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Original article

Effect of human parathyroid hormone on the cAMP production and the endocrine functions of trophoblast cells from first trimester placenta

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Summary — Our previous study on teratocarcinoma cells suggested the role of human parathyroid hormone (hPTH) in early development of the placenta. The purpose of this study was to evaluate the possible role of hPTH on the functions of first trimester trophoblast cells. Adenylate cyclase activity in crude membranes from first trimester human placental villous tissue is stimulated 2-fold by hPTH (1-34) (10^{-6} mol.l^{-1}) from 265 ± 32 to 532 ± 80 pmol of cAMP/mg protein/15 min. A similar stimulation of adenylate cyclase is observed in human term placental villous tissue but not in 3 different choriocarcinoma cell lines.

In order to evaluate the possible role of hPTH on the functions of first trimester human trophoblast cells, these cells were isolated by dispase and cultured (2 x 10^5 cells per plate) in DMEM supplemented with 20% fetal calf serum with or without 100 ng/ml of epidermal growth factor (EGF), for 4 d. On d 2 of culture, hPTH (10^{-7} mol.l^{-1}) stimulates cAMP production of these cells from 0.52 ± 0.2 to 2.58 ± 0.57 pmol.h^{-1} per 10^6 cells (mean ± SEM). As compared to control (30 ng/ml), the output of hCG is increased by 1.5- (NS), 2- ($P < 0.01$) and 3- ($P < 0.01$) fold by EGF, hPTH, and hPTH added with EGF, respectively. Dibutyryl cAMP (10^{-3} mol.l^{-1}) increased hCG secretion by 3-fold ($P < 0.05$). EGF and hPTH added separately or together significantly stimulated ($P < 0.01$) the secretion of free α subunit 2-fold from 35 ng/ml to 70 ng/ml. In contrast, hPTH and EGF added separately did not change the secretion of free βhCG. However, added together, they significantly increased ($P < 0.01$) the secretion of free β hCG after 48 h of culture, maximal stimulation (2.5 fold) being observed at d 4 of culture. In conclusion, human trophoblast cells are target cells for hPTH. hPTH acts in association with EGF in promoting expression of endocrine activity of these cells, such as hCG secretion. Trophoblast cells provide a model for the study of the cooperative effect between a peptide hormone and a growth factor in the regulation of endocrine function.

human placenta / parathyroid hormone / cAMP / trophoblast secretions

Résumé — Effet de la parathormone humaine sur la production d'AMPc et sur les fonctions endocrines des cellules trophoblastiques de placenta du 1er trimestre. *Notre précédente étude sur les cellules de tératocarcinome suggérait que la parathormone humaine (hPTH) jouait un rôle sur le développement précoce du placenta. Le but de la présente étude était d'évaluer le rôle pos-*

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sible de la hPTH sur les fonctions des cellules trophoblastiques du premier trimestre. L'activité de l'adénylcyclase dans les membranes issues de villosités humaines placentaires du 1er trimestre est doublée par la hPTH (1-34) (10^{-6} mol.l^{-1}) de 265 ± 32 à 532 ± 80 pmol d'AMPc par mg de protéines/15 min. Une stimulation identique de l'adénylcyclase est observée dans le tissu de villosités de placenta humain à terme, mais pas dans 3 lignées cellulaires différentes de choriocarcinomes. Afin d'évaluer le rôle possible de la hPTH sur les fonctions des cellules humaines du trophoblaste du 1er trimestre, de telles cellules ont été isolées par de la dispase et cultivées (2.10^5 cellules/boîte) dans du DMEM supplémenté en sérum de veau fœtal (20%) et en facteur de croissance épidermique (EGF) (100 ng/ml), sauf pour les témoins, pendant 4 j. Le 2e jour de la culture, la hPTH (10^{-7} mol.l^{-1}) stimule la production d'AMPc de ces cellules de 0,52 ± 0,2 à 2,58 ± 0,07 pmol.h^{-1} pour 10^6 cellules (moyenne ± écart type). Par rapport à la production des cellules témoins (30 ng/ml), la production de hCG est multipliée respectivement par 1,5 (NS), 2 (P < 0,01) ou 3 (P < 0,01), par la présence d'EGF, de hPTH, ou de hPTH + EGF. Le dibutyryl AMPc (10^{-3} mol.l^{-1}) multiplie le taux de sécrétion de hGC par 3 (P < 0,05). L'EGF et l'hPTH ajoutés séparément ou simultanément stimulent significativement (P < 0,01) la sécrétion de la sous-unité α (x 2) (70 ng/ml contre 35 ng/ml). En revanche, la hPTH et l'EGF ajoutées séparément ne changent pas la sécrétion de la βhCG libre. Cependant, ajoutés ensemble, ils augmentent significativement (P < 0,01) la sécrétion de la βhCG libre après 48 h de culture, la stimulation maximale (x2,5) étant observée au 4e jour de la culture. En conclusion les cellules trophoblastiques humaines sont des cellules cibles pour la hPTH. La hPTH agit en association avec l'EGF pour promouvoir l'expression des activités endocrines de ces cellules telle que la sécrétion de la hCG. Les cellules trophoblastiques fournissent un modèle pour l'étude de l'effet coopératif entre une hormone peptidique et un facteur de croissance sur la régulation d'une fonction endocrine.

