Biosynthesis and Processing of Endogenous Parathyroid Hormone Related Peptide (PTHRP) by Rat Leydig Cell Tumor H-500†

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ABSTRACT: We have examined in vitro the biosynthesis and processing of endogenous PTHRP in cultured rat H-500 Leydig tumor cells. Cells were grown to confluence and pulse labeled with [^{3}H]Ile, 50μCi/mL, in Ile free culture medium for 2 min to 6 h. In some experiments incubations were carried out in culture medium alone in the presence of 0.3 mM cycloheximide or 20 μg/mL unlabeled Ile. Cell extracts and culture media were analyzed by affinity chromatography employing an antibody directed against the bioactive NH_2-terminal region, PTHRP(1–34), followed by gel-permeation or reversed-phase high-performance liquid chromatography. Incorporation of [^{3}H]Ile into PTHRP in cell extracts increased over 20 min during pulse labeling and then remained constant throughout the incubation period up to 6 h. In contrast, the release of [^{3}H]PTHRP into culture medium increased progressively over 6 h. Addition of cycloheximide or unlabeled Ile almost completely blocked incorporation of [^{3}H]Ile into newly synthesised PTHRP. Three molecular forms of PTHRP were seen which comigrated with PTHRP(1–36), PTHRP(1–86), and PTHRP(1–141) standards in both chromatographic systems employed. After 20 min these species comprised approximately 63%, 30%, and 7% of newly synthesized PTHRP, respectively. These three molecular forms of PTHRP were observed both intra- and extracellularly, and no further metabolism of these species was seen after release into conditioned medium. Pulse–chase studies demonstrated a rapid decrease of newly synthesized PTHRP forms within cells after 20 min; there was, however, a progressive increase in [^{3}H]PTHRP in conditioned culture medium. The same molecular species of PTHRP were observed during pulse–chase studies as were seen during continuous pulse labeling. The presence of these molecular forms was also confirmed by chromatographic analysis of endogenous unlabeled PTHRP using an NH_2-terminal radioimmunoassay as a detection system. These studies demonstrate rapid processing of endogenous PTHRP into three major NH_2-terminal species and show rapid constitutive release of these forms into the extracellular milieu. Tumors such as the rat H-500 Leydig cell tumor which cause hypercalcemia in vivo may therefore produce multiple NH_2-terminal PTHRP entities, each potentially capable of eliciting biological activity.

Parathyroid hormone related peptide (PTHRP) is a recently isolated peptide which is known to be pathogenetic in the hypercalcemia associated with malignancy (Moseley et al., 1987; Stewart et al., 1987; Strewler, 1987; Mangin et al., 1988; Suva et al., 1987; Yasuda et al., 1989a). It can be produced by a variety of cancers, normal tissues, primary cultures, and tumor cell lines (Ikeda et al., 1988; Selvanyagam et al., 1991; Danks et al., 1989; Kremer et al., 1990; Henderson et al., 1991). Human PTHRP is the product of a single gene and can occur in three isoforms of 139, 141, and 173 amino acids as a result of alternate mRNA splicing (Yasuda et al., 1989b; Mangin et al., 1989). The rat PTHRP gene results in a single mRNA species which encodes a peptide of 141 amino acids (Karaplis et al., 1990). On the basis of an analysis of the amino acid sequence of PTHRP several posttranslation modifications of PTHRP can potentially occur, and O-glycosylated NH_2-terminal forms of PTHRP have been reported to be secreted by nonmalignant human keratinocytes (Wu et al., 1991). Characterization of serum of patients with hypercalcemia of malignancy showed the presence of multiple forms of PTHRP both by bioassay and by radioimmunoassay (Goltzman et al., 1981; Henderson et al., 1990). Furthermore, several human and rat cell lines secrete PTHRP of different molecular weights, and secretion of a novel midregion fragment by tumor cell lines has also been reported (Brandt et al., 1991; Soifer et al., 1992). Many of the biological consequences of PTHRP overproduction are due to the interaction of its NH_2-terminal domain with receptors in bone and kidney; however, a potential role for other regions of the molecule has also been reported (Rabbani et al., 1988; Orloff et al., 1989; Juppner et al., 1988; Nissenson et al., 1988; Care et al., 1990; Fenton et al., 1991; Kaiser et al., 1992; Horiuchi et al., 1987; Kemp et al., 1987; Stewart et al., 1988; Yates et al., 1988; Fukayama et al., 1988). To date, no detailed examination of endogenous PTHRP biosynthesis and processing by any cell type has been reported, yet knowledge of the metabolism of PTHRP clearly is critical for understanding its mechanism of action and for developing improved methods of detection. The Rice H-500 rat Leydig cell tumor, when implanted into male Fischer rats, produces a well-characterized animal model of malignancy-associated hypercalcemia which closely mimics the human syndrome and which is due to overproduction of PTHRP (Sica et al., 1983; Rizzoli & Boujour, 1989; Liu et al., 1993). We have therefore examined endogenous biosynthesis and processing of PTHRP(1–141) using this model system.

