Functional parathyroid hormone receptors are present in an umbilical vein endothelial cell line

CARLOS M. ISALES,1,2 BAUER SUMPIO,3 RONI J. BOLLAG,1 QING ZHONG,1 KE-HONG DING,1 WEI DU,3 JOSE RODRIGUEZ-COMMES,3 RAQUEL LOPEZ,1 OSCAR R. ROSALES,3 JOSE GASALLA-HERRAIZ,1 RICHARD MCCARTHY,3 AND PAULA Q. BARRETT4

1Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, and 2Augusta Veterans Affairs Medical Center, Augusta, Georgia 30912; 3Yale University School of Medicine, New Haven, Connecticut 06520; and the 4University of Virginia, Charlottesville, Virginia 22908

Received 28 September 1999; accepted in final form 20 April 2000

Isales, Carlos M., Bauer Sumpio, Roni J. Bollag, Qing Zhong, Ke-Hong Ding, Wei Du, Jose Rodriguez-Commes, Raquel Lopez, Oscar R. Rosales, Jose Gasalla-Herraiz, Richard McCarthy, and Paula Q. Barrett. Functional parathyroid hormone receptors are present in an umbilical vein endothelial cell line. Am J Physiol Endocrinol Metab 279: E654–E662, 2000.—Acute parathyroid hormone exposure induces vascular smooth muscle relaxation. In contrast, continuous infusion of parathyroid hormone leads to vasoconstriction and an elevation of blood pressure. Despite the known effects of parathyroid hormone on vascular smooth muscle, possible direct effects on the vascular endothelium have not previously been investigated. Using a human umbilical vein endothelial cell line, we found that parathyroid hormone increased both intracellular calcium and cellular cAMP content in these endothelial cells. Furthermore, exposure of these cells to increasing concentrations of parathyroid hormone stimulated both [3H]thymidine incorporation and endothelin-1 secretion. Parathyroid hormone/parathyroid hormone-related peptide receptor mRNA could be detected at low levels in these cells. In summary, these data demonstrate that endothelium-derived cells contain functional parathyroid hormone receptors. The potential physiological role of these receptors remains to be determined.

PARATHYROID HORMONE (PTH) is known to have multiple cardiovascular actions, including direct effects on cardiac myocytes and a positive chronotropic and inotropic effect. In addition, PTH in vitro, or by short-term infusion, has been shown to induce smooth muscle relaxation (26–28).

The PTH/PTH-related peptide (PTHRP) receptor (PTH1R) was originally cloned from an opossum kidney library and subsequently from a variety of cell lines (21). When this cloned receptor is expressed in COS cells, a cell line in which it is not normally expressed, the addition of high doses of PTH results in an increase in the content of both cAMP and inositol 1,4,5-triphosphate ([inositol 1,4,5-triphosphate (IP3)], as well as a transient rise in the intracellular Ca2+ concentration ([Ca2+]i) (1). The PTH1R is present in both smooth muscle and endothelial cells. In addition, the endothelium makes and secretes PThrP. Because PThrP inhibits the release of the potent vasoconstricting peptide, endothelin-1 (ET-1) from endothelial cells (20), a paracrine loop ensues that results in vasodilation.

A second receptor for PTH has been cloned, called the PTH 2 receptor (PTH2R) (36). This receptor has a distribution different from that of PTH1R in that PTH2R is expressed predominantly in the brain and the pancreas but also in the endothelium (35), whereas PTH1R is expressed predominantly in bone and kidney. Moreover, whereas PTH1R accommodates PTH and PTHrP equivalently, PTH2R binds PTH preferentially over PTHrP. Additional information supporting a role for an effect of PTH on the endothelium is the observation that patients with primary hyperparathyroidism (1°-HPT) have a higher incidence of essential hypertension than does the general population (5). Although the mechanism responsible for the elevation of blood pressure in these patients is not known, the combined evidence suggests the possibility of a direct effect of PTH on the endothelium. Despite the fact that PTH receptors are known to be present in endothelial cells, the effects of PTH binding to this type of cells has, to our knowledge, not been previously reported. To address these questions, we utilized a human umbilical vein endothelial cell line and tested whether PTH could activate signal transduction pathways and cellular responses in these cells.