placenta humain / hormone parathyroïdienne / AMPc / sécrétion trophoblastique

INTRODUCTION

Human chorionic gonadotropin (hCG) is a placental glycoprotein hormone necessary for the maintenance of pregnancy (Simpson and Mac Donald, 1981). The production of hCG is highest and of greatest significance in early pregnancy, but little is known of the factors regulating the production of this hormone at the present time.

The approach to these questions has been facilitated by culturing human trophoblast cells (Kliman *et al,* 1986, 1987). Isolated by enzymatic digestion from the villous tissue, the mononuclear cytotrophoblasts cultured *in vitro* aggregate and through cell fusion form a syncytiotrophoblast with specifc functions (Kliman *et al,* 1986).

The secretion of hCG is increased *in vitro* by 8-bromo-cAMP (Feinman *et al,* 1986; Ulloa-Aguirre *et al,* 1987). This cAMP analogue promotes hCG synthesis by increasing the mRNA levels encoding the α and β subunits of the hormone (Jameson *et al,* 1986). In addition, activation of the adenylate cyclase by cholera toxin or forskolin increases the hCG secretion by human cytotrophoblasts from term placenta (Nulsen *et al,* 1988).

Epidermal growth factor (EGF) is a polypeptide of 6040 Da, isolated from mouse submaxillary glands (Cohen, 1962) and human urine (Cohen and Carpenter, 1975). Receptors for EGF have been described predominantly on the syncytiotrophoblasts in term placenta (Maruo *et al,* 1987) and on the membranes of purified trophoblast cells in early and term placentas (Chen *et al,* 1988; Mirlesse *et al,* 1991). In addition, EGF has been reported to influence the differentiation of human cytotrophoblasts in culture (Truman and Ford, 1986). The effects of EGF on hCG secretion described in mid-term or full-term organ cultures are conflicting; Lai and Guyda (1984) reported hCG secretion stimulated by EGF, whereas Huot *et al* (1981) observed no such effect.

Murine teratocarcinoma cells differentiate *in vitro* following the first step of embryogenesis (Martin, 1980). They offer a model to study the role of polypeptide hormones during early embryogenesis. Thus, adenylate cyclase activity of these parietal endoderm cells in culture is stimulated by parathyroid hormone (PTH) (Evain Brion *et al,* 1981; Liapi *et al,* 1987). These previous results suggested a role for this hormone in placental development. In agreement with this hypothesis, recent studies demonstrate that the human placenta is a target organ for PTH as shown by the characterization of a specific receptor for PTH in purified membranes from term placenta (Lafond *et al,* 1988).

The purpose of this investigation was therefore the determination of the effect of PTH on the endocrine functions of trophoblast cells from first trimester placenta. In this study we demonstrate that human trophoblast cells from early gestation are specific target cells for PTH. Indeed, PTH stimulates adenylate cyclase activity of trophoblast cell membrane and fails to stimulate the adenylate cyclase activity of membranes isolated from either cultured choriocarcinoma or placental mesenchymal cells. Moreover, PTH together with EGF stimulates the secretion of hCG, and modulates the secretion of free α and β subunits to a different extent, as measured by specific monoclonal immunoradiometric assays.

