EVIDENCE FOR A PRO-CALCITONIN

B. A. Roos, K. Okano, and L. J. Deftos
Department of Medicine (Endocrinology)
University of California, San Diego and
Veterans Administration Hospital, La Jolla, California

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ABSTRACT

The biosynthesis of calcitonin was studied using radioimmunochemical methods and suspensions of calcitonin-producing cells derived from trout ultimobranchial glands. [^{14}C]leucine was incorporated into cell proteins in a linear fashion for up to 36 hrs. Acid-extracted cellular radioactivity could be precipitated by trichloroacetic acid and calcitonin antiserum. Chromatography of the cell extracts revealed two distinct peaks of radioimmunoassayable and immunoprecipitable calcitonin activity. One peak co-eluted with radioiodinated calcitonin, the other as a higher molecular weight species. The relative incorporation of [^{14}C]leucine into the higher and lower molecular weight peaks during "pulse-chase" experiments was consistent with a precursor-product relationship between them.

INTRODUCTION

The existence of multiple hormonal forms has been well-documented for several peptide hormones (2-10). In vitro studies of biosynthesis have demonstrated that, for some hormones, the presence of a biosynthetic precursor (pro-hormone) for the hormone can account for its multiple forms (2-5); for other hormones, the size heterogeneity of glandular and circulating forms are thought to be manifestations of metabolism, polymerization, and/or noncovalent binding to other proteins (6-10). We have recently demonstrated that such size and immunochemical heterogeneity also pertains to human calcitonin from patients with calcitonin-producing tumors (11-15). To further pursue our studies of calcitonin and its secretion and

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ABBREVIATIONS
CT: Calcitonin; SCT: salmon calcitonin; TCT: trout calcitonin; and, C-cell: calcitonin-producing cell.

biosynthesis, we have developed in vitro preparations of both human and trout calcitonin-producing cells (C-cells) (13-18). In this paper, we describe preliminary experiments with trout C-cell suspensions which suggest the presence of a biosynthetic precursor to calcitonin, a pro-calcitonin.

MATERIAL AND METHODS

Trout C-cell suspensions were obtained by a previously reported method employing serial enzymatic digestion of minced trout ultimobranchial glands (13-19). The cell suspensions (1-2 x 10^6 cells per ml) were incubated in Hank's salt solution containing 20 mM N-2-hydroxyethylpiperazine, N'-2-ethanesulfonic acid buffer, essential vitamins and amino acids with leucine excluded, 200 units/ml penicillin and 200 µg/ml streptomycin (Gibco), and 5% fetal calf serum. Incubations were at 20°C, pH 7.3. Cell viability was assessed by trypan blue dye exclusion (19). After a 14-16 hr. pre-incubation, [^{14}C]leucine (300 mC/mMole, uniformly labelled, Schwarz/Mann), was added to the cell suspension (7.5 µc/ml) and the incubation continued for a similar period of time. In "chase" experiments, after 5 hrs. incubation with [^{14}C]leucine, cell suspensions received a 100-fold excess of [^{12}C]leucine ("cold") and the incubation continued for 8 hrs. At the end of incubation, cell viability was again determined. (In all experiments shown, cell viability never fell below 75%.) Cells were washed 3 times with cold Hank's-[^{12}C]leucine solution and then homogenized and extracted in ice-cold 0.1 N HCl (0.2 ml/10^6 cells) for 2 hrs. at 20°C. Aliquots of the homogenate were withdrawn for determination of radioactivity in TCA-precipitable protein (10-20 µl) and for chromatography (0.2-0.4 ml) on a 1 x 15 cm Bio Gel P-2 column (0.25 ml fractions) after centrifugation at 10,000 g x 5 min. (3, 20). The excluded volume of this column was then chromatographed on a 1 x 60 cm Bio Gel P-10 column (1.1 ml fractions). In some experiments, the samples were treated for 2 hrs. at room temperature with 8M urea and 10mM mercaptoethanol before chromatography. The columns were

developed with phosphate buffer containing 0.1% fetal calf serum (v/v). Total calcitonin of each column fraction was determined by a radioimmunoassay based on salmon calcitonin which had previously been shown to be suitable for measurements of trout calcitonin (13-17, 21). Total [^{14}C] leucine radioactivity of each fraction was determined by measurement of [^{14}C]radioactivity in an aliquot dissolved in Aquasol (Amersham-Searle). Radioactivity incorporated into trout calcitonin, [^{14}C]TCT, was determined by immunoprecipitation employing rabbit antiserum to salmon calcitonin that cross-reacts with trout calcitonin and goat antiserum to rabbit gamma globulin (13-17, 21). For immunoprecipitation, 200 µl samples of P-10 column fractions or 25 µl of acid extract of C-cells were added to 10 x 75 test tubes along with either 25 µl of SCT-immune serum or control normal rabbit serum. Enough phosphate buffer was then added so that the final

