

Effect of PTH (1-34) and PTHrP (1-34) on EGFR Number

Specific EGF binding activity increased when trophoblast cells were exposed to PTH or PTHrP. As shown in Figure 1, the effect was concentration-dependent. Maximal EGF binding activity was reached with 10^{-9} M PTH or PTHrP. The half-maximal effective concentrations were 1×10^{-12} and 1×10^{-11} M for PTH and PTHrP, respectively. Under these experimental conditions, PTH stimulated EGF binding capacity four- to fivefold, while PTHrP gave a three- to fourfold increase. Scatchard plots of EGF binding to trophoblast cells in culture were curvilinear and compatible with two classes of binding site. PTH- and PTHrP-treated cells showed an increase in the number of high-affinity sites, but no change in their affinity (Table I): mean B_{max} values for control cells were 0.60 ± 0.07 compared to 1.24 ± 0.10 and 1.09 ± 0.09 pmol/mg protein for PTH- and PTHrP-treated cells, respectively ($P < 0.01$). Neither the mean B_{max} value (3.50 ± 0.45 pmol/mg protein) nor the apparent K_d (3.8 nM) of low-affinity sites was altered by PTH or PTHrP.

As shown in Figure 2A, a labeled band with an estimated molecular mass of 170 kDa appeared when ^{125}I-EGF was affinity cross-linked to trophoblast cells and analyzed by means of SDS-PAGE. The labeling was abolished when ^{125}I-EGF was bound in the presence of 0.5 μM unlabeled EGF. More-intense bands were observed with affinity-labeled protein from cells cultured in the presence of 0.1 μM PTH or PTHrP. Relative to control cells, PTH and PTHrP treatment increased the amount of affin-

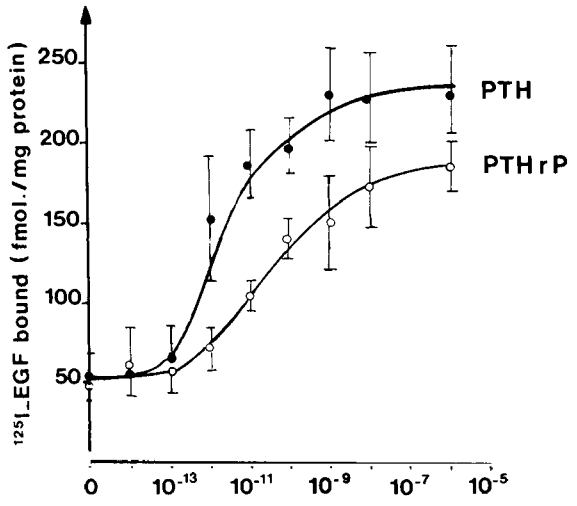

Fig. 1. *Effect of PTH* and PTHrP on the ^{125}I-EGF binding activity of trophoblast cells. Concentration-response curves: monolayer cultures were incubated in DMEM containing FCS (20%) with increasing concentrations of PTH or PTHrP for 48 h. Specific ^{125}I-EGF binding activity was then determined as indicated in Materials and Methods. Data are expressed in fmol of ^{125}I-EGF bound per mg of cell protein and are the mean ± S.D. of triplicate (PTH) or quadruplicate (PTHrP) determinations in a representative experiment.

TABLE I. Effect of 0.1 μM PTH and PTHrP on the K_d and B_{max} Values of High-Affinity EGF Binding Sites in 48 H Cultured Trophoblast

Addition	K_d (10^{-10} mol/l)	(pmol/mg protein)

†Values are the means ± S.E.M.
*$P < 0.01$ (significantly different from control cells).

ity cross-linked ^{125}I-EGF 3- and 2.2-fold, respectively, as estimated by densitometry.

This increase in EGFR number after exposure of trophoblast cells to 0.1 μM PTH or PTHrP was further assessed by Western immunoblotting using a polyclonal anti-EGFR antibody against the C-terminal fragment (Fig. 2B).

