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CALCITONIN IS NOT CONTAINED WITHIN THE COMMON PRECURSOR TO CORTICOTROPIN AND ENDORPHIN IN THE RAT

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SUMMARY

The suggestion that calcitonin is contained within the structure of the common precursor to ACTH and endorphin was examined. Immunohistochemical staining demonstrated calcitonin in thyroid parafollicular cells, and ACTH and 16K fragment in ACTH/endorphin cells of pituitary. No 16K fragment immunostaining was detected in thyroid parafollicular cells; no calcitonin staining was detected in pituitary. Immunoprecipitation of [^{35}S]methionine-labeled molecules synthesized by rat intermediate pituitary cells demonstrated that neither 30K precursor, 16K fragment nor any other major labeled cell product was recognized by calcitonin antiserum. Analyses of tryptic peptides of 30K precursor indicated that peptides expected from calcitonin were not present in 30K precursor.

INTRODUCTION

There is a substantial amount of clinical data which shows a frequent (but not invariant) correlation between the occurrence of immunoreactive calcitonin and immunoreactive corticotropin (ACTH) in hormone-producing tumors (1-5). In addition, recent immunohistochemical staining studies have reported the co-existence of calcitonin and ACTH or endorphin in the same cells of the anterior and intermediate pituitary (4,5). These observations have led to the suggestion (4,5) that the sequence of calcitonin is contained within the structure of the common precursor to ACTH and endorphin (6-8). This work directly examines the possibility that calcitonin is part of the common precursor to ACTH and endorphin.

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METHODS AND MATERIALS

Immunohistochemistry: Male rats (200g) were killed by cervical dislocation after pentobarbital anesthesia; tissues were fixed by immersion in picric acid-formaldehyde for 16h at room temperature (9). Tissues were dehydrated and embedded in EPON 812 with propylene oxide as intermediate solvent. Resin was removed from 1 μm thick sections using sodium methoxide (10); sections were then stained by the peroxidase-antiperoxidase method as described (11, 12). Blocking experiments were performed by incubating antisera at 4° for 24h with samples (0.2 - 20 μg/ml) of the appropriate peptides.

Immunoprecipitations: Rat intermediate pituitary cell suspensions were prepared and incubated in complete culture medium containing 100 μM [^{35}S]-methionine (New England Nuclear; 647 Ci/mmol) as described (13). Cells were extracted in 5N acetic acid with protease inhibitors (13,14); immunoprecipitates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (14). Tryptic digests were analyzed by gel filtration in 6M guanidine HCl (7,15).

Antibodies: The affinity-purified middle ACTH rabbit antibody (Bertha) and the 16K fragment rabbit antibody (Georgie) have been described (13,14); 16K fragment is the non-ACTH, non-β-lipotropin part of the common precursor molecule (6-8). The goat antibody to synthetic human calcitonin was kindly provided by Dr. R. Ziegler (Universitat Ulm, Ulm, F.R.G.) (16).

Peptides: We have used several commercially available synthetic peptides in this work. In addition, we received the following gifts of synthetic peptides: human ACTH(1-39) (Ferring AB); ACTH(1-24) and ACTH(25-39) (Dr. J. Gelzer, CIBA-GEIGY); met-enkephalin (Dr. E. Wunsch, Munich). Highly purified human β-lipotropin (Dr. C.H. Li, San Francisco), sheep β-lipotropin and porcine β-melanotropin (Dr. M. Chretien, Montreal) and mouse tumor cell 16K fragment (13) were also used.

RESULTS AND DISCUSSION

When rat thyroid sections were stained with antiserum to calcitonin, specific labeling of the parafollicular cells (C-cells) was observed (Fig. 1A); the staining was blocked by preincubation of the antiserum with synthetic calcitonin (Fig. 1B). Staining by the calcitonin antiserum was not blocked by preincubation with an excess of mouse pituitary tumor cell 16K fragment (Fig. 1A). No specific staining of the C-cells was seen when using 16K fragment antiserum (Georgie). In addition, no immunoprecipitation of ^{125}I-labeled calcitonin by three different antisera to 16K fragment was observed [antisera Georgie (13), Bertha (8), and Alice, raised against highly purified mouse tumor 16K fragment].