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MATERIALS AND METHODS

Materials. Leydig cell tumor (Rice H-500) was obtained from EG and G Mason Research Institute (Worcester, MA) and the Breast Cancer Animal and Tumor Bank, NCI, NIH, (Bethesda, MD). Culture medium RPMI-1640, penicillin, streptomycin, fungizone, and fetal bovine serum were from Gibco, Canada (Burlington, Ontario, Canada). [^{3}H]Ile was purchased from NEN Du Pont (Mississauga, Ontario, Canada), and CNBr Sepharose 4B was from Pharmacia and LKB (Uppsala, Sweden). Gel-permeation HPLC standards were from Boehringer Mannheim (Montréal, Québec, Canada). [Tyr^{36}]PTHRP(1–36) and PTHRP(1–86) were from Bachem (Torrance, CA). PTHRP(1–34) was synthesized by a solid-phase method (Henderson et al. 1990). Recombinant PTHRP(1–141) was prepared in our laboratory as described (Rabbani et al., 1991). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. The Leydig cell tumor (Rice H-500) was maintained by subcutaneous transplantation. After approximately 14 days, when serum calcium was 13–14 mg/dL, tumors were removed. Portions of tumor tissue were mechanically dispersed into single-cell suspensions, which were cultured in RPMI 1640 medium containing an antibiotic–antimycotic solution (100 μg/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone) and 10% fetal bovine serum. Cells were grown in 75-cm^2 tissue culture flasks as previously described (Rabbani et al., 1986). Biosynthetic labeling was performed when the cultures were 75% confluent. For analysis of unlabeled PTHRP, cultures at 70% confluence were changed to serum-free RPMI 1640 medium, and after 24 h cells and conditioned medium were harvested for study.

Biosynthetic Labeling. Rat Leydig tumor cell medium was replaced with Ile-deficient RPMI 1640 culture medium containing [^{3}H]Ile (50 μCi/mL) and 200 KIU/mL Trasylol (KIU, kallikrein international unit). In view of the fact that the purpose of the study was to examine the biosynthesis and processing of NH_2-terminal forms of PTHRP, [^{3}H]Ile was employed as a preferred amino acid because of its abundance in the NH_2-terminal region of the molecule. Cells were cultured in this medium for 2 min to 6 h (Chiba et al., 1988). In some studies the incubations were carried out in the presence of cycloheximide (0.3 mM) or Ile (20 μg/mL). In separate pulse–chase experiments, the medium with [^{3}H]Ile was removed after 20 min, and the cells were incubated in culture medium containing nonradioactive Ile for various time periods.

Extraction of Cells and Conditioned Medium. On completion of pulse labeling or after pulse–chase incubations cells were washed twice in phosphate-buffered saline (PBS), scraped in RPMI medium, and pelleted by centrifugation at 200*g* for 5 min. Cells were resuspended in a solution of 1 M guanidine hydrochloride, 0.1 N acetic acid, 10 mM dithiothreitol, 0.25 mg/mL protamine sulfate, 1 mM EDTA, 0.1 mg/mL bacitracin, 0.2 mM phenylmethanesulfonyl fluoride, 2 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 0.1% Tween-20 (w/v) (Brandt et al., 1991). Cell pellets were sonicated for 30 s and clarified by centrifugation at 12000*g* using an SS-34 rotor in an RC 5C centrifuge (Sorvall Instruments, Newtown, CT). Unlabeled cell cultures and conditioned medium for analysis were similarly extracted. All cell extracts and conditioned culture media were then applied to affinity columns.