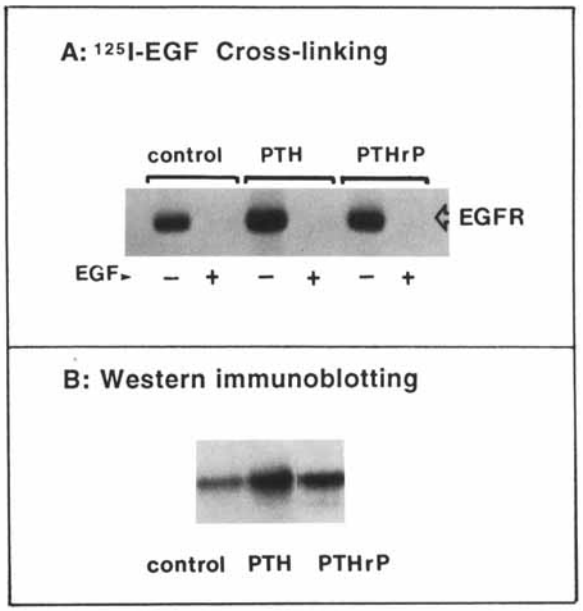

Fig. 2. Effect of PTH and PTHrP on EGFR protein levels. A: Cross-linking of ^{125}I-EGF to control cells and to PTH- and PTHrP-treated cells. After plating, cells were treated for 24 h with 0.1 μM of PTH or PTHrP, or with DMEM alone (control). Affinity labeling of ^{125}I-EGF to trophoblast cells in the presence (+) and absence (−) of an excess of unlabeled EGF and identification of labeled EGFR protein after SDS-PAGE were carried out as described in Materials and Methods. Autoradiograms of the gels were scanned using a microdensitometer. B: Immunoblotting of EGFR. Membrane proteins (5 μg) from control cells and from PTH- or PTHrP-treated cells were subjected to 7.5% SDS-PAGE and their EGFR content was analyzed by immunoblotting using a polyclonal anti-EGFR antibody against the C-terminal domain (diluted 1:200). Antibody complexes were visualized with anti-rabbit IgG HRP-linked antibody (diluted 1:10,000) and chemiluminescence (ECL, Amersham). These experiments (A,B) were performed twice with similar results.

active phosphorylation of 2 major proteins of 170 and 35 kDa. The 170 kDa species corresponded to EGFR itself, as assessed in specific immunoprecipitation experiments using a polyclonal antibody against the C-terminal fragment of EGFR (not shown). Phosphorylation of EGFR was increased 2.5-fold in cells treated for 24 h with 0.1 μM PTH and by 2-fold in cells treated with PTHrP in the same conditions. Thus, for a given amount of membrane protein, EGFR-tyrosine kinase activity was higher in PTH- and PTHrP-treated cells.

As shown in Figure 4, the effect of PTH on EGFR autophosphorylation was time-dependent. Increase in relative ^{32}P incorporation into EGFR was detectable after 1 h of exposure of trophoblast cells to 0.1 μM PTH (Fig. 4B) and was maximal (2.5-fold increase) at 3 h. A similar time course was observed with 0.1 μM PTHrP

