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BIOSYNTHESIS OF HUMAN CALCITONIN : EVIDENCE FOR A PROHORMONE.

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SUMMARY : Medullary carcinoma tissue was incubated in vitro in Eagle's medium containing labeled cysteine or lysine. After extraction and purification by affinity chromatography using antibodies raised against synthetic human calcitonin (hCT), two major peaks of radioactivity were detected after SDS polyacrylamide gel electrophoresis. The estimated molecular weights were respectively 11 800 for the first peak and identical to that of hCT for the second. Chase experiments reduced drastically both peaks. In the incubation medium, a single labeled product was observed, comigrating with hCT. These results suggest that hCT is liberated prior to its secretion from a larger prohormone, 11 800 Mr (Apparent molecular weight).

We have reported the presence of several immunoreactive forms of calcitonin (CT) with a larger molecular size than monomer CT in medullary carcinoma of the thyroid (MCT) (1). In two of these forms CT is likely to be covalently linked to a peptide as they resisted both denaturation and rupture of S-S bonds (2) and therefore could represent biosynthetic precursors. Prohormones of CT have been reported in two animal species, trout (MW 7 000) (3) and chicken (MW 13 500) (4).

Translation of mRNA from animal species and MCT have yielded conflicting results as regards the molecular weight of the primary translation product : 65 000 (5) and 15 000 (6) (7).

In the present work, we have investigated the biosynthesis of human CT in vitro by following the incorporation of labeled amino acids in order to identify possible precursors of human CT.

MATERIAL AND METHODS

MCT tissue was obtained at surgery and transported to the laboratory at 4°C in a balanced salt solution (Earle). The tissue was sliced and incubated at 37°C for 30 minutes in Eagle's medium containing 5% foetal calf serum but devoid of either leucine or cysteine.

At time 0 the tissue were transferred to the same medium containing either [^{3}H] leucine or [^{35}S] cysteine and further incubated for 4 hours in an atmosphere of 5% CO_2 in O_2 (under constant agitation) in a shaking water bath.

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At the end of this period, half of the tissue was immediately removed for extraction and the remaining half, transferred to the same incubation medium devoid of labeled amino acid and containing 2mM of the appropriate non radioactive amino acid. After four hours incubation, the tissue was removed for extraction and the incubation medium was frozen and kept at -80°C.

The tissues corresponding to pulse and chase were homogenized and extracted in 0.1 N HCl at 4°C. The extracts were centrifuged and the supernatant lyophilised and stored at -80°C.

Affinity chromatography : Extracts were dissolved in 1 ml of 0.1 M sodium phosphate buffer pH 7.4 containing 0.05% human albumin and 0.1% sodium azide and chromatographied on an affinity column prepared by coupling antibodies raised in the sheep against synthetic human CT (hCT) to Sepharose 4B. After 30 minutes of contact, the column was washed with 100 ml of buffer until radioactivity of the effluent had decreased to background, then with ammonium acetate buffer 0.1 M, pH 6.6. Bound immunoreactive CT was eluted using 1 M acetic acid. Fractions containing the peak of radioactivity were pooled and lyophilised.

Polyacrylamide Gel Electrophoresis (PAGE) : 40 µl of samples of the extract dissolved in distilled water were added to 40 µl electrophoresis buffer (0.09 M tris, 0.08 M borate, 2.5 mM Na_2 EDTA pH 8.35) containing 10% glycerol and 0.2 SDS. 2 µl of 0.2 M DTE (dithioerythritol) were added and the samples heated for 2 minutes at 100°C. Alkylation was carried out in the dark by adding 5 µl of 0.25 M iodoacetamide for 15 minutes at 50°C and the reaction stopped by cooling. After addition of 5 µl bromophenol blue, electrophoresis was carried out in polyacrylamide gel 5% or 15% in electrophoresis buffer containing 0.1 M SDS at 6 mA per tube until the dye reached the end of the tube. [^{14}C] phosphorylase B 92 500 MW, [^{14}C] bovine albumin 69 000 MW, [^{14}C] ovalbumine 46 000 MW, [^{125}I] bovine parathyroid hormone 9 250 MW and [^{125}I] hCT 3 400 MW were denatured, alkylated and separated by electrophoresis under the same conditions.