Characterization of Biosynthetic Products. (i) Affinity Chromatography. Polyclonal rabbit anti-PTHRP(1–34) serum was raised as described previously (Henderson et al., 1990). A PTHRP(1–34) affinity chromatography column was prepared by coupling 10 mg of PTHRP(1–34) to cyanogen bromide activated Sepharose 4B. Polyclonal anti-PTHRP serum was applied to the column, and antibodies bound to immobilized PTHRP(1–34) were eluted with 50 mM glycine, pH 3.0, and 100 mM sodium chloride and adjusted to pH 8.0. This affinity-purified PTHRP(1–34) antibody was then coupled to cyanogen bromide activated Sepharose 4B according to the manufacturer's instructions. Aliquots (500 μL) of affinity resin were poured into 0.6-cm-i.d. columns and stored in 0.2% sodium azide at 4 °C until use and also between experiments. A total of six such columns were used for these studies (Stewart et al., 1991). All columns were characterized for their specificity by affinity chromatography using ^{125}I-labeled PTHRP(1–36)NH_2, ^{125}I-labeled PTHRP(67–86), and ^{125}I-labeled urinary plasminogen activator uPA(1–143). Only ^{125}I-labeled [Tyr^{36}]PTHRP(1–36) bound to these columns. All cell extracts and culture media were adjusted to pH 7.5 and applied to affinity columns. This loading step was repeated three times, and then columns were washed with a mixture of PBS and 0.1% BSA until basal levels (approximately 30 cpm) of radioactivity were obtained. Columns were eluted with a solution of 50 μM glycine, pH 3.0, and 100 mM NaCl and counted by liquid scintillation counting. In some experiments affinity eluates were adjusted to pH 5.0 with 0.1% trifluoroacetic acid (F_3CCOOH) and passed through cartridges of octadecyl silica (ODS) (C_{18} Sep-Pak, Waters Associates, Mississauga, Ontario, Canada), which were washed with 0.1% F_3CCOOH and eluted with 80% acetonitrile (CH_3CN). All Sep-Pak eluates were dried in a speed vac (Savant Instruments, Hicksville, NY) and stored at −70 °C until further characterization (up to 1 week).

(ii) Gel-Permeation Chromatography. Size-exclusion HPLC was carried out on an ^{125}I gel-permeation HPLC column (Waters Associates) connected in series with a Protein Pak 300-SW gel-permeation column (Waters Associates), eluting at a flow rate of 1 mL/min with 40% aqueous CH_3CN containing 0.1% CF_3COOH. Calibration was achieved by injection of a mixture of 0.5 μg each of trypsin inhibitor, cytochrome *c*, aprotinin, insulin B chain (molecular weights of 21 500, 12 500, 6500, and 3400, respectively) (all from Boehringer Mannheim, Montréal, Québec, Canada), [Tyr^{36}]-PTHRP(1–36), PTHRP(1–86), and PTHRP(1–141).

(iii) Reversed-Phase HPLC. Reversed-phase (RP) HPLC was performed, as previously described (Rabbani et al., 1986; Rabbani et al., 1990), on C_{18} μBondapak columns (Waters Associates). Samples for loading were diluted with 0.1% CF_3COOH, and columns were developed over 60 min at a flow rate of 1.5 mL/min with linear gradients of CH_3CN containing 0.1% CF_3COOH as a counterion. Column fractions were counted by liquid scintillation counting.

PTHRP Radioimmunoassay. Column fractions were dried and assayed for PTHRP by radioimmunoassay as previously described (Henderson et al., 1990) using PTHRP(1–34) as a standard. Results are expressed therefore as nanogram equivalents (ng equiv) relative to PTHRP(1–34).

Statistical Analysis. All results were statistically analyzed by performing a *t* test, with paired analysis or multiple comparisons when appropriate.

RESULTS

Time Course of [^{3}H]Ile Incorporation into PTHRP in Rat Leydig Tumor (H-500) Cells. A progressive increase in affinity-purified nascent [^{3}H]PTHRP was seen in cell extracts during 20 min of labeling; the level then remained constant for the remainder of the labeling period up to 6 h. The release