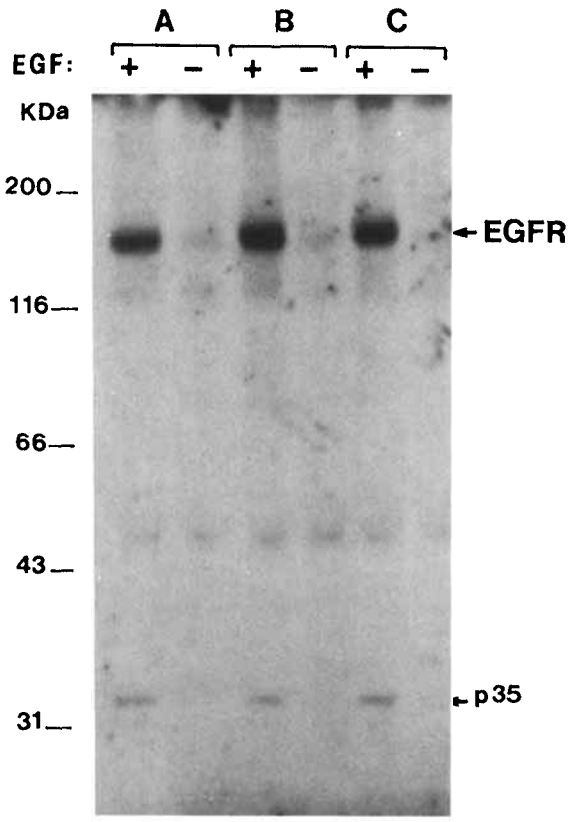

Fig. 3. Effect of PTH and PTHrP on EGF-dependent phosphorylation of EGFR in trophoblast cells in culture. Monolayer cultures were incubated in DMEM containing FCS (20%) in the absence (**A**) or presence of 0.1 μM PTH (**B**) or 0.1 μM PTHrP (**C**) for 24 h at 37°C. The cells were harvested and crude membranes were prepared. Membrane proteins (5 μg) were incubated with (γ-^{32}P) ATP (0.5 × 10^6 cpm) in 16 mM HEPES buffer (pH 7.4) in the presence (+) or absence (−) of 0.16 mM EGF. After 10 min at 4°C, the reaction was stopped by the addition of Laemmli sample buffer and boiling for 3 min. Phosphoproteins were then analyzed by means of 7.5% SDS gel electrophoresis and autoradiography.

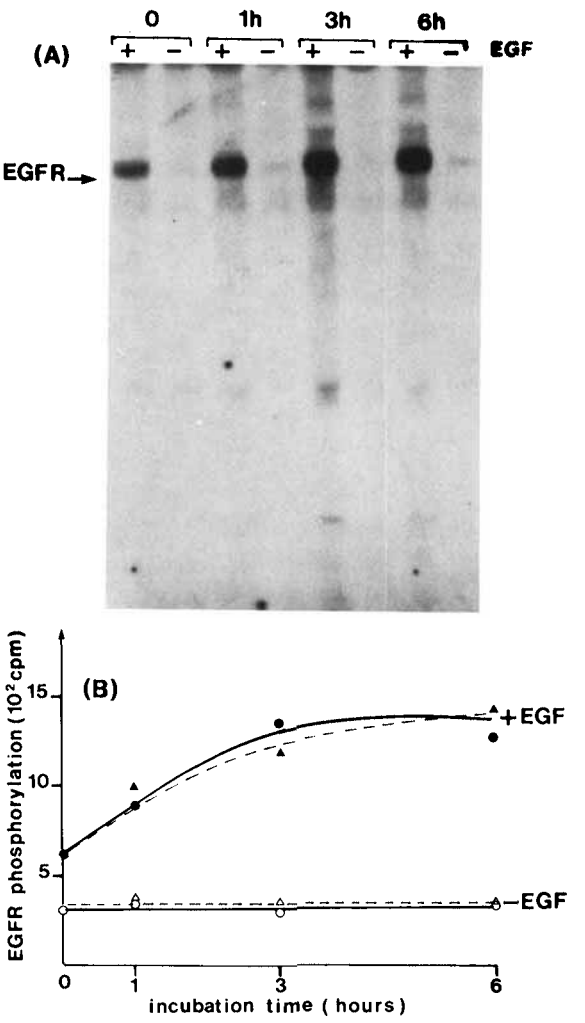

Fig. 4. Time course of EGFR autophosphorylation induced by PTH and PTHrP. Cultured (24 h) trophoblast cells were incubated for the times indicated with 0.1 μM PTH or PTHrP. Cell membranes were isolated and membrane proteins (5 μg) were incubated with 5 μM (γ-^{32}P) ATP (0.5 × 10^6 cpm) in 16 mM HEPES buffer in the presence (+) or absence (−) of 0.16 mM EGF. After 10 min at 4°C, Laemmli sample buffer was added and proteins were analyzed by means of 7.5% SDS gel electrophoresis and autoradiography. **A:** Autoradiogram. **B:** Relative ^{32}P incorporation into the 170 kDa protein (EGFR) was evaluated by counting each labeled spot of the dried gel in a liquid scintillation counter. These results are representative of three independent phosphorylation time course analyses with PTH (—)- or PTHrP (---)-treated cells.

(Fig. 4B, dashed line). Phosphorylation of EGFR in control cells remained stable.