MATERIALS AND METHODS

First-trimester placentas were obtained from voluntary elective abortions at 6 to 12 wk of amenorrhea, and term placentas from spontaneous full-term deliveries. Placental villous tissues were dissected free of chorionic membranes and incubated at 4 °C for 1 h in phosphate-buffered saline pH 4.7 (NaCl 137 mM, KCl 3 mM, Na_2HPO_4 8 mM, KH_2PO_4 1.5 mM) containing 100 μg/ml gentamycin and 2.5 μg/ml amphotericin.

Cell isolation, characterization and culture

Trophoblast cells were isolated by using a previously described dispase dispersion method (Dodeur *et al,* 1990). The villous tissue was incubated with a solution of dispase (2.4 U/ml), a neutral protease from *Bacillus polymyxa* (EC 3.4.24.4) in Puck's solution (Boerhinger–Mannheim) for 3 h at 4 °C. Afterwards the dispase solution was discarded and the tissue incubated overnight in medium A: DMEM containing 25 mM glucose, 4 mM glutamine, 25 mM HEPES, 100 μg/ml gentamycin, 2.5 μg/ml amphotericin (Seromed Biochrom, Lyon, France) and 20% heat-inactivated fetal calf serum (Biological industries) in humidified 5% CO_2 and 95% air at 37 °C.

Trophoblast cells were spontaneously released from villous tissue into the medium. The trophoblast cells were plated at 2.5 x 10^5 cells/per 35 mm plates coated with human fibronectin (1 mg/ml, Sigma). At 24 h of culture, cytotrophoblast cells were positively immunostained by antibodies against cytokeratin (Pan cytokeratin, undiluted antibody, Amersham-France) and against α hCG (Mab 326-2-1, Immunotech). Cells were treated with these antibodies at appropriate dilutions and then with biotinylated secondary antibodies (Amersham-France), the binding of which was visualized with an avidin–biotin–peroxidase complex detection method (Hsu and Raine, 1981). The avidin–biotin–peroxidase complex detection kit was purchased from Amersham (France). In contrast, no immunostaining was obtained using the same methodology and a monoclonal anti-vimentin antibody (undiluted, Amersham-France). At 72 h of culture, multinucleated syncytiotrophoblast was predominant as studied by electron microscopy.

Mesenchymal cells were obtained from undigested villous tissue by explant cultures; these cells were grown in medium A and used to their second or third passages.

The choriocarcinoma cell lines JAR, JEG, BEWO, were cultured as previously reported (Patillo and Gey, 1968; Patillo *et al,* 1971).

Secretion of hCG by trophoblast cells in culture

Trophoblast cells were cultured during 96 h in medium A with or without EGF (100 ng/ml, Collaborative Research), human PTH (hPTH)1-34 (10^{-6} M, a generous gift of Rorer Laboratory, Levallois, France), hPTH plus EGF, or dibutyryl cAMP (10^{-3} M, Sigma). The medium was changed daily and stored at –80 °C until analyzed.

Crude membrane preparation

Cultured cells were washed with ice-cold buffer (50 mM Tris–HCl, pH 7.8, 0.33 M sucrose, 1 mM $MgCl_2$), harvested by scraping and homogenized by 20 strokes with a Dounce homogenizer. Homogenates were centrifuged at 12 000 *g* for 20 min at 4 °C to isolate crude membranes. Placental villous tissue was cut in small pieces and homogenized by 50 strokes in the ice-cold buffer as described above.

Adenylate cyclase activity

Adenylate cyclase activity was determined by measuring the conversion of [α^{32}P]-ATP (Amersham) to [^{32}P]-cAMP in crude membranes (12 000 *g* pellet) at 37 °C as previously described (Evain Brion *et al*, 1981). The standard incubation mixture contained 25 mM Tris–HCl, pH 7.8, 10 mM phosphoenolpyruvate, 4 μg of pyruvate kinase, 5 mM $MgCl_2$, 0.2 mM ATP, 0.5 mM EGTA, 4 x 10^6 cpm [α^{32}P]-ATP and 50 μg of membrane protein. The hormonal stimulation by human PTH (hPTH) 1-34 (10^{-6} M) was measured in presence of 10^{-5} M GTP. The effect of PTH analog (Nle^{8-18} Tyr^{34}) PTH 3-34 amide (Boerhinger–Mannheim) was tested in 10-fold excess as compared to hPTH 1-34.

