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CLONAL RAT PARATHYROID CELL LINE EXPRESSES A PARATHYROID HORMONE-RELATED PEPTIDE BUT NOT PARATHYROID HORMONE ITSELF

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A novel parathyroid hormone-related peptide has been identified in tumors associated with the syndrome of humoral hypercalcemia of malignancy. Subsequently, mRNAs encoding this peptide have been found to be expressed in a number of normal tissues, including the parathyroids. Using Northern blotting, RNase protection, and immunochemical techniques, we examined a clonal rat parathyroid cell line originally developed as a model system for studying parathyroid cell physiology. We found that this line expresses the parathyroid hormone-related peptide but not parathyroid hormone itself. Secretion of the parathyroid hormone-related peptide varied inversely with extracellular calcium concentration, but neither calcium nor 1,25-dihydroxyvitamin D_3 appeared to influence steady-state parathyroid hormone-related peptide mRNA levels. This clonal line may prove to be an interesting system for studying the factors responsible for tissue-specific parathyroid hormone and parathyroid hormone-related peptide gene expression.

A parathyroid hormone-related peptide (PTHRP) has been purified (1) and its cDNA cloned (2-4) from tumors associated with the syndrome of humoral hypercalcemia of malignancy (HHM). Chromosomal localization and structural data indicate that the PTHRP and parathyroid hormone (PTH) genes arose by duplication from a common ancestral chromosome and therefore represent members

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Abbreviations: PTHRP, parathyroid hormone-related peptide; PTH, parathyroid hormone; PT-r, clonal rat parathyroid cell line; 1,25-$(OH)_2$D, 1,25-dihydroxyvitamin D_3; HHM, humoral hypercalcemia of malignancy; RIA, radioimmunoassay; h, human; b, bovine; r, rat.

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of a gene family (3,5). The human PTHRP gene has been recently isolated and found to be a complex transcriptional unit which appears to use multiple promoters (5), in contrast to the relatively simple organization of the PTH gene (6). In addition, whereas the PTH gene seems to be expressed exclusively in parathyroid cells (7), PTHRP transcripts have been identified in a variety of endocrine and non-endocrine tissues (3,4,8). PTHRP mRNAs have also been identified in low abundance in normal parathyroid tissue and appear to be over-expressed in human parathyroid adenomas (8).

In the present study, we examined a clonal rat parathyroid cell line (PT-r) initially developed from hyperplastic rat parathyroid glands (9). These cells do not display a transformed phenotype and secrete PTH-like activity as an inverse function of the extracellular calcium concentration (9). We found that this PTH-like activity represents the expression of PTHRP and not PTH by these cells. In additional experiments, we examined the effects of calcium and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$D) on steady-state PTHRP mRNA in PT-r cells, since both agents are known to influence PTH gene expression in normal parathyroid tissue (10,11).

MATERIALS AND METHODS

Cell Culture. PT-r cells were cultured in a growth medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's minimal essential medium (DMEM) and Coon's modified Ham's F12 (Sigma) with supplements as described (9) and a total calcium concentration of 1.6 mM. Subconfluent cells were passaged at high dilution (>1:50). For regulation experiments, cells were plated at a density of 4 x 10^5/10-cm dish (RNA studies) or 1.5 x 10^5/6-cm dish (secretion studies) and maintained in growth medium until subconfluent. Medium was then replaced with calcium-free medium (9) supplemented with $CaCl_2$ to the required concentrations, or, in some experiments, with growth medium containing 1,25-$(OH)_2$D or ethanol vehicle (<0.1%). Ionized calcium was measured using a Radiometer ICA 1 analyzer. Medium was changed at 48 hr in experiments extending beyond this time. For RNA studies, cells were harvested in guanidinium solution at the times indicated. For the secretion experiments, medium was harvested at the indicated times, centrifuged, and stored at -20°C for assay; cells were then detached using trypsin-EDTA and counted using a Coulter counter.