Effect of PTH and PTHrP on EGFR mRNA Expression

In order to assess the possible effect of PTH and PTHrP on EGFR synthesis, the specific expression of EGFR mRNA was analyzed by means of Northern blotting in trophoblast cells treated with PTH and PTHrP using a 64-2 EGFR cDNA probe. As shown in Figure 5, 2 prominent EGFR transcripts of 10.5 and 5.8 kb were identified; their expression increased in hormone-treated cells, with the highest degree of expression induced by PTH (Fig. 5B). The elevation in transcript levels after 24 h of treatment paralleled the increase in EGF binding to trophoblast cells after 24 h of incubation with PTH, indicating that the increase in receptor transcript levels could account for the enhanced EGF binding. The ribosomal RNA content in each extract, determined by ethidium bromide staining, indicated equal RNA loading per lane (not shown).

Possible Role of PKC in PTH-Stimulated EGFR Number and Autophosphorylation

Recent evidence indicates that interaction of PTH with its receptors induces the activation of both PKA and PKC. In order to assess which of these enzymes was involved in PTH-stimulated EGFR number and phosphorylation, trophoblast cells were treated with specific inhibitors of PKA or PKC for 3 h. As shown in Figure 6A, in the presence of PKA inhibitor (0.1 mM) EGFR protein level was increased more than 2-fold by further addition of PTH (lane D) compared to untreated control cells or to cells treated with the PKA inhibitor alone (lanes A and B, respectively). Similarly, treatment of trophoblast cells with PKA inhibitor (lane B) modified neither the control cell level (lane A) nor the PTH-induced increase (lane D) in EGFR phosphorylation (Fig. 6B). This does not point to a role of PKA but rather supports the possible involvement of a Ca^{2+}/PKC pathway in the PTH-induced EGFR increase in trophoblast cells. Therefore, the cells were treated with a specific PKC inhibitor (5 μM): PKC inhibitor alone induced an increase in EGFR protein level (Fig. 6A) and EGFR phosphorylation relative to control cells (Fig. 6B, lanes C and A, respectively). In the presence of PKC inhibitor, addition of PTH (lanes E) did not induce a further increase in EGFR protein level and EGFR autophosphorylation relative to cells treated with PKC inhibitor alone (lanes C).

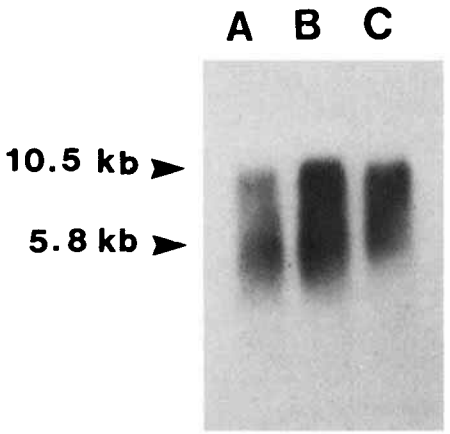

Fig. 5. Northern blot analysis of total RNA from syncytiotrophoblasts cultured in the absence **(A)** or presence of 0.1 μM PTH **(B)** or 0.1 μM PTHrP **(C)** for 24 h. Fifteen micrograms of total RNA was denatured, blotted, and hybridized with the ^{32}P-labeled EGFR-cDNA probe (64-2 fragment) as described in Materials and Methods. The size of EGFR species was estimated from the migration of molecular weight markers (0.24–9.5 kb RNA ladder from Gibco BRL, Cergy Pontoise, France) in the same run.

DISCUSSION

In the chorionic villi, cytotrophoblast epithelial cells play a major role in placental development. These cells divide and invade the gravid