Determination of cAMP in the culture medium

After acetylation, cAMP was measured by a specific radioimmunoassay in the culture medium (Steiner *et al*, 1972). cAMP antibody was a generous gift of J Saez (Inserm U 307, Lyon, France).

Determination of hCG and its free α and β subunits

MAb-based specific IRMAs were used for the determination of hCG (Bellet *et al*, 1986), α hCG and β hCG (Ozturk *et al*, 1987) levels in cell culture media. The production of MAbs with defined epitope specificity, the development of multisite IRMAs and the testing of their specificty have been previously described (Bellet *et al*, 1986; Ozturk *et al*, 1987).

Protein concentration

Protein concentration was determined by the method of Bradford (1976).

Statistical analysis

Statistical analysis of the data was carried out by the paired Student's *t*-test to compare the effects of the added factors to the effect of fetal calf serum (FCS) alone at each time.

RESULTS

Effect of PTH on placental adenylate cyclase activity

Adenylate cyclase activity was assayed in membranes prepared from either villous tissue (crude membranes) from early gestation and term placenta, or cultured cells from first trimester placenta and from choriocarcinoma cell lines. The results are summarized in table I. PTH (10^{-6} M) induced a significant increase ($P < 0.025$) in adenylate cyclase activity of crude membranes from first trimester and full-term placenta,

Table I. Effect of hPTH (1-34) on adenylate cyclase activity of placental villous tissue and cultured cell membranes.

	Basal	*GTP* 10^{-5} *M*	*NaF* 10^{-2} *M*	*PTH* 10^{-6} *M*	*PTH antagonist*
Villous tissue					
1st trimester	201 ± 22	265 ± 32	1 093 ± 100	532 ± 80*	
Term	154 ± 25	202 ± 27	1 620 ± 140	338 ± 33*	
Cultured cells					
Mesenchymal cells	515 ± 90	644 ± 100	2 320 ± 386	644 ± 40	
Trophoblastic cells	140 ± 15	185 ± 20	940 ± 100	270 ± 20*	180 ± 10
JAR cells	57 ± 10	80 ± 10	1 700 ± 180	90 ± 20	
JEG cells	30 ± 12	50 ± 20	646 ± 100	52 ± 20	
BEWO cells	69 ± 10	90 ± 25	1 230 ± 150	90 ± 25	

Enzyme activity was measured as described in *Materials and Methods* with GTP (10^{-5} M), GTP (10^{-5} M) + hPTH (10^{-6} M). PTH antagonist (Nle^{8-18} Tyr^{34}) PTH 3-34 was tested in 10-fold excess as compared to hPTH 1-34. The results are expressed in pmol of cAMP/mg of protein/15 min and are the means ± SD of triplicate incubations from 3 different experiments. Values* are significantly greater than control values (GTP 10^{-5} M), ($P < 0.025$).

as compared to corresponding control values (GTP 10^{-5} M). Similar results were observed for PTH effect on membrane adenylate cyclase activity of isolated trophoblastic cells, cultured for 2 d ($P < 0.025$). The specificity of this stimulation was demonstrated using a PTH analog (Nle^{8-18} Tyr^{34}) PTH (3-34) amide –able to compete with the PTH receptor– which fails to stimulate the adenylate cyclase activity in membranes from cultured trophoblastic cells. Furthermore, in comparison with control values (GTP 10^{-5} M), the enzyme activity was not modified by PTH in membranes from placental mesenchymal cells and from 3 different choriocarcinoma cell lines (JAR, JEG and BEWO), indicating that PTH-induced stimulation of adenylate cyclase was specific for normal trophoblastic cells.

In addition, as shown in figure 1, PTH stimulated cAMP production by intact isolated trophoblast cells in culture. The effect was dose-dependent with a maximal stimulation (≈ 5-fold) occurring at 10^{-7} M of hPTH ($P < 0.01$), as compared to the cAMP production by control cells (0.50 pmol/60 min/10^6 cells).

Effect of PTH on hCG secretion by trophoblast cells in culture

Having demonstrated the PTH stimulated adenylate cyclase activity in the membranes of trophoblast cells, we next determined whether this activation would promote the expression of one endocrine activity of these cells which is stimulable by cAMP, the secretion of hCG.