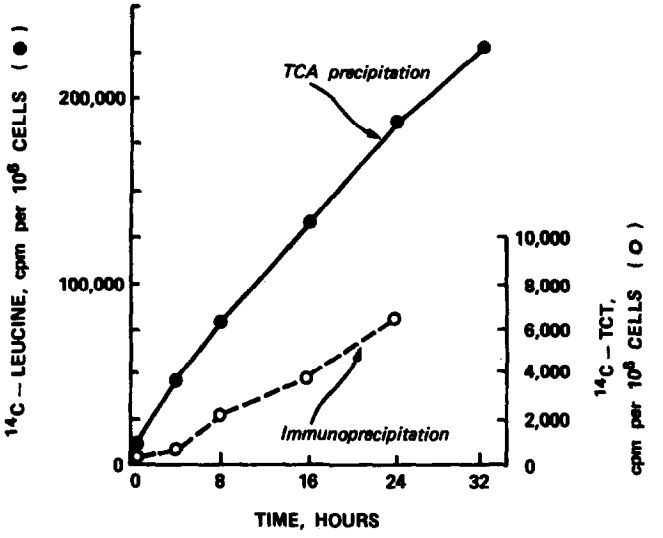

Figure 1

Time course of [^{14}C]leucine incorporation into newly synthesized C-cell proteins. C-cell suspensions (10^6 cells/ml) derived from trout ultimobranchial glands were preincubated 15 hrs. and then incubated with [^{14}C]leucine (7.5 µc/ml) for the indicated times. After homogenization in 0.1 N HCl, aliquots of the homogenate were removed for determination of radioactivity incorporated into TCA-precipitable protein (●) and for determination of newly synthesized trout calcitonin ([^{14}C]TCT) by immunoprecipitation (o) as described in the text. Each point represents the mean of 3 determinations.

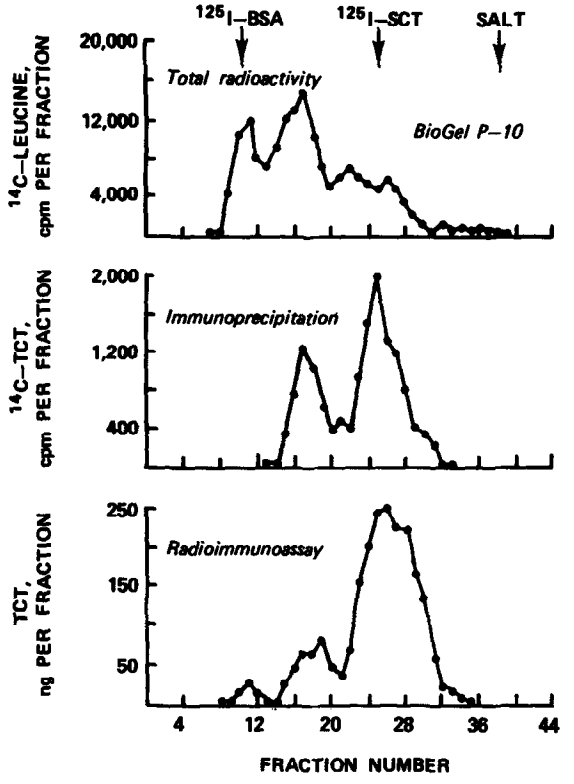

Figure 2

Gel chromatography of acid-soluble C-cell proteins. After preincubation for 16 hrs., 2 x 10^6 trout C-cells were continuously labelled with [^{14}C] leucine for 16 hrs. Acid-soluble proteins were extracted with 0.1 N HCl and 0.4 ml of extract was applied to a Bio Gel P-2 column which was eluted with phosphate buffer containing 0.1% (v/v) fetal calf serum. The excluded volume (pool of fractions 16-22) was then similarly chromatographed on a Bio Gel P-10 column. Aliquots of each 1.1 ml fraction of this column were analyzed for TCT by radioimmunoassay (bottom panel), [^{14}C]TCT by immunoprecipitation (middle panel) and total [^{14}C]leucine radioactivity (top panel) as described in the text. Each determination was done in duplicate. The elution positions of bovine serum albumin ([^{125}I]BSA), salmon calcitonin ([^{125}I]SCT) and [^{14}C]leucine are indicated by labelled arrows.