1 mm sections of the gel were sliced mechanically and either eluted in 3% protosol econofluor (V/V) for radioactive determination or extracted with phosphate buffer 0.1 M pH 7.4 containing 0.2% human albumin and 0.1 sodium azide for the assay of cold CT. Radioimmunoassay (RIA) of hCT was carried out in the same buffer using synthetic hCT as a tracer and standard, and an antiserum raised against hCT at a final dilution of I/200 000 (2).

All results of PAGE were expressed as the ratio of mobility of unknowns to the mobility of synthetic hCT (Rf/RfCT).

RESULTS

Specificity of the affinity column was established by the retention of hCT, while salmon and porcine CT, labeled amino acids or proteins were not retained.

Figure 1 shows the profile of unlabeled immunoreactive CT present in extracts from the pulse experiment as measured by radioimmunoassay. Three forms of CT are present, A, B and C, the last one co-migrating with synthetic hCT.

Incorporation of [^{35}S] - cysteine is very high in fractions B and C (figure 2) and much lower in forms corresponding to fraction A or having an apparently larger molecular weight.

After four hours of chase, a considerable reduction in these two peaks B and C was observed (figure 2). The supernatant from both pulse and chase experiments was analysed on 5% PAGE (not shown) and 15% PAGE (figure 3). A single peak of radioactivity was observed, migrating in the position of synthetic CT. Radioimmunoassay of the same fractions showed also a single important peak of immunoreactivity in the same position.

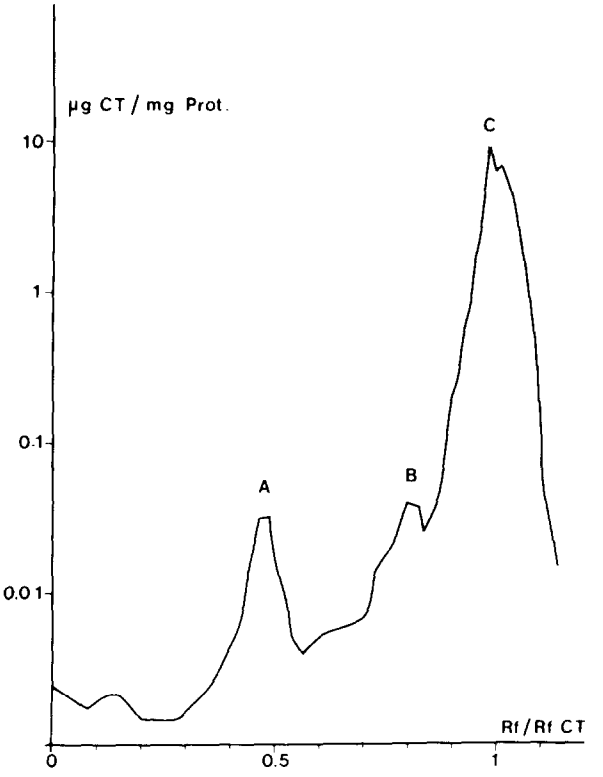

Figure 1. CT content as determined by RIA of affinity purified extract of pulse.SDS PAGE.

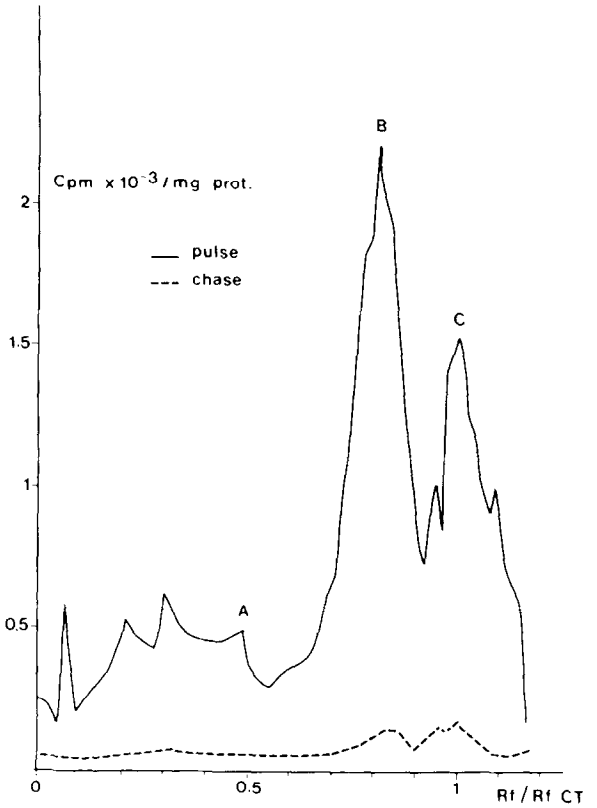

Figure 2. Affinity purified ^{35}S labeled material in extracts of pulse and chase experiments. SDS PAGE.