MATERIALS AND METHODS

Endothelial cell culture. Immortalized human umbilical vein endothelial cells (ECV 304), a gift of Dr. K. Takahashi (National Defense Medical College, Tokyo, Japan) (34) were

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
used at passages 84–94 for these studies. This strain is characterized by a cobblestone monolayer growth pattern, high proliferative potential, and anchorage dependency with contact inhibition. Ultrastructurally, endothelium-specific Weibel-Palade bodies were identified. Immunocytochemical staining for human endothelium and di-1-acetylated low-density lipoprotein was detected as well as angiotensin-converting enzyme activity. Cells were grown to confluence in Medium 199 (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS, vol/vol) (HyClone laboratories, Logan, UT), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (3 µg/ml) and were utilized 3–7 days postconfluence.

**Materials.** Parathyroid hormone-1—84 was from Bachem (Torrance, CA). Fura 2-AM and EGTA-AM were purchased from Molecular Probes (Eugene, OR). The diastereoisomer of adenosine 3’,5’-phosphorothioate (Rp)-cAMPS was from CalBiochem (La Jolla, CA). The riboprobe for the PTH1R was kindly provided to us by Dr. H. Jüppner (Harvard Medical School, Boston MA).

**Calcium measurements with fura 2.** Intracellular calcium measurements with fura 2 were performed as previously described (13). Briefly, ECV 304 cells grown on glass coverslips were loaded with the calcium-sensitive probe fura 2-AM (Molecular Probes, Eugene, Oregon), 1 µM for 45 min at room temperature. The cells were washed with a modified Krebs-Ringer bicarbonate (KRB) buffer (containing 25 mM sodium bicarbonate, 120 mM sodium chloride, 4 mM potassium chloride, 1.2 mM magnesium sulfate, 1.2 mM sodium bisphosphate, 5.6 mM dextrose, 1.25 mM calcium chloride and 2 mg BSA/ml) and were left for an additional 30 min at room temperature to allow esterase cleavage of fura 2-AM to fura 2. The cells were then placed in a cuvette in a dual-wavelength spectrofluorimeter (Photon Technologies International, South Brunswick, NJ). Fluorescence was then measured using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Autofluorescence was measured in unloaded cells, and this value was subtracted from all the measurements. For experiments wherein “nominally free calcium” medium was used, the buffer was prepared with deionized water (<1 µM calcium) but did not have EGTA. For these experiments, the cells were placed in the calcium-free medium for 10 min before stimulation.

**Measurement of inositol phosphates by HPLC.** Inositol phosphates were measured as previously described (16). Briefly, cells were seeded in 6-well plates and grown to confluence. Two to seven days postconfluence, the cells were placed in KRB (inositol free) with 0.1% FCS and labeled with [3H]myo-inositol (50 µCi/well) for an additional 48 h. At the end of this time, the cells were carefully washed three times with the same medium, and the appropriate agonist was added to the cells for 20 s. The medium was then removed, and the reaction was stopped by addition of 500 µl of a 6% perchloric acid solution containing phytic acid (500 µg/ml). The cells were incubated in this solution for 20 min on ice, and the supernatant was removed and neutralized (pH 7.5) with a saturated potassium bicarbonate solution. The solution was then centrifuged (15,000 g), and the supernatant was kept for analysis.

Inositolts were separated and identified by HPLC (Rainin Systems, Woburn, MA). A SAX hydroprop column was used (1.4 mm × 10 cm, 1.6 ml) at a flow rate of 1 ml/min with a stepped gradient from 10–25 mM NH₄H₂PO₄ (pH 3.8) for 5–30 min, followed by a linear gradient from 0.25–1.4 M NH₄H₂PO₄ (pH 3.8) for 30–40 min. Radioactivity was detected with an in-line scintillation counter (Packard Instruments, Meriden, CT). The column was calibrated using radioactive inositol standards (Amersham, Arlington Heights, IL). Before each new sample, the column was washed for 10 min with water at 1 ml/min. Each sample was run with ATP for identification of the IP₃ peak; the area under the curve was integrated and expressed as total counts.