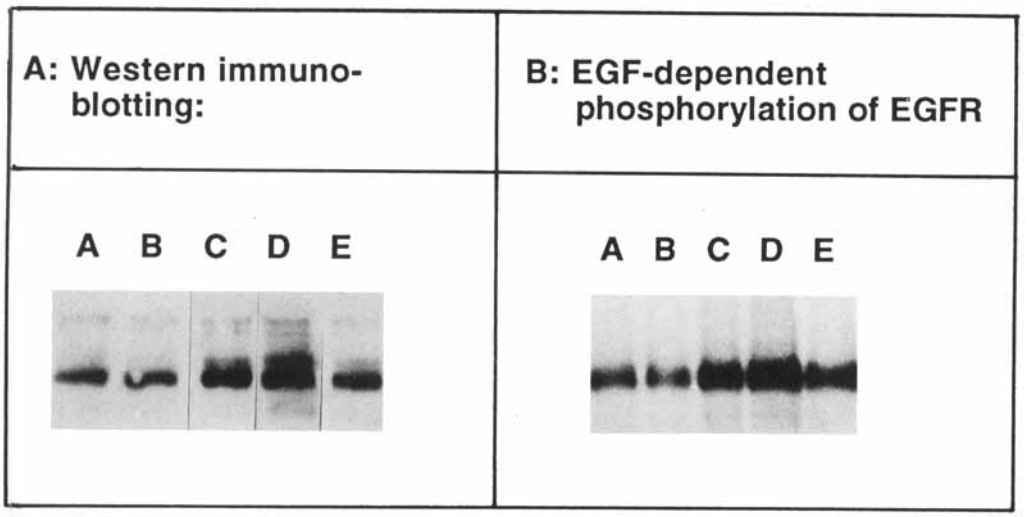

Fig. 6. Effect of PKA and PKC inhibitors on the PTH-induced increase of EGFR protein level **(A)** and autophosphorylation **(B)**. Cultured (24 h) trophoblast cells were incubated for 3 h with DMEM alone (control, **lanes A**) or with 0.1 mM PKA inhibitor **(lanes B)** or together with 0.1 μM PTH **(lanes D)**, and with 5 μM PKC inhibitor alone **(lanes C)** or together with 0.1 μM PTH **(lanes E)**. Cell membrane proteins (5 μg) were used for immunoblotting in A or for EGF-dependent phosphorylation of EGFR in B as described in the legends to Figures 2–4. Relative ^{32}P incorporation was evaluated by counting the corresponding labeled spot of the dried gel: 443 (A), 415 (B), 858 (C), 1,165 (D), and 699 (E) cpm.

uterus during the first trimester of pregnancy and also fuse and form a syncytium with specific hormonal production. This last process is predominant in the term placenta [Kliman et al., 1986]. During the last few years the development of techniques for trophoblast cell culture has allowed the syncytiotrophoblast formation to be followed in vitro. Mononuclear cytotrophoblasts first aggregate in vitro, a process requiring the presence of serum [Kao et al., 1988] and divalent cations [Babalola et al., 1990] and associated with the detection of cellular adhesive molecules such as E cadherin [Coutifaris et al., 1991]. The cytotrophoblasts then fuse within 24 to 48 h to form syncytial cells in which groups of nuclei appear scattered within a single, large body of cytoplasm. This syncytia formation is associated with an increase in the number of EGFR that coincides with enhanced EGFR mRNA content [Alsat et al., 1993]. These cells in culture offer a model of spontaneous upregulation of EGFR expression with cell differentiation and may be helpful for studying the role of factors which modulate EGFR expression.

In this study, we found that human trophoblast cell EGFR is upregulated by both PTH (1-34) and PTHrP (1-34); PTH was on average 1.5 times more effective than PTHrP (average stimulation vs. control cells: 3.6- and 2.4-fold, respectively). This result was predictable given the strong homology between the amino-terminal domains of the two peptides [Orloff et al., 1989] which also share the same receptor in numerous cells [Jüppner et al., 1988]. However, quantitative tissue-specific differences in bioactivity between these two peptides have been reported [Orloff et al., 1989; Stewart and Broadus, 1990]. Some tissues in which PTH seems inactive can display clear responses to PTHrP. Thus, calcium transport in lamb placenta is enhanced by PTHrP but not by PTH [Rodda et al., 1988]. In cultured human trophoblast cells, PTH (1-34) is at least as effective as PTHrP (1-34) in increasing the level of biologically active EGFR. Both peptides produce a concentration-related increase in EGF binding capacity, with a maximal effect at 1 nM. This is in agreement with the receptor K_d values (0.1–10 nM) reported for PTH and PTHrP in target cells [Orloff et al., 1989] and particularly those of the placental PTH receptor recently described in purified syncytiotrophoblast brush border and basal plasma membranes [Lafond et al., 1988]. Scatchard analysis of the binding data with PTH and PTHrP suggested that the increase in EGF binding was due to a rise in the number of receptor sites rather than to altered receptor affinity. The increased numbers of EGFR in PTH- and PTHrP-treated cells was further substantiated by affinity labeling of EGF to its receptor and by immunoblotting and EGFR autophosphorylation studies. These results indicate that PTH and PTHrP both induce an increase in the number of biologically active receptors for EGF.