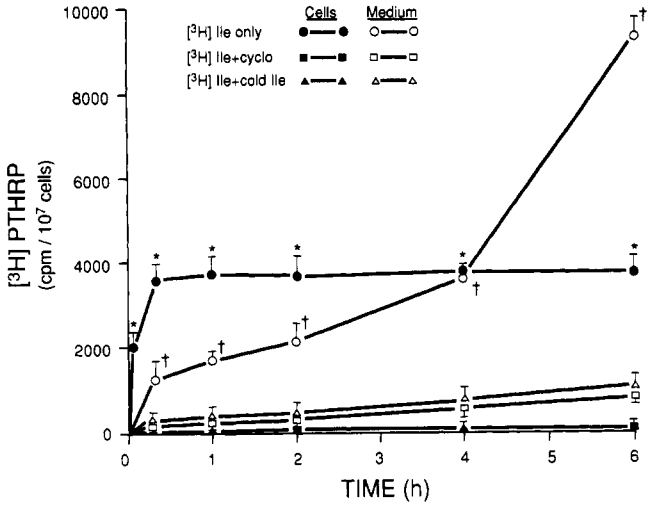

FIGURE 1: Time course of incorporation of [^{3}H]Ile into PTHRP in H-500 rat Leydig tumor cells. Intracellular content at each time point of affinity-purified [^{3}H]PTHRP from cells cultured in the absence (●) or presence of 0.3 μM cycloheximide (■) or of 20 μg/mL nonradioactive Ile (▲). Significant differences, at each time point, compared to incubations performed with nonradioactive Ile are denoted by asterisks ($p < 0.01$). Medium content, at each time point, of affinity-purified [^{3}H]PTHRP from incubations performed in the absence (○) or presence of 0.3 uM cycloheximide (□) or of 20ug/mL nonradioactive Ile (△). Significant differences, at each time point, compared to incubations performed with nonradioactive Ile are denoted by crosses ($p < 0.01$). Results are expressed as cpm/10^7 cells and represent the mean ± SE of triplicate results. The data is representative of three different experiments.

of newly synthesized [^{3}H]PTHRP into the culture medium, on the other hand, continued to increase over 6 h of labeling. Incorporation of [^{3}H]Ile into PTHRP was prevented both by inhibition of protein synthesis using cycloheximide and by incubation with excess unlabeled Ile (Figure 1).

Analysis of Intracellular and Extracellular [^{3}H] PTHRP. Analysis of affinity-purified [^{3}H]PTHRP in cell extracts by gel-permeation HPLC showed the presence of three molecular forms which eluted in the positions of the [Tyr^{36}]PTHRP(1–36), PTHRP(1–86), and PTHRP(1–141) standards (Figure 2). Labeling periods up to 20 min resulted in a predominant increase in radioactivity in the species of PTHRP eluting in the position of [Tyr^{36}]PTHRP(1–36).

Three NH_2-terminal moieties were also seen in the medium, and these also eluted in the positions of the [Tyr^{36}]PTHRP(1–36), PTHRP(1–86), and PTHRP(1–141) standards (Figure 3). Release of [^{3}H]PTHRP, especially of the smaller species, into culture medium progressively increased over 4 h.

Reversed-phase HPLC analysis of affinity-purified [^{3}H]-PTHRP from cells and conditioned culture medium, after 4 h of continuous labeling, also demonstrated three [^{3}H]PTHRP forms which co-eluted with the standards PTHRP(1–36)NH_2, PTHRP(1–86), and PTHRP(1–141) in this chromatographic system (Figure 4).

After 20 min, equilibrium between the rate of intracellular [^{3}H]PTHRP production, particularly of the smaller species, and the rate of extracellular release of these species may have occurred, resulting in the relatively constant intracellular profile of [^{3}H]PTHRP species observed (Figure 2).

Pulse–Chase Analysis. Rat Leydig tumor cells were pulse labeled for 20 min and then chased for 1–4 h. Under these conditions a rapid decrease of newly synthesized affinity-purified [^{3}H]PTHRP was seen within the cells; little newly synthesized [^{3}H]PTHRP remained intracellularly when the chase period was extended beyond 1 h (Figure 5). Secreted [^{3}H]PTHRP was seen in the conditioned culture medium at

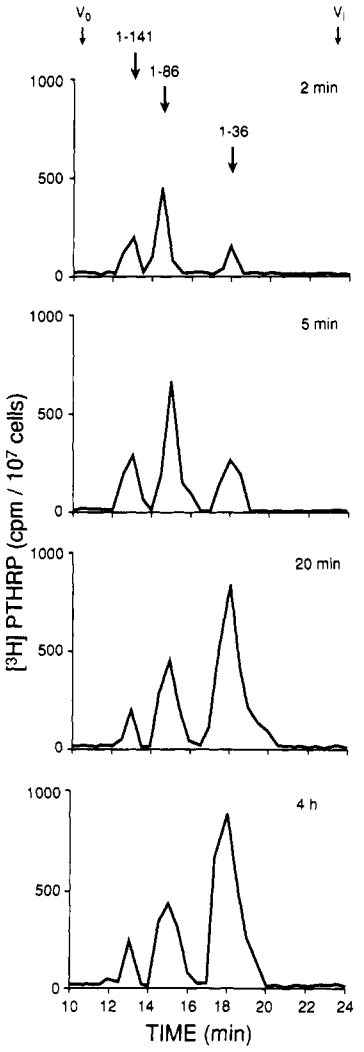

FIGURE 2: Gel-permeation HPLC profiles of [^{3}H]PTHRP biosynthesized by H-500 rat Leydig tumor cells. After pulse labeling with [^{3}H]Ile for 2 min to 4 h, [^{3}H]PTHRP in H-500 Leydig cell extract was affinity purified and then analyzed by gel-permeation HPLC. Cells were extracted, affinity purified, and passed through C_{18} Sep-Pak cartridges as described in Materials and Methods. Eluates were then analyzed by gel-permeation HPLC using an isocratic gradient of 40% CH_3CN/0.1% CF_3COOH at a flow rate of 1 mL/min. Fractions (0.5 min) were collected and counted for ^{3}H radioactivity by liquid scintillation counting. Elution positions of PTHRP(1–141), PTHRP(1–86), and [Tyr^{36}]PTHRP(1–36) are shown. V_0 and V_i denote the exclusion and inclusion volumes, respectively. All results are expressed as cpm/10^7 cells, and representative results from three such experiments are shown.

the end of the pulse labeling of 20 min, and a progressive accumulation of extracellular newly synthesized [^{3}H]PTHRP was seen during the chase period of 1 h–4 h (Figure 5).