RNA Preparation, Northern Blotting and RNase Protection Analysis. Total RNA and poly $(A)^+$ RNA were prepared as described (8,12,13) and quantitated by A_{260}. Northern blot analysis was performed as reported previously (8,13). The RNA probes included a PvuII-SacI fragment of a human PTHRP cDNA clone (3), a 330-bp PvuII-BglII fragment of a rat PTHRP cDNA clone (courtesy M. Thiede) (4), and a PstI-XbaI genomic fragment corresponding to the coding region of rat PTH (Courtesy G. Heinrich) (14). The DNA probes included a BamHI fragment of the rat cyclophilin cDNA (courtesy T. Van Itallie) (15) and a 2.1-kb EcoRI fragment of the human vitamin D receptor cDNA (courtesy W. Pike) (16). Riboprobes and DNA probes were prepared as reported previously (8,13). RNase protection analysis was performed as described (17,18) using 2 x 10^5 cpm of [^{32}P]-labeled rat PTHRP Riboprobe.

Radioimmunoassays. Antiserum R14 was raised in a rabbit against synthetic human (h) PTHRP(1-74) (19) and used at a final dilution of 1:12,000 in a radioimmunoassay (RIA) employing HPLC-purified [^{125}I]hPTHRP(1-74) (lactoperoxidase method) as radioligand and hPTHRP(1-74) as standard (10-10,000 pM).

Conditioned media and antiserum were pre-incubated for 24 hr at 4°C and [^{125}I]hPTHRP(1-74) added and incubated for an additional 48 hr at 4°C; phase separation was carried out with dextran-coated charcoal. Assay sensitivity was 25 pM hPTHRP(1-74) (20 pgeq hPTHRP(1-74) per 100 µl). The RIA was equally sensitive to (Tyr^{36})hPTHRP(1-36) (20) but 10-fold less sensitive to hPTHRP(49-74). There was no cross-reactivity with up to 10,000 pM bovine PTH(1-34). The human and rat PTHRPs differ by only one residue through the first 74 amino acids (4).

The RIA employing a goat anti-hPTH antiserum (B6-6, 1:1000 final dilution) and [^{125}I] rat PTH (1-34) was performed as described previously (9).

RESULTS AND DISCUSSION

Initially, we examined PT-r RNA for PTHRP transcripts by Northern blotting using a rat PTHRP RNA probe. As shown in Fig. 1A, poly (A)$^+$ RNA prepared from PT-r cells contained a single 1.7-kb hybridizing band, which comigrated with that identified in poly (A)$^+$ RNA derived from a rat Leydig cell tumor, a well-characterized animal model of HHM (13,21). The PTHRP mRNA in the PT-r cells was relatively abundant, being in approximately the same range found in the Leydig tumor sample and far more impressive than the faint transcripts previously observed in poly (A)$^+$ RNA prepared from normal bovine parathyroid glands (8). This relative abundance provided a strong hybridization signal even when total RNA rather than poly (A)$^+$ RNA was used (Fig. 1A). RNase protection analysis using the same coding-region rat PTHRP RNA probe confirmed these results. As shown in Fig. 1B, the Leydig cell and PT-r RNAs strongly protected an identical 330-bp fragment. These protected fragments were of roughly the same intensity.

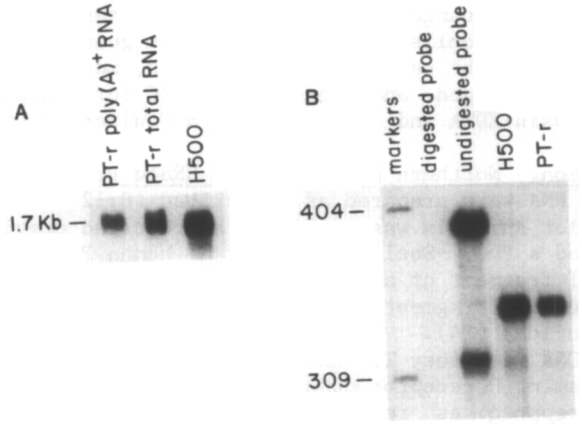


Figure 1. Identification of PTHRP mRNA in PT-r cells. (A) Northern analysis of poly (A)$^+$ RNA (3 µg) or total RNA (15 µg) from PT-r cells and poly (A)$^+$ RNA (2 µg) from the rat H500 Leydig cell tumor (21) using a rat PTHRP RNA probe (18-hr exposure). (B) RNase protection analysis of poly (A)$^+$ RNA (8 µg each) prepared from the H500 tumor and PT-r cells (8-hr exposure). The RNA probe contains some polylinker sequences from the vector and is about 390 bp in length.