Such an increase may result from decreased internalization or degradation of the receptor or from an increase in its synthesis. Northern blot analysis showed an increase in EGFR transcript levels in trophoblast cells after incubation with PTH or PTHrP, suggesting that the increase in receptor transcript levels could fully account for the enhanced numbers of bioactive EGFR. However, we cannot firmly conclude that these peptides regulate the rate of EGFR gene transcription, as mRNA levels may be enhanced by other processes, e.g., inhibition of mRNA degradation.

The rate of transcription of the EGFR gene is regulated by EGF itself, leading to an increase in EGFR mRNA levels [Clark et al., 1985; Earp et al., 1988] and by other factors or hormones such as phorbol esters [Clark et al., 1985], retinoic acid [Thompson and Rosner, 1989], angiotensin II, epinephrine, (Arg^8) vasopressin [Earp et al., 1988], TGF β [Thompson et al., 1988], and testosterone [Noguchi et al., 1991]. The mechanism through which EGF (or other factors) stimulates the synthesis of its own receptor is still not clear. Agents activating the phosphoinositide/PKC signal pathway increase receptor mRNA content. The phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which activates PKC, stimulates EGFR synthesis in a human breast carcinoma cell line [Bjorge and Kudlow, 1987] and in rat liver epithelial cells [Earp et al., 1988].

Recent evidence indicates that once PTH interacts with its receptor in osteosarcoma cells, both cAMP/PKA and phospholipase C/PKC systems are independently activated [Fujimori et al., 1992; Jouishomme et al., 1992]. PTH has been shown to downregulate EGFR in clonal osteoblastic mouse calvarial cells [Ohta et al., 1989] apparently via a cAMP-dependent cytoskeleton mechanism. This signal transduction pathway does not appear to be directly involved in the increased expression of biologically active EGFR in placental cells. Indeed, cAMP production is stimulated by PTH concentration 100 times higher than that effective on EGF binding [Do-

deur et al., 1991], and the inhibition of PKA, the intracellular mediator of cAMP, does not block the PTH-induced increase in EGFR. This higher concentration of PTH required to increase cAMP levels is consistent with the data obtained by Jouishomme et al. [1992], who found that physiological concentrations of PTH (1–50 pM) increased PKC activity, while at least 100-fold higher concentrations (5–50 nM) were needed to activate adenylate cyclase.

In this study, we found that inactivation of PKC by a specific inhibitor abolished the effect of PTH on the level of biologically active receptors relative to cells treated with PKC inhibitor alone. This suggests that a PKC-dependent pathway might be involved in the PTH-induced effect on EGFR protein expression. However, as shown in this study, the PKC inhibitor that we used induces alone an increase in the amount of EGFR protein and EGFR autophosphorylation. Therefore, we cannot rule out from these experiments that the stimulatory effect of PTH on EGFR number may be obscured by the effect of PKC inhibitor alone. This effect of PKC inhibitor could be related to the previously described post-transcriptional modulation of EGFR by PKC: EGFR have been shown to be phosphorylated by PKC at threonine-654, which is located in the cytoplasmic juxtamembrane domain [Cochet et al., 1984; Hunter et al., 1984; Davis and Czech, 1985]. Phosphorylation of EGFR by PKC appears to be responsible for several cellular effects, including a decrease in EGFR affinity and kinase activity [reviewed by Ullrich and Schlessinger, 1990]. A decrease in EGFR affinity results in lowered EGFR synthesis due to the fact that EGF is able to induce its own receptor synthesis, as demonstrated in cytotrophoblasts by DePalo and Das [1988]. Thus, inactivation of this negative feedback control by PKC inhibitor results in the observed increase in EGFR protein expression.

In conclusion, our findings demonstrate that PTH (1-34) and PTHrP (1-34) increase the expression of biologically active EGFR in human placental cells. Given the predominant role of EGFR in modulating placental cell growth and differentiation, these data suggest that PTHrP, which is synthesized in both human placenta [Asa et al., 1990; Kramer et al., 1991] and the human uterus [Casey et al., 1992], might play a role in placental development.

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