Trophoblast cells were cultured during 96 h in the presence of PTH (10^{-6} M) or EGF (100 ng/ml) alone or in the presence of PTH together with EGF and their effect on the secretion of hCG (fig 2) and its free α (fig 3A) and β (fig 3B) subunits was stud-

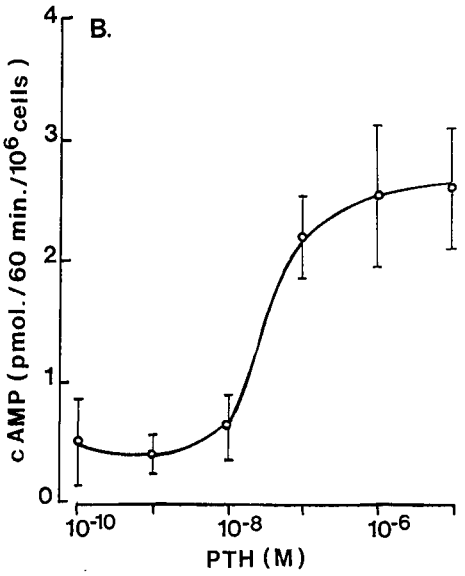

Fig 1. Effect of increasing concentration of PTH (1-34) on the cAMP production by human trophoblast cells in culture. Trophoblast cells isolated as described in *Methods* were cultured for 2 d in medium A. The culture medium was then changed and the cells were incubated in medium A in presence of different concentrations of hPTH. After 1 h incubation, the medium was collected for cAMP determination. Each value is a mean of triplicate determinations of 2 different experiments (mean ± SEM).

ied. As compared to control (20% serum supplemented medium), the secretion of hCG or of its free β subunits was not significantly modified by EGF at any time of the cell culture. The secretion of free α subunit seemed significantly enhanced by EGF during the first 2 d of culture. Parathyroid hormone significantly stimulated the secretion of intact hCG and its free α subunit by ≈ 2-fold ($P < 0.01$) but did not modify the secretion of β subunit.

The addition of EGF and hPHT to the culture medium increased by ≈ 3-fold the secretion of hCG and of its free α subunit during the first 3 d of culture and in a similar manner to the addition of dibutyryl cAMP (10^{-3} M) (fig 2, 3A).

Of interest is the fact that the association of EGF and PTH which did not have any effect on the secretion of β subunit during the first 2 d of culture increased significantly ($P < 0.01$) by ≈ 2-fold the output of free β subunit at 72 and 96 h of culture (fig 3B).

DISCUSSION

We have established in this study that human trophoblast cells from early gestation placenta possess an adenylate cyclase activity which is stimulated by hPTH. The specific activity of early gestational adenylate cyclase activity is in the range reported in term placenta villous homogenates (Menon and Jaffe, 1973; Milewich *et al*, 1982) or in term placenta trophoblast cells in culture (Nulsen *et al*, 1988). PTH stimulates cAMP production in intact human trophoblast cells in a dose-dependent manner similar to that reported in other fetal and adult organs (Nissenson and Arnaud, 1979; Silve *et al*, 1982).

Our results are in agreement with the recent report on: i), the study of adenylate cyclase in the membranes of cultured trophoblast cells from term placenta (Nulsen *et al*, 1988); and ii), the activity of adenylate cyclase stimulated by PTH in the basal plasma syncytiotrophoblastic membranes of term placenta (Lafond *et al*, 1988). In addition, PTH stimulates phosphate transport in the placenta (Brunette *et al*, 1989) and increases the number of EGF receptors in cultured human trophoblast cells (Alsat *et al*, 1991). These results suggest that PTH stimulates adenylate cyclase activity of syncytiotrophoblast cells throughout pregnancy. Moreover, the pres-