Analysis of affinity-purified [^{3}H]PTHRP from cells and conditioned culture medium from pulse–chase studies also revealed the presence of three molecular forms of PTHRP which eluted in the positions of [Tyr^{36}]PTHRP(1–36), PTHRP(1–86), and PTHRP(1–141) on gel-permeation HPLC. These three entities were seen in both cells and conditioned culture medium and comprised 67%, 30%, and 7%, respectively, of the newly synthesized and secreted PTHRP (Figure 6). Although these molecular species progressively decreased intracellularly and progressively increased extracellularly, the relative proportion of the three forms remained constant after the 20-min pulse.

Analysis of Immunoreactive PTHRP. Analysis of cells and conditioned culture medium of unlabeled rat Leydig tumor

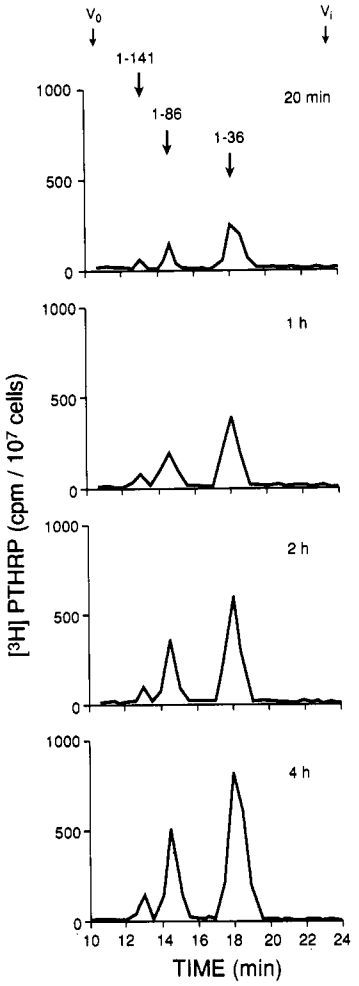

FIGURE 3: Gel-permeation HPLC profiles of biosynthetic [^{3}H]-PTHRP released by H-500 rat Leydig tumor cells. After pulse labeling with [^{3}H]Ile for 20 min to 4 h, [^{3}H]PTHRP in extracts of conditioned medium from cultures of H-500 Leydig cells was affinity purified and then analyzed by gel-permeation HPLC. Medium was extracted, affinity purified, and passed through C_{18} Sep-Pak cartridges as described in Materials and Methods. Eluates were then analyzed by gel-permeation HPLC using an isocratic gradient of 40% CH_3CN/0.1% CF_3COOH at a flow rate of 1 mL/min. Fractions (0.5 min) were collected and counted for ^{3}H radioactivity by liquid scintillation counting. Elution positions of PTHRP(1–141), PTHRP(1–86), and [Tyr^{36}]PTHRP(1–36) are shown. V_0 and V_i denote the exclusion and inclusion volumes, respectively. All results are expressed as cpm/10^7 cells, and representative results from three such experiments are shown.