**Cyclic AMP measurements.** Endothelial cells were grown to confluence in 60-mm dishes and changed to medium supplemented with 0.1% FCS for 24 h before use. Experiments were performed as previously described (18). To facilitate the measurement of cAMP production, isobutyl-1-methylxanthine (IBMX, 1 mM) was added before agonist addition for 10 min, followed by incubation with agonist for 10 min. Incubations were stopped by addition of 5% TCA and left on ice for 15 min, and the cell extract was then collected. The extracts were neutralized by addition of a 1:1 solution of ice-cold Freon/tri-N-ctylamine (1:1, vol/vol). Each sample was vortexed for ≥30 s to ensure adequate mixing; the mixture was then centrifuged at 2,500 rpm for 20 min (4°C). The samples then consisted of two phases, and the top aqueous phase containing the cAMP was collected. The pH of the upper phase was checked to ensure adequate neutralization. The samples were stored at ~80°C until analysis. cAMP was measured with a commercially available radioimmunoassay kit (Biomedical Technologies, Stoughton, MA).

**Thymidine incorporation studies.** Endothelial cells were seeded at 2 × 10⁵/well and allowed to attach and reach confluence. Cells were then placed in medium containing 0.1% FCS for 48 h. The medium was then changed, and the appropriate agonist was added for 24 h. During the last 6 h of incubation, 1 µCi/well of [3H]thymidine was added. Cells were then washed four to five times with Hank’s medium, and the reaction was stopped by addition of 10% TCA (ice cold) for 1 h. The sample was then washed three times with 5% TCA, and then 1 N NaOH (500 µl) was added to each well, which was left to incubate overnight. Subsequently, a 250-µl aliquot from each well was placed in scintillation vials, and radioactivity was determined in a β-counter (Beckman Instruments).

**ET-1 measurements.** Preliminary time course experiments (30 and 60 min; 2, 3, 6, 20, and 24 h) revealed that ET-1 tends to accumulate in the culture medium with time, reaching maximum level at 20 h. In view of these data, subsequent measurements of ET-1 secretion were made after 20 h of agonist stimulation. In brief, EC were grown in 60-cm² dishes, placed in 0.1% FCS-containing medium for 24 h before use, and then incubated for an additional 20 h with the appropriate agonist. The cell medium was then removed, and ET-1 was measured with a commercially available radioimmunoassay kit (Amersham), which detects ET-1, ET-2, and Big ET-1, but not ET-3.

**RNA isolation and RT-PCR.** ECV 304 were grown to confluence in 75-cm² flasks. Cells were placed in medium containing 0.1% FCS for 24 h, followed by incubation with the appropriate agent for an additional 24 h. Cells were washed with PBS, and RNA was isolated by a guanidinium isothiocyanate-phenol extraction. Optical density (260/280 nm) was measured for RNA quantitation, and a coupled RT-PCR was performed as previously described (18). To facilitate the measurement of ET-1 secretion, we used at 2 × 10⁵/well and allowed to attach and reach confluence. Cells were then placed in medium containing 0.1% FCS for 24 h before use. Experiments were performed as previously described (18). To facilitate the measurement of cAMP production, isobutyl-1-methylxanthine (IBMX, 1 mM) was added before agonist addition for 10 min, followed by incubation with agonist for 10 min. Incubations were stopped by addition of 5% TCA and left on ice for 15 min, and the cell extract was then collected. The extracts were neutralized by addition of a 1:1 solution of ice-cold Freon/tri-N-ctylamine (1:1, vol/vol). Each sample was vortexed for ≥30 s to ensure adequate mixing; the mixture was then centrifuged at 2,500 rpm for 20 min (4°C). The samples then consisted of two phases, and the top aqueous phase containing the cAMP was collected. The pH of the upper phase was checked to ensure adequate neutralization. The samples were stored at ~80°C until analysis. cAMP was measured with a commercially available radioimmunoassay kit (Biomedical Technologies, Stoughton, MA).