All ACTH/endorphin cells in the intermediate and anterior lobes of the rat pituitary contained immunoreactive 16K fragment (Figs. 2A,B,E,F). Immuno-

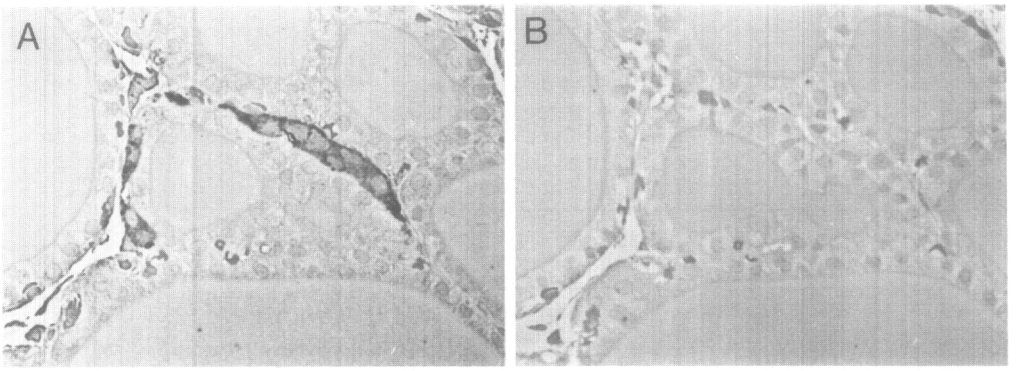


Fig. 1. Immunostaining of Rat Thyroid Tissue

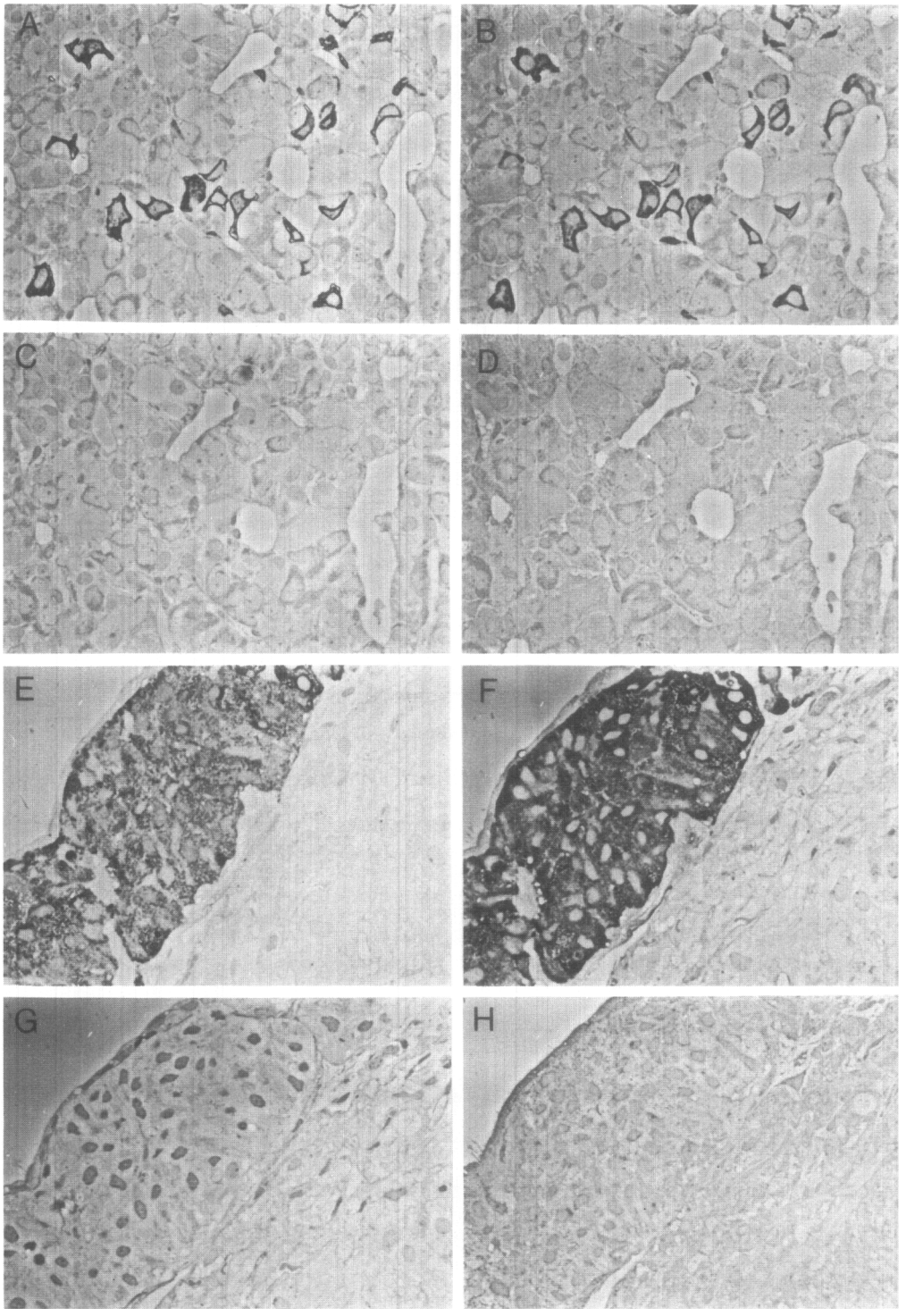
Sections were stained with calcitonin antiserum (at 1:1000) preincubated with (A) 20 μg/ml purified mouse tumor cell 16K fragment or with (B) 20 μg/ml synthetic calcitonin. A modification of the classical peroxidase-antiperoxidase method of immunostaining was developed in preliminary experiments: sections were incubated with the goat antiserum to calcitonin, washed, incubated with rabbit anti-goat-IgG (Behring Werke, Marburg; 1:8000 for 10 min at room temperature), washed and finally incubated with swine anti-rabbit-IgG (1:100, Dakopatts, Copenhagen) and soluble rabbit peroxidase-antiperoxidase complex (1:50; Dakopatts).