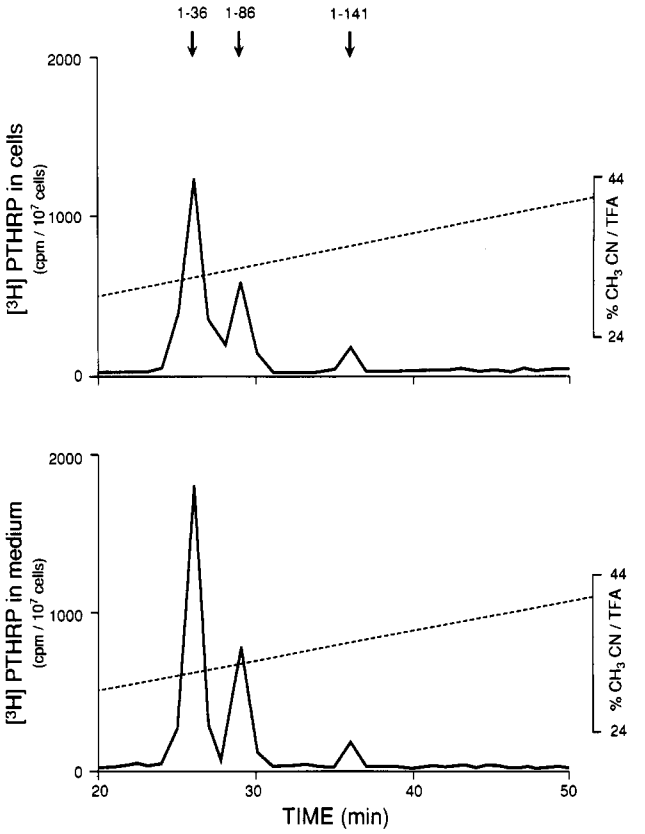

FIGURE 4: Reversed-phase HPLC profiles of biosynthetic [^{3}H]-PTHRP from rat Leydig tumor H-500 cell cultures. Reversed-phase HPLC profiles of affinity-purified [^{3}H]PTHRP from an H-500 Leydig cell extract (upper panel) and from conditioned culture medium (lower panel) after 4 h of pulse labeling with [^{3}H]Ile are shown. Cells and culture medium were extracted, affinity purified, and passed through C_{18} Sep-Pak cartridges as described in Materials and Methods. Eluates were analyzed by reversed-phase HPLC on a C_{18} μBondapak column using a linear gradient of 24–44% acetonitrile/0.1% CF_3COOH (CH_3CN/TFA) over 60 min. Fractions (1 min) were collected and counted for ^{3}H radioactivity by liquid scintillation counting. Elution positions of [Tyr^{36}]PTHRP (1–36), PTHRP(1–86), and PTHRP(1–141) are shown. V_0 and V_i denote the exclusion and inclusion volumes, respectively. All results are expressed as cpm/10^7 cells, and representative results from three such experiments are shown.

cells revealed the presence of three major NH_2-terminal immunoreactive forms in cells and culture medium (Figure 7). The relative quantities of the intracellular and extracellular entities and their elution position on gel-filtration HPLC was the same as that seen by pulse labeling and pulse–chase studies with [^{3}H]Ile.

DISCUSSION

In the present study, steady-state levels of molecular forms of [^{3}H]PTHRP were achieved intracellularly within 20 min. Rapid release and relatively little intracellular storage of newly synthesized molecular species of [^{3}H]PTHRP were also observed. This latter pattern is highly suggestive of a constitutive mode of secretion and is in keeping with the finding of the production and release of PTHRP by cells such as keratinocytes (Kremer et al., 1990) and osseous cells (Rodan et al., 1989), which do not have a well-developed system of secretory granules. Consequently, regulation of biosynthesis and processing, rather than regulation of the exocytic process by secretagogues, may well be the predominant (although perhaps not the exclusive) mechanism involved in the control of secretion of PTHRP forms in many, if not most, cell types.

In our studies, we identified the production of three major NH_2-terminal forms of PTHRP which, on two chromatographic systems, coeluted with the standards PTHRP(1–141), PTHRP(1–86), and [Tyr^{36}]PTHRP(1–36). The predicted translation product encoded by rat PTHRP mRNA contains a signal sequence and a pro sequence, as well as a mature peptide of 141 amino acids. Although the precise length of the pro sequence is unclear, it appears to be at least 6 and possibly 13 amino acids in length (Yasuda et al., 1989a) with a highly basic COOH-terminal pentapeptide. The basic hexapeptide pro sequence of parathyroid hormone (PTH) readily permits the resolution of proPTH (–6→+84) and proPTH(–6→+34) from their corresponding mature forms, PTH(1–84) and PTH(1–34), on the chromatographic systems we have employed (Rabbani et al., 1990). Therefore, it is possible that these systems would similarly resolve any proforms of PTHRP(1–86) and [Tyr^{36}]PTHRP(1–36) from these mature forms. Nevertheless, we cannot exclude that pro forms of these two moieties and of PTHRP(1–141) might