**ET-1 measurements.** Preliminary time course experiments (30 and 60 min; 2, 3, 6, 20, and 24 h) revealed that ET-1 tends to accumulate in the culture medium with time, reaching maximum level at 20 h. In view of these data, subsequent measurements of ET-1 secretion were made after 20 h of agonist stimulation. In brief, EC were grown in 60-cm² dishes, placed in 0.1% FCS-containing medium for 24 h before use, and then incubated for an additional 20 h with the appropriate agonist. The cell medium was then removed, and ET-1 was measured with a commercially available radioimmunoassay kit (Amersham), which detects ET-1, ET-2, and Big ET-1, but not ET-3.

**RNA isolation and RT-PCR.** ECV 304 were grown to confluence in 75-cm² flasks. Cells were placed in medium containing 0.1% FCS for 24 h, followed by incubation with the appropriate agent for an additional 24 h. Cells were washed with PBS, and RNA was isolated by a guanidinium isothiocyanate-phenol extraction. Optical density (260/280 nm) was measured for RNA quantitation, and a coupled RT-PCR was utilized (11). The sense (SN) and antisense (ASN) oligonucleotide primers for ET-1 and the probe (P) for a constitutively expressed internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized based on reported sequences. Tm is the melting temperature.

**Endothelin 1:**

SN 5′-AGAGTGTGCTCTATTGCGC-3′ 183-202 Tm = 60 exon2
P 5′-GGAACACCTAAGACAAACCAGG-3′ 513-534 Tm = 66
exon4-5
ASN 5′-TTGTGGGTCACTAACAAGC-3′ 623-695 Tm = 56
exon5
product length: 441 bp (4, 19)

GAPDH:
SN 5′-CCATGGAGAAGGCTGGG-3′ 371-388 Tm = 60
P 5′-CTAAAGCTTGTTGGTG-5′ 532-514 Tm = 58
ASN 5′-CAAAGTTGTCATGGATGACC-3′ 565-546 Tm = 58
product length: 195 bp (3)

Briefly, reverse transcription of 2 μg total RNA for both ET-1 and GAPDH was performed simultaneously using the Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories (BRL), Gaithersburg, MD), excess nucleotide substrates, and specific antisense primers for ET-1 and GAPDH. The resulting ET-1 and GAPDH cDNA samples were then coamplified by the PCR utilizing a thermostable polymerase (ampli-TAQ, Perkin Elmer-Cetus, Norwalk, CT), excess nucleotides, and the specific sense and antisense primers for ET-1 and GAPDH in a thermocycler (Perkin Elmer-Cetus). The PCR products were fractionated by electrophoresis on a 1.5% agarose gel and then transferred overnight to a nylon membrane (Zetabind, American Bioanalytical, Natick, MA). The membranes were probed simultaneously for ET-1 and GAPDH using their respective oligonucleotide probes end-labeled with 32P (125 μCi for each probe) by T4 polynucleotide kinase (BRL). Autoradiographs were exposed with an intensifying screen at ~80°C for 4 h with Kodak XAR film. Autoradiographs were quantified by densitometric analysis (Visage, BioImage), and results were normalized to the GAPDH lanes. Preparation of RNA and Northern blot analysis. Total RNA was extracted from cells with TRizol (BRL). RNA was stored at −70°C until use. RNA (20 μg) was electrophoresed on a 1.2% agarose-formaldehyde gel and transferred to a nylon filter. The blots were hybridized overnight at 65°C with a 32P-labeled probe (10^6 cpm/ml) labeled by the random priming method and washed at maximum stringency. Hybridization was carried out in a solution of 7% SDS, 1% BSA, 1 mM EDTA, and 250 mM Na2HPO4. The hybridized filters were washed with four 5-min washes of 2X standard sodium citrate (SSC) and 0.1% SDS at room temperature and twice more in 0.1X SSC and 0.1% SDS for 30 min at 65°C. The blots were then exposed to Kodak XAR 5 film.