staining was specific for 16K fragment because it was blocked by preabsorption of the antiserum with purified 16K fragment (Figs. 2C and G), but not by any other segments of the 30K precursor (ACTH, αMSH, β-endorphin, β-lipotropin, or β-melanotropin). Importantly, no inhibition of staining occurred when the 16K fragment antibody was preincubated with as much as 200 μg/ml calcitonin. The calcitonin antibody did not produce any specific immunostaining in either lobe of the pituitary (Figs. 2D,H).

Figures 1 and 2 together indicate that the calcitonin antibody used reacts with rat calcitonin [which differs from human calcitonin at only two residues (17)], but does not detect calcitonin in fixed pituitary tissue. In addition,

Fig. 2. Immunostaining of Rat Pituitary Tissue

Serial sections through the anterior lobe (A-D) and the intermediate-posterior lobe (E-H) of a rat pituitary were stained with several different antisera: antiserum to middle ACTH (at 1:100; A,E), antiserum Georgie to 16K fragment (at 1:4000; B,F), 16K fragment antiserum preincubated with 10 μg/ml 16K fragment (C,G), and antiserum to calcitonin (at 1:1000; D,H). The slight background staining in H was not blocked by preincubation of the antiserum with calcitonin; it may be the histochemical correlate of the nonspecific radioactivity at 62K and 26K in Fig. 3B.


A
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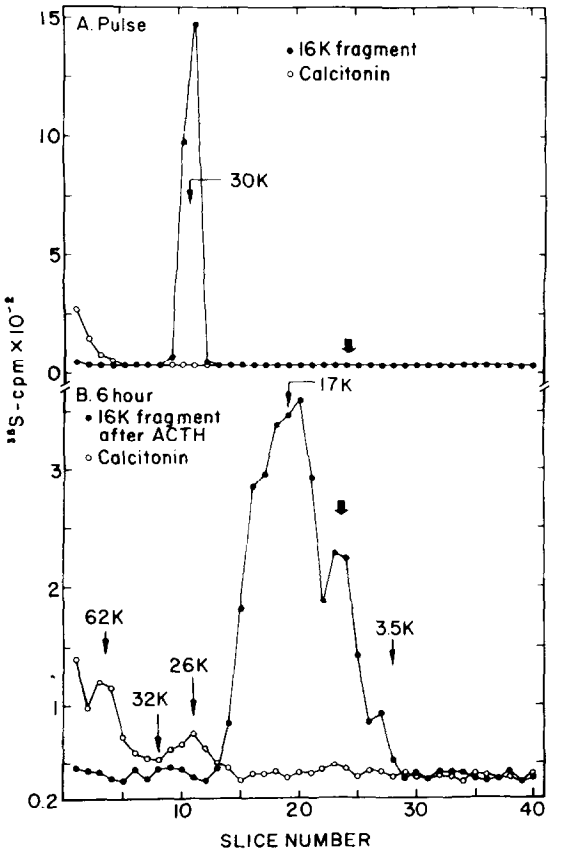