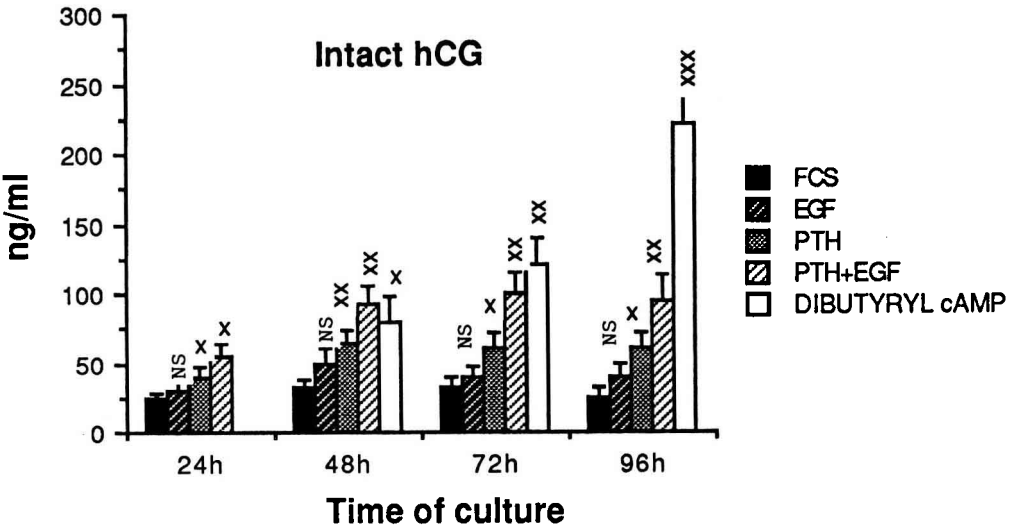

Fig 2. Effect of EGF (100 ng/ml), hPTH (10^{-6} M), hPTH added together with EGF and dibutyryl cAMP (10^{-3} M) on hCG secretion by trophoblast cells in culture. The results are expressed as ng of hCG released into 1 ml of medium / 24 h. Each value is the mean ± SD of triplicate incubations from 5 different experiments. Statistical analysis of the data was carried out by the Student's *t*-test to compare the effects of the added factors to the effect of fetal calf serum alone (control) at each time of cell culture. NS = not significant; x = $P < 0.05$; xx = $P < 0.01$; xxx = $P < 0.001$.

ence of PTH receptors in membranes of trophoblast cells appeared to be specific for the normal cells since we failed to detect any activation in the membranes of 3 choriocarcinoma cell lines.

In this study we also investigated the secretion of hCG and of its free α and free β subunits by cultured trophoblast cells. In our experimental conditions the secretion of hCG did not vary significantly from 24 to 96 h of culture. As compared to control (20% FCS), EGF alone had no significant effect on the stimulation of hCG secretion. This might be related to the concentration of EGF already present in the fetal calf serum. PTH alone increased significantly ($P < 0.01$) hCG secretion by 2-fold, maximum stimulation being observed at 48 h of culture. Of interest is the fact that PTH in association with EGF induced a level of secreted hCG similar to that obtained by addition of dibutyryl cAMP alone during the mid-stages of the culture (48 and 72 h). This result suggested that PTH in association with EGF might have a specific role in the regulation of endocrine functions in trophoblast cells. In contrast, PTH alone or in association with EGF stimulated the secretion of α subunit in a similar way. No stimulation of free β subunits was observed during the first 2 days of culture but a significant ($P < 0.01$) increase in free β subunits secretion was observed at 72 and 96 h of culture in the presence of PTH together with EGF.

The apparent synergism of action between PTH and EGF on hCG secretion and especially on its free β subunit secre-