Statistics. Results are expressed as means ± SE. Data were analyzed using either ANOVA (with Bonferroni post hoc analysis) or unpaired t-test where appropriate. A computer software statistics program (GraphPad InStat 2.03, GraphPad Software, San Diego, CA) was used for all calculations. Experiments were performed a minimum of three separate times unless otherwise stated.

RESULTS

PTH induces a rise in intracellular calcium concentrations. In Fig. 1, ECV 304 cells were loaded with the calcium-sensitive probe fura 2 and stimulated with increasing concentrations of PTH. The data are reported as intracellular calcium concentration ([Ca2+]i), nanomolar; peak calcium values were used for statistical analysis. PTH increased [Ca2+]i dose dependently with elevations of [Ca2+]i, seen at PTH concentrations as low as 10−12 M, with a maximum rise in [Ca2+]i at PTH concentrations of 10−8 M, followed by a decline in [Ca2+]i at the higher PTH concentrations. The magnitude of the elevation of intracellular calcium induced by 10−10 M PTH was similar to that induced by ATP (10−8 M), an agonist known to mobilize intracellular calcium stores in endothelial cells (8, 15). The pattern of rapid rise in intracellular calcium in ECV cells following 10−10 M PTH (Fig. 1B) is suggestive of a mobilization of intracellular calcium stores, rather than an influx of extracellular calcium. To evaluate further the source of PTH-induced elevations in intracellular calcium, we placed endothelial cells in either a nominally calcium-free extracellular medium (Fig. 1C) or a calcium-containing KRB buffer (Fig. 1B). The elevations in intracellular calcium induced by PTH showed similar calcium peaks, but in the absence of extracellular calcium, there is no sustained elevation, consistent with PTH inducing an increase in calcium from both intracellular and extracellular sources. Shown in Fig. 1, B and C, are representative traces of cells stimulated with 10−8 M PTH; the other PTH concentrations tested showed similar responses (data not shown). In view of these results and because the PTH receptor in kidney cells is known to be linked to the phosphoinositol (PI)-phospholipase C (PLC) pathway, we next examined whether PTH had effects on phosphoinositol turnover.

PTH stimulates phosphoinositol turnover. ECV 304 cells were loaded with [3H]myoinositol and were stimulated with increasing doses of PTH. The phosphoinositols were separated by high-pressure liquid chromatography. Doses of PTH as low as 10−11 M caused an elevation of IP3 (Fig. 2). PTH induced an elevation of IP3 comparable to that of ATP, an agonist known to stimulate phosphoinositol turnover (15). These data are consistent with PTH inducing a mobilization of calcium from intracellular stores. Nevertheless, PTH could also stimulate calcium influx through calcium-permeable channels, which have been observed previously in endothelial cells (2).

PTH stimulates cAMP accumulation in ECV 304. PTH has been shown to elevate cAMP levels in other cells; therefore, we examined the effect of PTH on cAMP content in the ECV 304 cells. As shown in Fig. 3, PTH dose dependently increased cAMP content of endothelial cells, becoming significant at a dose of 10−8 M. The effect of PTH was compared with that of forskolin. As is shown in Fig. 3, forskolin at 10−6 M increased cAMP accumulation to a much greater extent than did PTH, even at very high concentration (10−8 M). Thus these data demonstrate that PTH activates two signaling pathways in these cells. We next wished to evaluate whether the activation of these pathways resulted in a physiological response. Two important endothelial cell functions are proliferation and secretion of ET-1.