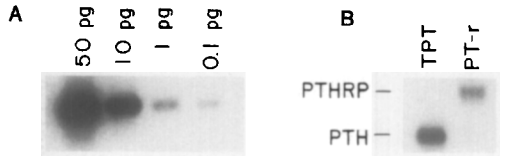
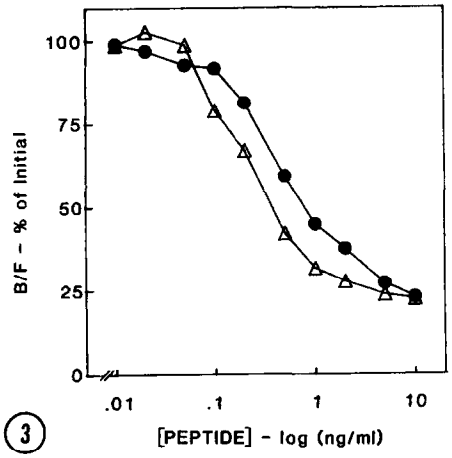


Figure 2. Absence of PTH mRNA in PT-r cells. (A) Northern analysis of graded amounts of synthetic rat PTH sense RNA added to 2 μg of control (human renal carcinoma) poly $(A)^+$ RNA; rat PTH cRNA probe (16-hr exposure). (B) Northern analysis of rat thyroparathyroid total RNA (20 μg) and PT-r cell poly $(A)^+$ RNA (2 μg) using rat PTHRP and PTH cRNA probes added simultaneously (10^6 cpm/ml) to the hybridization solution (16-hr exposure).

We next examined PT-r cell RNA for PTH transcripts. In the first such experiments, we found no detectable hybridization using a human PTH DNA probe (data not shown). This question was subsequently explored in a more sensitive and quantitative fashion using a rat PTH RNA probe. As shown in Fig. 2A, this probe could easily detect 0.1 pg of synthetic sense rat PTH RNA added to 2 μg of control poly $(A)^+$ RNA, defining a detection limit of a 0.000005% mRNA species. PTH mRNA represents an approximately 1% mRNA species in normal parathyroid tissues (10,11). As shown in Fig. 2B, no detectable PTH mRNA was identified on Northern analysis of PT-r cell poly $(A)^+$ RNA using this RNA probe, whereas an abundant 800-base PTH transcript was observed in rat thyroparathyroid total RNA (14). We confirmed this result in RNA prepared from PT-r cells cultured independently at the NIH and Yale. We concluded that the PTH gene is either not expressed in PT-r cells or is expressed at an extremely low level.

In the initial description of the PT-r clonal line, secretory PTH-like activity was detected both by bioassay and by RIA using a goat anti-hPTH antiserum. The bioassay employed in these initial studies would not distinguish between PTH and PTHRP, but we were puzzled by the RIA findings in view of the data described above. We therefore examined displacement of $[^{125}I]$ rat (r) PTH(1-34) in this assay by (Tyr 36)hPTHRP(1-36) and rPTH(1-34). We found that this antiserum actually has a higher affinity for (Tyr 36)hPTHRP(1-36) than rPTH(1-34), by a factor of about 1.5 (Fig. 3). This cross-reactivity explains the initial RIA results (9) and further substantiates the conclusion drawn above concerning the lack of PTH expression by PT-r cells.

Given the relationship of the PTH and PTHRP genes, the well-established regulation of PTH gene transcription by calcium and 1,25-$(OH)_2D$ (10,11), and the expression of the PTHRP gene in PT-r cells, we were particularly interested in whether calcium and/or 1,25-$(OH)_2D$ might influence PTHRP mRNA levels in these cells. We initially examined steady-state PTHRP mRNA levels in PT-r cells maintained in low (0.8 mM) or high (4.8 mM) extracellular calcium for 15 to 72 hr. The high calcium concentration seemed to have little effect,



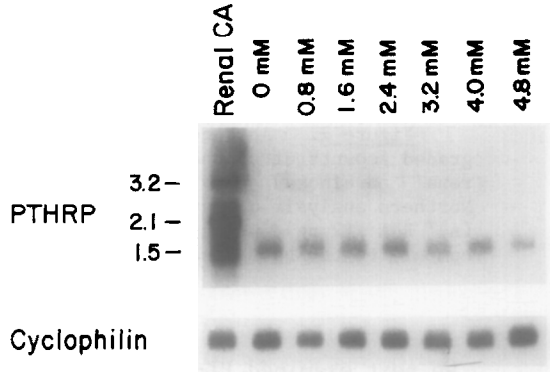


Figure 3. Displacement of [^{125}I] rat PTH(1-34) by (Tyr 36) human PTHRP(1-36)(Δ) and rat PTH (1-34) (●) using an anti-human PTH antiserum. Results were expressed as a percentage of the initial B/Fo, which ranged from 0.39-0.48 for the curves shown. In separate experiments, up to 100 ng/ml ACTH, lysine- or arginine-vasopressin, vasoactive intestinal peptide, or gastrin (all from Peninsula) were not detected in the assay.