DISCUSSION

After separation by affinity chromatography, a single radioactive and immunoreactive peak comigrating with synthetic CT was detected in the incubation media of pulse and chase experiments (fig. 3). This strongly suggests that CT was rapidly synthetized and secreted by the cells. This is further substantiated by the small amount of residual radioactive material present in the tissue in the chase experiment. The severe reduction in peak B during the chase (figure 2) is consistent with a precursor role, while the reduction of peak C could be accounted for by its secretion. Some degradation of both forms cannot be excluded. Molecular weight estimation of peak B, the presumed prohormone, is of the order of 11 800 daltons. This value is higher than that reported for trout prohormone, 7 000 MW (3), and lower than the one of chicken prohormone, 13 500 MW.

Peak B could represent the human prohormone cleaved from a translation precursor of 15 000 MW (7) after removal of a leader sequence. It is of interest to note that the processing of rat mRNA translation product by pancreatic ribosomal membranes has been reported to yield a molecule of 12 000 (6) (7).

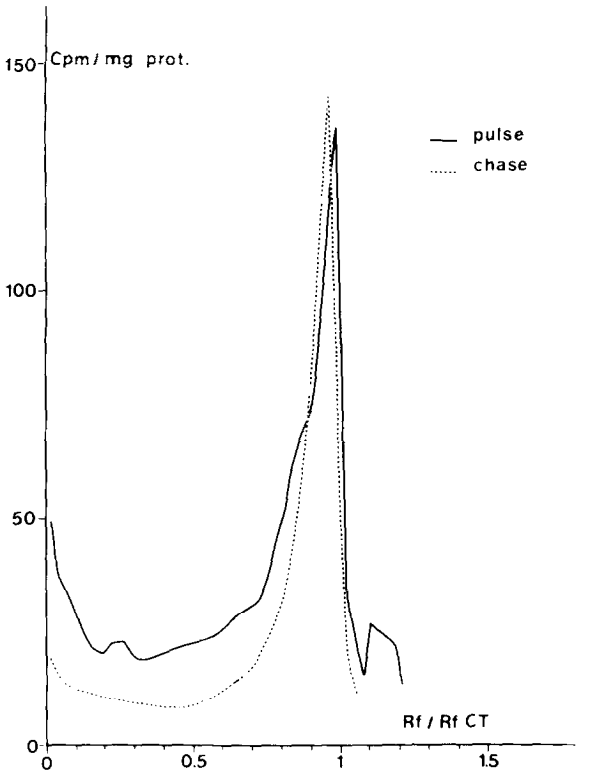

Figure 3. Affinity purified ^{35}S labeled material in supernatant pulse and chase experiments.SDS PAGE.

We have not found any significant evidence for the presence of cleavage products consistent with a primary precursor of 65 000 MW (5) : the small peak of immunoreactive cold CT (A), 54 500 Mr is not associated with a distinct peak of immunoreactive labeled molecules and furthermore labeled molecules with higher MW than 65 000 are present. A likely explanation for the presence of these large forms could be :

a) incomplete denaturation, in as much as peak A represents but a small fraction of total immunoreactivity applied to the gels,

b) the presence of molecules cross reacting with CT, which would therefore be retained by the affinity columns and detected by the RIA used. The last hypothesis is in part substantiated by the demonstration of the presence of thyroglobulin in C-cells of both animal species (8) and MCT (9) and by evidence for the presence of common antigenic sites (8).

c) abnormal synthesis of a small amount of big CT by the cancer cell.

CONCLUSION

The biosynthesis of human CT by MCT tissue *in vitro* proceeds through a prohormone of 11 800 MW.

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