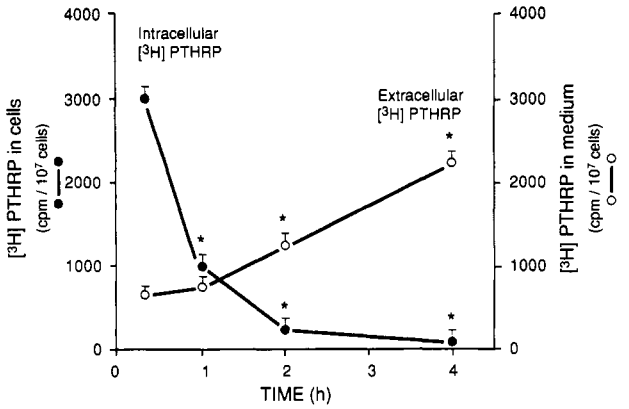

FIGURE 5: Pulse–chase studies of [^{3}H]Ile incorporation into PTHRP by H-500 rat Leydig tumor cells. Cells were pulse labeled with [^{3}H]Ile for 20 min and then transferred to radioactivity-free culture medium for indicated periods of time. Cells (●) and culture media (○) were extracted and affinity purified as described in Materials and Methods. Affinity eluates were collected, and an aliquot was counted by liquid scintillation counting. Results are expressed as cpm/10^7 cells and are the means ± SE of triplicate determinations. Significant differences from cellular [^{3}H]PTHRP, at each time point, are denoted by asterisks ($p < 0.01$). Representative data from three such experiments are shown.

coelute on one or both chromatographic systems we employed. Further studies will therefore be required to define the precise residue comprising the NH_2 terminus of these entities.

In addition to the presence of basic residues for propeptide-to-peptide conversion at the NH_2-terminus of PTHRP, basic residues which might be substrates for intrapeptide enzymatic cleavage occur at positions 37 and 88. Cleavage at the former site would yield the peptide PTHRP(1–36). Inasmuch as cleavage at the Pro-Gly-Lys sequence at positions 86, 87, and 88 would produce a COOH-terminal glycine, this would be predicted to yield the amidated peptide PTHRP(1–86)NH_2. Indeed these may be the entities identified and which eluted by both size-exclusion and reversed-phase HPLC in the position of the PTHRP(1–36) and PTHRP(1–86) standards, respectively.

The molecular species coeluting with PTHRP(1–141) was least abundant in our system after 5 min, suggesting that after synthesis it is rapidly converted intracellularly to other forms. By 5 min the most prominent species appeared to be the form coeluting with PTHRP(1–86). By 20 min, however, the moiety coeluting with [Tyr^{36}]PTHRP(1–36) predominated and continued to be the most abundant form over the subsequent 4 h. After 20 min intracellular processing of these entities appeared to have ceased, and the same relative proportion of these species was maintained for several hours. All species could be released into the conditioned medium, and little if any extracellular metabolism occurred, inasmuch as it appeared by pulse–chase analysis that the extracellular content of each radiolabeled species increased in proportion to the decrease in the intracellular content of the corresponding radiolabeled species.

Our studies examined only the biosynthesis and processing of NH_2-terminal species of PTHRP in view of the known importance of this region of the molecule in mediating many well-characterized biological effects of the hormone (Rabbani et al., 1988; Yates et al., 1988; Fukayama et al., 1988). Consequently, the fate of the corresponding COOH-terminal moieties generated in our studies is unclear. Nevertheless, recent work examining the processing of exogenous PTHRP overexpressed by stable transfection of PTHRP cDNA in human and rat tumor cell lines has reported the existence of a midregion fragment starting at amino acid 38 (Soifer et al., 1992). This could generate a corresponding 1–37 fragment, which after trimming of the COOH-terminal Arg residue by carboxypeptidase activity would leave a 1–36 entity in agreement with our results. Numerous additional sites exist for potential internal cleavage at basic residues in the middle and COOH regions of PTHRP(1–141), and indeed it has been suggested that cleavage at such sites might release bioactive peptides with non-PTH-like bioactivity (Care et al., 1990; Fenton et al., 1991). We have recently postulated that growth-inhibiting effects of PTHRP observed in keratinocytic cell cultures might involve a bipolar nuclear localization sequence containing highly basic peripheral domains (Kaiser et al., 1992). Consequently, some basic sequences within the molecule might participate in intracellular trafficking events, and not all may serve as candidate sites for internal cleavage. Overall, therefore, considerable further work is required before analysis of the production and characterization of endogenous midregion and COOH fragments of PTHRP is completed.