Figure 4. Effect of extracellular calcium on PTHRP mRNA in PT-r cells. Poly $(A)^+$ RNA (3 μg) from PT-r cells maintained for 72 hr in the total calcium concentrations indicated was analyzed by Northern blotting. Poly $(A)^+$ RNA (1 μg) from an HHM-associated human renal carcinoma was included as a positive control (3,8,13). The membrane was hybridized to a human PTHRP cRNA probe (8-hr film exposure) and a rat cyclophilin DNA probe (6-hr exposure).

although there appeared to be a modest decrease in PTHRP mRNA at 72 hr (not shown). We next examined mRNA prepared from cells maintained for 72 hr in a wide range of calcium concentrations. Again, we observed an apparent decrease in PTHRP mRNA at 4.8 mM total calcium, but this effect was slight and did not appear to be dose-related (Fig. 4). We confirmed appropriate concentrations of ionized calcium in this experiment by measurement and also analyzed the samples by RNase protection assay, with equivalent results (not shown). Short-term (2-12 hr) experiments at low or high calcium showed no convincing calcium effect. Thus, any putative calcium effect on steady-state PTHRP mRNA levels appeared to be modest and occurred only in cells maintained in very high calcium for prolonged periods.

In the next set of experiments, we examined PTHRP mRNA levels in PT-r cells exposed to 100 nM 1,25-$(OH)_2$D for 6 to 72 hr. As shown in Fig. 5A, 1,25-$(OH)_2$D was without apparent effect. We also explored the possibility of additive calcium and 1,25-$(OH)_2$D effects by performing experiments with 100 nM 1,25-$(OH)_2$D at low and high extracellular calcium. Again, we saw no apparent effect (Fig. 5B). Since we have observed a quite striking reduction in steady-state PTHRP mRNA levels in a cultured human C cell line in response to 1,25-$(OH)_2$D (K. Ikeda, C. Lu, and A. Broadus, unpublished observations), we were

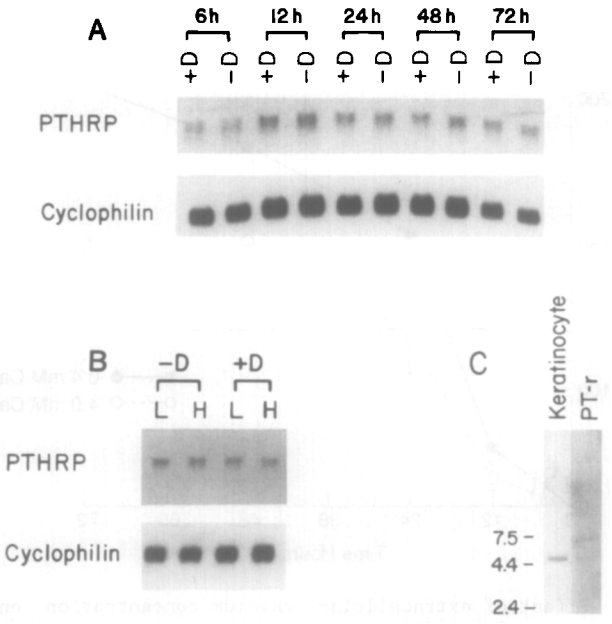


Figure 5. Absence of a 1,25-$(OH)_2D$ effect on PTHRP mRNA levels in PT-r cells. (A) Northern analysis of total RNA (10 μg) from PT-r cells cultured in 1.6 mM calcium with (+D) or without (-D) 100 nM 1,25-$(OH)_2D$ for the times indicated. The membrane was hybridized with the human PTHRP (36-hr film exposure) and rat cyclophilin (8-hr exposure) probes. (B) Northern analysis of total RNA (20 μg) from PT-r cells pretreated with 100 nM 1,25-$(OH)_2D$ for 48 hr and then maintained in low (0.8 mM) or high (4.0 mM) calcium with 100 nM 1,25-$(OH)_2D$ for an additional 24 hr. The probes and film exposures were as described above. (C) Northern analysis of poly $(A)^+$ RNA from human keratinocytes (2 μg) and PT-r cells (8 μg) using a human vitamin D receptor DNA probe (72-hr film exposure). The 7-kb hybridizing transcript in the PT-r specimen probably represents an incompletely processed mRNA (J.W. Pike, personal communication).