Fig. 3. Immunoprecipitation of [^{35}S]Methionine-Labeled Pituitary Extracts

Rat intermediate pituitary cells were incubated in medium containing [^{35}S]methionine for 15 min (A) or 6h (B). In A, equal aliquots of cell extract were immunoprecipitated with antisera to 16K fragment (Georgie: ●) or calcitonin (○) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (14). In B, equal aliquots were immunoprecipitated with calcitonin antiserum (○) or with 16K fragment antiserum after ACTH-containing molecules had been removed in an initial immunoprecipitation step (7) (●). The radioactive material at 62K and 26K in the calcitonin immunoprecipitate was not competed from the precipitate by addition of an excess of unlabeled synthetic calcitonin, and is therefore not calcitonin-related material.

16K fragment does not contain antigenic determinants capable of inhibiting staining by the calcitonin antiserum. It seemed possible that fixation of a putative pituitary calcitonin or calcitonin precursor eliminated its immunoreactivity, while not destroying immunoreactivity of thyroid calcitonin. Therefore, the ability of the calcitonin antibody to immunoprecipitate pituitary molecules which had not been subjected to fixation was examined (Fig. 3).

Neither the common precursor molecule (Fig. 3A) nor 16K fragment (Fig. 3B) reacted with the calcitonin antibody. Control experiments showed that the calcitonin antibody precipitated greater than 90% of the ^{125}I-labeled calcitonin added to a pituitary extract; thus sufficient calcitonin antibody was employed to precipitate any [^{35}S]methionine-labeled calcitonin-like molecules that might have been present in the extracts. Furthermore, the exogenous ^{125}I-labeled calcitonin immunoprecipitated from pituitary extracts was intact when examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The calcitonin sequence might be hidden within a putative precursor molecule in a form that is inaccessible to calcitonin antibodies. Therefore, tryptic digests of rat [^{35}S]methionine-labeled 30K precursor and 16K fragment were examined. Raulais et al (17) have determined the sequence of rat calcitonin; the sequence predicts that any putative precursor to rat calcitonin should produce a methionine-containing tryptic peptide of at least 18 residues (Fig. 4A). Analysis of a pool of the three methionine-containing tryptic peptides of rat 30K precursor or of the single methionine-containing tryptic peptide of rat 16K fragment showed that all of the methionine-containing peptides were only 8 to 9 amino acid residues in length. Thus a molecule identical to rat calcitonin is not contained within the structure of the rat 30K precursor molecule. Since cell free synthesis of the common precursor molecule yields a protein very similar to the intracellular product studied here (18), rapid post-translational cleavage of the putative calcitonin-like segment is unlikely.

It is not clear how the apparent immunostaining of calcitonin in pituitary tissue could have been observed (4,5). Sequencing of purified mouse tumor 16K fragment clearly demonstrates the occurrence of a pair of cysteine residues separated by 5 amino acid residues; thus a disulfide bridge similar to that found in all calcitonins could be present in 16K fragment (H.T. Keutmann, B.A. Eipper, and R.E. Mains; in preparation). Calcitonin has been detected in radioimmunoassays of pituitary extracts (19), but calcitonin levels