PTH stimulates [3H]thymidine incorporation. ECV 304 cells were serum deprived (0.1% FCS) for 24 h and then stimulated with PTH for an additional 24 h. Cells demonstrated an increase in thymidine incorporation when exposed to either a low or a high dose of PTH (Fig. 4A). These results are consistent with PTH acting as a mitogen in these cells. However, when forskolin (an agent which, like PTH, raises cAMP) was added to
these cells, no stimulation, but rather an inhibition, of [3H]thymidine incorporation was seen (Fig. 4B).

**PTH stimulates endothelin secretion.** The effects of PTH on ET-1 secretion from endothelial cells were examined next and compared with those of A23187, a calcium ionophore that is known to cause a Ca\(^{2+}\)-dependent stimulation of ET-1 secretion (39). As shown in Fig. 5A, PTH causes a dose-dependent increase in ET-1 secretion from ECV 304. At its highest concentration (10\(^{-8}\) M), PTH was comparable to the calcium ionophore A23187. However, concentrations of PTH <10\(^{-10}\) M had no effect on ET-1 secretion. In addition, we examined the effects of high extracellular calcium on ET-1 secretion. As seen in Fig. 5B, an extracellular calcium concentration between 1.5 and 2.25 mM led to a significant stimulation of ET-1 secretion; however, ET-1 secretion was found to increase in a biphasic manner in response to increasing calcium concentration. ET-1 secretion was found to reach maximum at an extracellular calcium concentration of 2.0 mM and then began to drop at higher calcium concentrations (2.25 mM). Interestingly, at an extracellular calcium concentration of 2.25 mM, the addition of PTH (10\(^{-8}\) M) had an inhibitory rather than an additive effect on ET-1 secretion. Furthermore, as shown in Fig. 5B, the protein kinase C (PKC) inhibitor staurosporine (30 nM) was inhibitory to ET-1 secretion stimulated by Ca\(^{2+}\) (2.0 mM), inhibiting ET-1 secretion to a level below that of control cells.

**Role of calcium and cAMP in ET-1 secretion.** When ET-1 secretion was measured (Fig. 6A), forskolin, at a dose (10\(^{-6}\) M) that stimulated cAMP accumulation over fourfold higher than PTH, does not significantly stimulate ET-1 secretion. Comparison with Fig. 3 illustrates the fact that this concentration of forskolin induces a much greater effect on cellular cAMP content than does PTH. Nevertheless, this concentration of forskolin had little effect on either ET-1 secretion or ET-1 mRNA expression (Fig. 6C). As can be seen in Fig. 6C, a high dose of PTH (10\(^{-7}\) M) also caused a nearly twofold rise in ET-1 message levels (as measured using RT-PCR), whereas forskolin (10\(^{-6}\) M) had no effect on ET-1 message production. GAPDH was used as an internal control, and densitometry samples were normalized to it (38). The gel densitometry for the RT-PCR is shown in Fig. 6D. Thus PTH appears to activate
adenylate cyclase, but the increase in cAMP by itself does not appear to serve as the signal by which this peptide influences ET-1 synthesis or secretion.

To evaluate further the role of calcium vs. cAMP in PTH-stimulated ET-1 secretion, ECV 304 cells were stimulated with increasing concentrations of PTH by itself (Fig. 6B, E) or in the presence of an inhibitor of A-kinase (Rp-cAMPS, F). As shown in Fig. 6B, Rp-cAMPS had little effect on PTH-stimulated ET-1 secretion. Various doses of Rp-cAMPS were tested (between 1.0 and 10^(-8) M) in dosing studies, without any effect on PTH-induced secretion. Thus, although elevations in extracellular calcium alone were sufficient to stimulate ET-1 secretion, elevations in cAMP (induced by forskolin) were not, suggesting that calcium is a more important second messenger in PTH-induced ET-1 secretion.