puzzled by the lack of a 1,25-$(OH)_2D$ response in PT-r cells. Although there are a number of possible explanations for this lack of responsiveness, receptor binding studies using [^{3}H]1,25-$(OH)_2D$ indicate that vitamin D receptors are present in these cells in very low abundance (D. DeGrange, K. Sakaguchi, M. Brandi, and G. Aurbach, unpublished observations). We also examined poly $(A)^+$ RNA from PT-r cells with a human vitamin D receptor DNA probe, using human keratinocyte poly $(A)^+$ RNA as a positive control. As shown in Fig. 5C, the keratinocyte specimen contained a prominent 4.6-kb hybridizing mRNA (16), whereas only a faint 4.4-kb hybridizing transcript corresponding to the rat vitamin D receptor mRNA (22) was observed in the PT-r specimen.

We have recently developed a RIA for human PTHRP and used this assay to study PTHRP secretion by PT-r cells. Immunoreactive PTHRP was detected as early as 12 hr in media from PT-r cells and increased gradually with time. As shown in Fig. 6, a high (4.0 mM) medium calcium concentration was associated with a statistically significant reduction in PTHRP secretion from 12 through 72 hr. In preliminary dose-response experiments carried out at 48 hr, de-

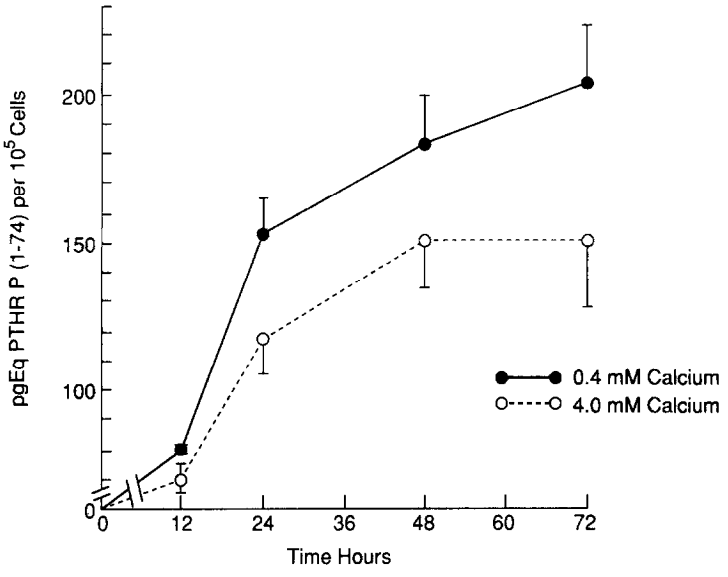


Figure 6. Effect of extracellular calcium concentration on PTHRP secretion by PT-r cells. Subconfluent cells were incubated for 12-72 hr in medium containing 0.4 mM (●) or 4.0 mM (○) total calcium; ionized calcium concentrations were 0.6 mM and 2.5 mM, respectively. Mean (± SEM) results of four experiments are shown. In each experiment, cell counts and PTHRP concentrations were determined in triplicate. The results were expressed as pgeq hPTH(1-74) per 10^5 cells. Analysis of all data using Wilcoxon's signed rank test for differences with paired treatments (23,24) showed significant ($p = 0.01$) suppression of PTHRP secretion at 4.0 mM calcium.

creases in PTHRP were observed in the range of 2.4 mM calcium (2.1 mM Ca^{2+}). The concentrations of calcium employed in these studies and our quantitative findings are very similar to those reported in the initial report describing the PT-r system (9).

We conclude that PT-r cells express PTHRP and not PTH itself. These cells secrete PTHRP as an inverse function of the extracellular calcium concentration, but neither calcium nor 1,25-$(OH)_2$D appears to significantly influence steady-state PTHRP mRNA levels. PT-r cells should prove useful for additional studies of PTHRP secretion and also for examining potential post-translational processing of the peptide. In addition, these cells represent a very interesting system for investigating the mechanisms responsible for tissue-specific PTH and PTHRP gene expression.

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