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volume of each sample was 500 µl. After incubation at 4°C for 17 hrs., each tube received 100 µl of goat anti-rabbit gamma globulin (Calbiochem) for an additional 3 hr. incubation at 4°C. The samples were then centrifuged at 1500 g x 30 min. and the supernatant discarded. The pellet was washed 3 times, dissolved in 0.5 ml of protein solubilizer (NCS-Amersham-Searle), and counted in a liquid scintillation spectrometer (Searle Analytic). Radio-

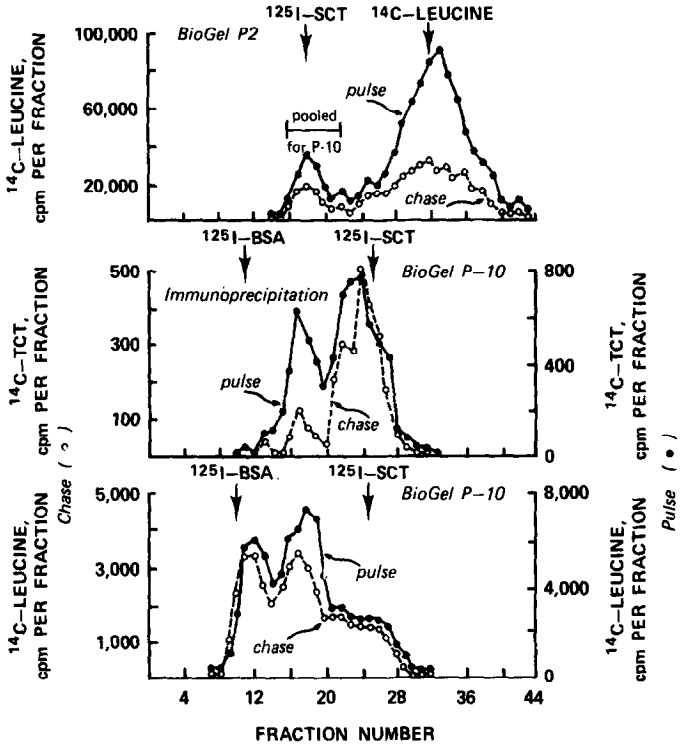

Figure 3

Gel chromatography of acid-soluble C-cell proteins obtained from pulse-chase experiments. After 14 hr. preincubation, duplicate tubes of trout C-cell suspensions (10^6 cells/tube) were labelled with [^{14}C]leucine (7.5 μc/ml) for 5 hrs. At this time 0.10 ml of 50 mM [^{12}C]leucine in Hank's salt solution was added to the "chase" tube, while the "pulse" tube received 0.10 ml of Hank's salt solution containing no leucine. Incubations were continued for an additional 8 hrs. before homogenization and extraction of acid-soluble proteins. The pulse (●) and chase (○) samples were separately chromatographed on Bio Gel P-2 and total [^{14}C]radioactivity of each column fraction was determined (top panel). The excluded volume from the P-2 column was then chromatographed on Bio Gel P-10 and total [^{14}C]radioactivity (bottom panel) and immunoprecipitable, radiolabelled trout calcitonin ([^{14}C]TCT) (middle panel) was determined for each fraction.

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activity non-specifically precipitated in the presence of normal rabbit serum was subtracted from that precipitated with immune serum.

RESULTS AND DISCUSSION

Linear incorporation of [^{14}C]leucine into the TCA-insoluble protein fraction of C-cell homogenates was observed for 36 hrs. (Fig. 1). When a

100-fold excess of [^{12}C]leucine was added, the rate of [^{14}C]leucine incorporated into C-cell proteins fell markedly. Linear incorporation of [^{14}C]leucine into immunoprecipitable CT was also observed; at least 3% of newly synthesized C-cell protein was TCT (Fig. 1). Two distinct peaks of TCT approximating molecular weights of 3000 and 7000 were detected by radioimmunoassay and immunoprecipitation of P-10 column fractions (Fig. 2). The greater specific activity of the earlier eluting immunoreactive calcitonin moeity (Fig. 2) and failure of urea (8M) and mercaptoethanol (10mM) to alter its elution pattern suggested a precursor-product relationship rather than simply dimerization of the CT monomer or noncovalent binding to other protein (22). The chase experiments, in which a 100-fold excess of [^{12}C]leucine was added after an initial pulse period, demonstrated that the [^{14}C]leucine showed decreased incorporation into the higher molecular weight CT species and increased incorporation in the lower molecular weight species (Fig. 3). This further supported a precursor-product relationship between the larger and smaller species of calcitonin. Although incomplete dissociation of calcitonin from cell organelles could not be ruled out, the results of treatment with acid-urea and mercaptoethanol make unlikely the type of protein-membrane interactions noted as pulse-chase artifacts in studies of growth hormone and prolactin biosynthesis; furthermore the non-dissociation observed with growth hormone and prolactin was with much higher molecular weight, presumably microsomal in part, cellular components (23).

In summary, our results suggest that calcitonin in trout C-cell suspensions has a higher molecular weight biosynthetic precursor. Further studies employing shorter labelling times and stronger dissociating conditions for CT extraction are necessary to substantiate these observations.

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