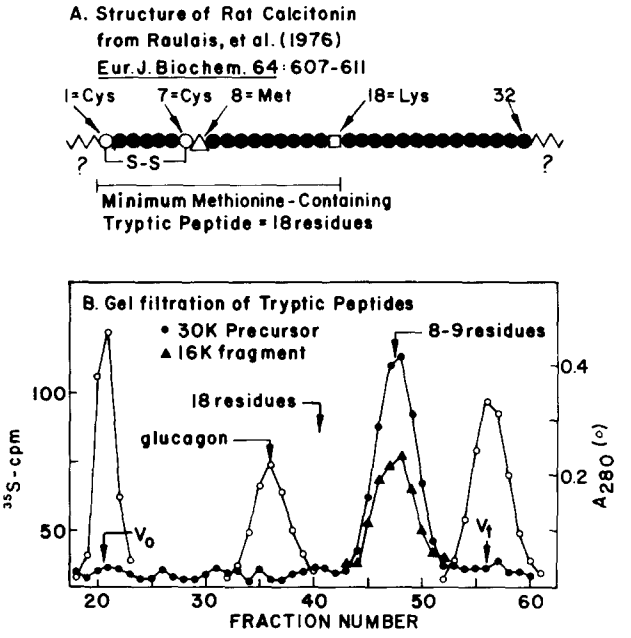

Fig. 4. Peptide Analyses of [^{35}S]Methionine-Labeled Molecules

A. The structure of rat calcitonin (17) is diagrammed; peptide extensions that might occur in a putative precursor are indicated. The size of the minimum methionine-containing tryptic peptide is also indicated.

B. Tryptic peptides of [^{35}S]methionine-labeled 16K fragment and 30K precursor (Fig. 3) were prepared and analyzed by gel filtration on Sephadex G-50 (superfine) in 6M guanidine HCl, 0.2 mg/ml bovine serum albumin (7,15); blue dextran, glucagon, and 2-mercaptoethanol were included as internal markers in each analysis.

are three orders of magnitude lower than levels of ACTH, β-endorphin or 16K fragment in similar extracts (13). Even in a human lung carcinoma cell line that secreted approximately equimolar amounts of immunoreactive calcitonin and ACTH, the calcitonin-related material appeared to be associated with a set of molecules distinct from the ACTH-related molecules (3).

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REFERENCES

1. Rosenberg, E.D., Hahn, T.J., Orth, D.N., Deftos, L.J., and Tanaka, K. (1978) J. Clin. Endocrinol. Metab. 47, 255-262.
2. Goltzman, D., and Tischler, A.S. (1978) J. Clin. Invest. 61, 449-458.
3. Bertagna, X.Y., Nicholson, W.E., Pettengill, O.S., Sorenson, G.D., Mount, C.D., and Orth, D.N. (1978) J. Clin. Endocrinol. Metab. 47, 1390-1393.
4. Deftos, L.J., Burton, D., Bone, H.G., Catherwood, B.D., Parthemore, J.G., Moore, R.Y., Minick, S., and Guillemin, R. (1978) Life Sci. 23, 743-748.
5. Deftos, L.J., Burton, D., Catherwood, B.D., Bone, H.G., Parthemore, J.G., Guillemin, R., Watkins, W.B., and Moore, R.Y. (1978) J. Clin. Endocrinol. Metab. 47, 457-460.
6. Mains, R.E., Eipper, B.A., and Ling, N. (1977) Proc. Natl. Acad. Sci. USA 74, 3014-3018.
7. Eipper, B.A., and Mains, R.E. (1978) J. Biol. Chem. 253, 5732-5744.
8. Mains, R.E., and Eipper, B.A. (1978) J. Biol. Chem. 253, 651-655.
9. Zamboni, L., and DeMartino, C. (1967) J. Cell Biol. 35, 148A.
10. Mayor, H.D., Hampton, J.C., and Rosario, B. (1961) J. Cell Biol. 9, 909-910.
11. Sternberger, L.A. (1974) Immunocytochemistry, Prentice-Hall, Englewood Cliffs, New York.
12. Weber, E., Voigt, K.H., and Martin, R. (1978) Proc. Natl. Acad. Sci. USA 75, 6134-6138.
13. Eipper, B.A., and Mains, R.E. (1978) J. Supramol. Struct. 8, 247-262.
14. Mains, R.E., and Eipper, B.A. (1976) J. Biol. Chem. 251, 4115-4120.
15. Eipper, B.A., and Mains, R.E. (1977) J. Biol. Chem. 252, 8821-8832.
16. Raue, F., Bayer, J.M., Rahn, K.H., Herfarth, Ch., Minne, H., and Ziegler, R. (1978) Klin. Wschr. 56, 697-701.
17. Raulais, R., Hagaman, J., Ontjes, D.A., Lundblad, R.L., and Kingdon, H.S. (1976) Euro. J. Biochem. 64, 607-611.
18. Roberts, J.L., and Herbert, E. (1977) Proc. Natl. Acad. Sci. USA 74, 5300-5304.
19. Deftos, L.J., Catherwood, B.D., Bone, H.G., Parthemore, J.G., Minick, S., and Guillemin, R. (1978) Clin. Res. 26, 629A.