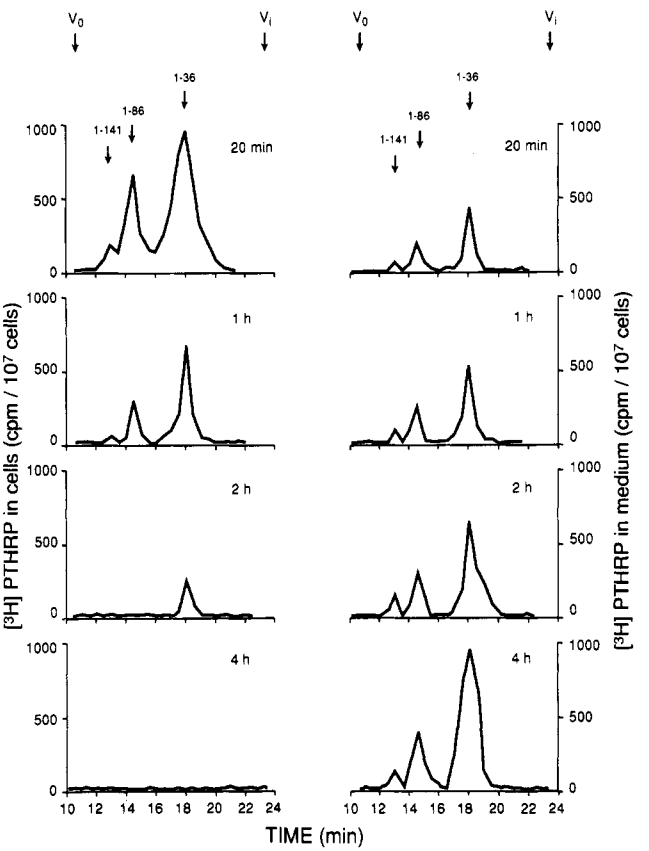

FIGURE 6: HPLC analysis of [^{3}H]PTHRP biosynthesized during pulse–chase studies in H-500 rat Leydig tumor cell cultures. Gel-permeation HPLC profiles of affinity-purified [^{3}H]PTHRP in H-500 Leydig cell extracts (left panel) and in conditioned culture medium (right panel) are shown. After pulse labeling for 20 min, cells were transferred to radioactivity-free culture medium for the indicated periods of time. Cells and culture medium were extracted, affinity purified, and passed through C_{18} Sep Pak cartridges as described in Materials and Methods. Eluates were analyzed by gel-permeation HPLC using an isocratic gradient of 40% CH_3CN in 0.1% CF_3COOH at a flow rate of 1 mL/min. Fractions (0.5 min) were collected and counted for ^{3}H radioactivity by liquid scintillation counting. Elution positions of PTHRP(1–141), PTHRP(1–86), and [Tyr^{36}]PTHRP(1–36) are shown. V_0 and V_i denote the exclusion and inclusion volumes, respectively. All results are expressed as cpm/10^7 cells, and representative results from three such experiments are shown.

Rat and human PTHRP(1–141) share 89% homology in the amino acid sequences of the prepro region and 98% homology between amino acids 1 and 111 of the mature peptides. Furthermore, recent preliminary results in human tissues suggest the production of NH_2-terminal forms similar

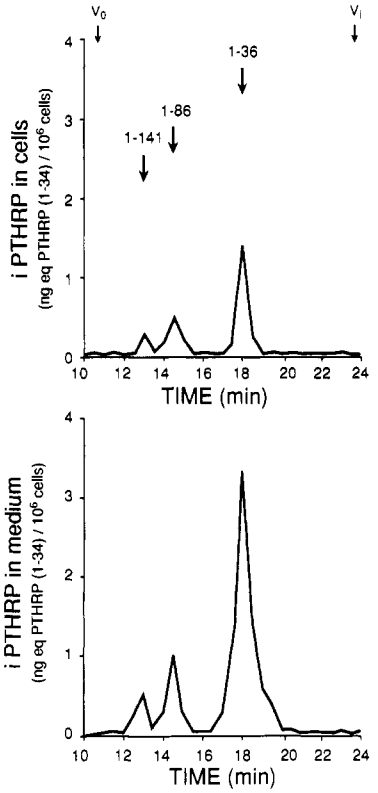

FIGURE 7: Gel-permeation HPLC profiles of immunoreactive PTHRP in H-500 rat Leydig tumor cell cultures. Rat H-500 Leydig tumor cells were cultured in radioactivity-free culture medium, and cells and culture medium were extracted, affinity purified, and passed through C_{18} Sep Pak cartridges as described in Materials and Methods. Eluates were analyzed by gel-permeation HPLC using an isocratic gradient of 40% CH_3CN in 0.1% CF_3COOH at a flow rate of 1 mL/min. Fractions (0.5 min) were collected and assayed for NH_2-terminal PTHRP immunoreactivity. V_0 and V_i denote the exclusion and inclusion volumes, respectively. All results are expressed as ng equiv of PTHRP(1–34)/10^6 cells. The data shown are representative of three such experiments.

to those seen in our system (Soifer et al., 1992). Consequently, our results may have relevance to the biosynthesis and processing of PTHRP in cell types and species beyond the rat Leydig tumor model. Nevertheless, processing of some secretory peptides may also be both tissue specific and developmentally regulated. Consequently, further work is required to determine whether additional processing patterns of PTHRP will be observed in other biological settings.

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