ECV 304 cells contain low levels of PTH1R mRNA. In an effort to evaluate whether the effects observed above in ECV 304 cells where related to activation of the PTH1R, we probed the cells for the cloned PTH1R (Fig. 7). Control cells (SaOS2) were found to contain the message for the cloned receptor, whereas the ECV 304 do not (n = 4). However, the presence of the PTH1R could be demonstrated by RT-PCR (n = 4; Fig. 7B). To evaluate whether PTH1R expression could be upregulated by the experimental maneuvers utilized to
study ET-1 secretion (because ET-1 was measured after 20 h of stimulation), ECV 304 cells grown to confluence in 10% FCS were either
1) kept in 10% FCS or
2) changed to 0.1% FCS by itself, or 0.1% FCS together
with one of the following:
   a) PTH $10^{-11}$ M,
   b) PTH $10^{-11}$ M,
   c) the phorbol ester 12-O-tetradecanoylphorbol 13-acetate $10^{-7}$ M,
   d) the calcium ionophore A23187 $10^{-7}$ M, or
   e) an extracellular calcium concentration of 2 mM for an additional 24 h. These maneuvers did not increase PTH receptor expression (data not shown, $n = 2$). Although the PTH2R has been reported to be present in endothelial cells (35), our preliminary exploration using the same PCR primers utilized by Usdin et al. (35) did not confirm its presence in ECV 304. RNA from endothelial cells was converted to cDNA via reverse transcription with an Invitrogen cDNA cycle kit. The resulting cDNA was subjected to amplification via PCR by use of primers CTRNYIH-F and LVEGLVL-R. PCR products were gel purified and introduced into pBluescript KS— (Stratagene). Inserts were amplified from this vector by PCR, and inserts of appropriate size were subjected to digestion with Sau3AI.

**DISCUSSION**

The data presented demonstrate that functional PTH receptors are present in a human umbilical vein endothelial cell line. PTH binding to these endothelial cells results in an activation of the calcium and cAMP signaling pathways in a matter of seconds/minutes, followed by an increase in [3H]thymidine incorporation and ET-1 secretion in hours/days.

The first well established effect of PTH on cell signaling was its ability to stimulate adenylate cyclase in kidney tubular cells (9). A similar effect was later demonstrated in bone cells (10). In the past decade, work from a
number of different laboratories has demonstrated that PTH in both renal and bone cells stimulates the production of inositol 1,4,5-trisphosphate and diacylglycerol, a rise in cytosolic Ca\(^{2+}\) concentration, and an activation of PKC (24, 33, 37). These results have led to the conclusion that PTH activates both the cAMP and PI-Ca\(^{2+}\) messenger systems when acting on many of its target cells. More recent studies have also demonstrated that PTH can stimulate calcium influx by inducing the appearance of voltage-sensitive calcium channels in an epithelial cell line (distal convoluted mouse cells) that does not normally contain voltage-sensitive calcium channels (14).

Our initial studies focused on PTH-induced changes in intracellular calcium. We report that PTH stimulates the PI-PLC pathway with mobilization of calcium from intracellular stores, which was responsible for the initial calcium transient (Fig. 1), as evidenced by the sustained elevation induced by PTH in the presence of extracellular calcium. In view of the fact that PTH increases intracellular calcium and stimulates inositol phosphate turnover (and presumably accumulation of diacylglycerol), it is likely that, as in other cell types, PTH activates PKC.

We also examined the effect of PTH on two specific endothelial cell functions, proliferation and ET-1 secretion. Although the role of PTH as a mitogen has been described for other cell systems (22), the regulatory properties of PTH and second messengers for PTH action on endothelial cells are unknown. The fact that increases in \(^{3}H\)thymidine incorporation are seen with the lower doses of PTH, similar to those in which increases in intracellular calcium are observed, and the fact that addition of forskolin (an activator of the catalytic subunit of adenylate cyclase) inhibits \(^{3}H\)thymidine incorporation suggest that the calcium signaling pathway is involved in this proliferative response.
The clinical significance of the observed PTH-induced increases in thymidine incorporation are not clear. Patients with 1°-HPT have a higher incidence of left ventricular hypertrophy and arterial hypertension. Whether this is related to a direct effect of PTH or to an indirect effect of ET-1 or ET-1-stimulated vascular endothelial growth factor secretion is not known (12, 25, 29, 30, 32). ET-1 is one of the most powerful and longest acting vasoconstrictors known. Numerous clinical studies support small changes in ET-1 levels as being physiologically important. Thus the changes in ET-1 levels induced by PTH reported here are likely to be clinically significant (17, 23).