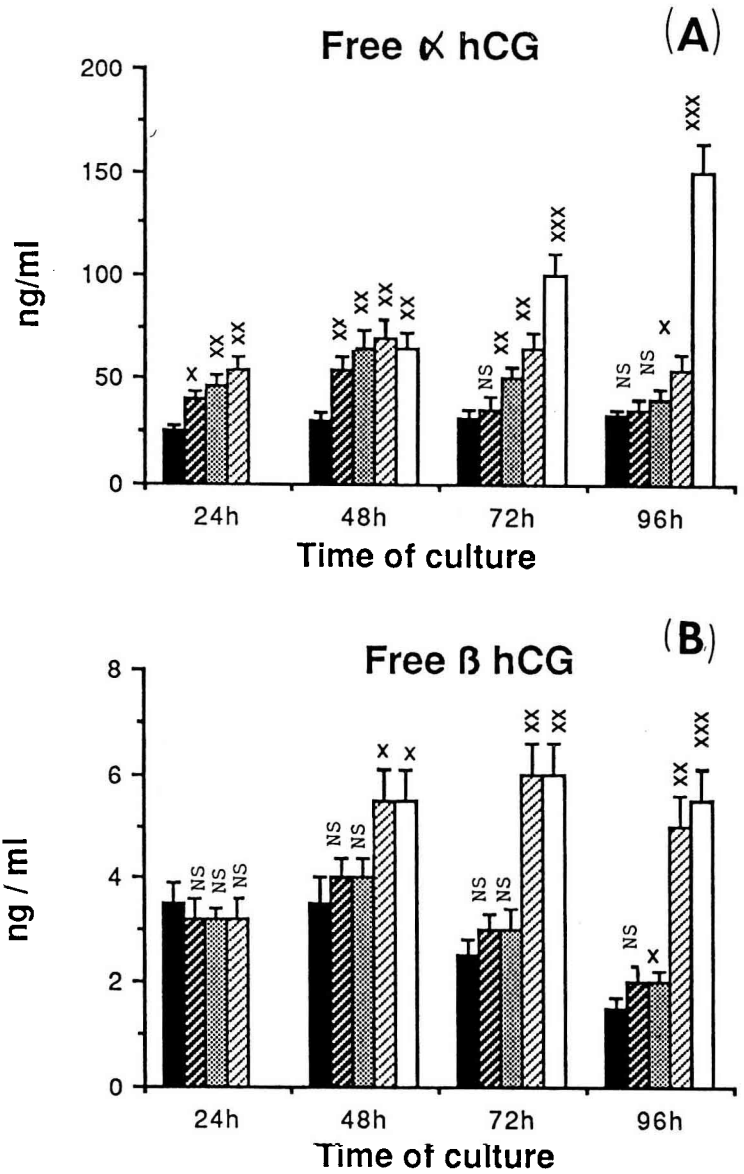

Fig 3. Effect of EGF (100 ng/ml), PTH (10^{-6} M), PTH added together with EGF and dibutyryl cAMP (10^{-3} M) on the secretion of free α hCG subunit (panel A) and free β hCG subunit (panel B) by trophoblast cells in culture. The results are expressed as ng of hCG subunit released into 1 ml of medium per 24 h. Each value is the mean ± SD of triplicate incubations from 5 different experiments. The statistical analysis was performed as indicated in the legend of figure 2.

tion could be related to a synergistic effect at the receptor or post-receptor levels of this growth factor and this hormone. A growth factor may regulate the binding of a specific hormone. For example, PTH alters the TGF β binding to its receptors in osteoblast in culture (Centrella *et al,* 1988). The interaction between hormone and growth factor may also be located at the coupling level of adenylate cyclase (Bernier *et al,* 1986; Jaillard *et al,* 1987). It is interesting to note that EGF has been shown to promote the phosphorylation of a protein which appears to be similar to the G protein subunit (Valentine-Braun *et al,* 1986).

Another explanation could be that PTH potentiated the role of EGF in the differentiation of cytotrophoblast to syncytiotrophoblast. Therefore in presence of PTH and EGF, the cytotrophoblasts which continuously synthesize α subunit became differentiated into syncytiotrophoblasts, more competent to express the β subunit of hCG inducing first an increase in hCG secretion followed by secretion of free β subunit. This hypothesis is in agreement with the recent report of Hoshina *et al* (1982) who showed by *in situ* hybridization different steps in the differentiation of cytotrophoblast into syncytiotrophoblast.

Recently the structure, location, and transcriptional pattern of a PTH-like protein (PTHLP), first described as tumoral products have been revealed (Orloff *et al,* 1989). This protein shares amino acid sequence homology with PTH and interacts with its receptors in classical target and other tissues. In sheep placenta, partially purified PTHLP appears to stimulate specifically calcium transport *in vivo* (Rodda *et al,* 1988) and PTHLP has been detected in human placenta (Asa *et al,* 1990). In our experimental conditions PTHLP was not significantly more effective than hPTH in stimulating cAMP production or hCG secretion (data not shown).

In summary, trophoblast cells are a target for PTH. This hormone acts in association with EGF to promote expression of endocrine activities of these cells, such as hCG secretion. Trophoblast cells provide a model for studying the cooperative effect between a peptide hormone and a growth factor in the regulation of endocrine function.

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