The relative contribution of the signaling pathways activated by PTH in stimulating ET-1 secretion was assessed. Low concentrations of PTH (10^{-11} M, Fig. 5), which mobilize calcium from intracellular stores, do not stimulate ET-1 secretion, and increasing extracellular calcium or adding a calcium ionophore (A23187) increases ET-1 secretion by the same magnitude as PTH, but its effect is not additive to that of PTH.

An unexpected finding was that ET-1 secretion showed a biphasic response when cells were stimulated with increasing concentrations of extracellular calcium (Fig. 5B). However, these data are consistent with that reported by Brunner and colleagues (6, 7). In those studies, porcine aortic endothelial cells maximally secreted ET-1 when [Ca^{2+}]_i was between 190 and 470 nM (initial extracellular calcium 1.8 mM), and ET-1 secretion was inhibited when the calcium concentration was higher or lower than that. Because, in our experiments, ET-1 secretion was already dropping at a calcium concentration of 2.25 mM, the further addition of PTH (which raises [Ca^{2+}]_i further) leads to an inhibitory rather than an additive effect of these two agonists. Clearly, however, either PTH or extracellular calcium can by themselves stimulate ET secretion. These data would suggest that, with regard to stimulation of ET-1 secretion, there is an optimal [Ca^{2+}], that theoretically may be achieved in vivo with a range of PTH and calcium concentrations.

In contrast, adenylate cyclase activation by PTH appears to play only a minor role in ET-1 secretion, because forskolin does not stimulate secretion. Higher concentrations of PTH were also required to increase cAMP compared with PTH effects on intracellular calcium. Nevertheless, it is possible that PTH might induce small elevations of cAMP and still stimulate ET-1 secretion by A-kinase activation. To evaluate this possibility, endothelial cells were incubated with PTH in the presence of the A-kinase inhibitor Rp-cAMPS (31) (Fig. 6). Rp-cAMPS had no effect on PTH-induced ET-1 secretion, consistent with calcium being the main second messenger involved in PTH effects on endothelial cells. Whether PTH activation of adenylate cyclase contributes even a minor role to its effects on ET-1 is unclear. One caveat is that the PTH-induced signaling events occur in a very short time frame compared with the cellular responses studied (ET-1 secretion and proliferation); thus it is difficult to prove a definitive causal relationship.

Although our studies demonstrate that the PTH receptor is in fact present in endothelial cells, it is expressed only at very low levels. Whether the coupling of the PTH receptor on G protein to PLC is different in ECV cells than it is in other cell types, or whether the effects we are observing are through a PTH2 or even a third PTH receptor that remains to be identified, is an issue we are currently exploring further.

In summary, the present study demonstrates that PTH has distinct effects on endothelium-derived cells. PTH is a hormone with diverse functional properties besides those usually associated with its action. Of note is that the low concentrations of PTH used in this study are within the physiological range, whereas the higher concentrations are not. Thus it is likely that, under physiological conditions, PTH plays only a minor role in modulating endothelial cell function. However, in pathological states such as primary, secondary, or tertiary hyperparathyroidism, the circulating levels of PTH are within the range utilized in this study; 10^{-10} M.

In conclusion, our finding that PTH has effects on an endothelial cell line suggests that PTH may have direct effects on the endothelium in addition to its effects on vascular smooth muscle. The physiological relevance of these findings remains to be defined.

We would like to thank Dr. Howard Rasmussen for helpful discussions and suggestions.

This study was supported by National Institutes of Health Grant DK-19813. O. R. Rosales is a Research Fellow of the American Heart Association (Connecticut Affiliate).
REFERENCES


