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Calcemic Fraction-A: Biosynthetic Peptide Precursor of Parathyroid Hormone

(bovine/109-aminoacid precursor/molecular weight/gel electrophoresis/calcium)

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ABSTRACT **Calcemic fraction-A (CF-A) is a biologically active, hypercalcemic and bone resorptive peptide, which was detected in, and isolated from, bovine parathyroid glands [Hamilton *et al.* (1971) *Endocrinology* 89, 1440–1447]. It has been further purified, and its relationship to parathyroid hormone clarified. The peptide is present in fresh glands at a concentration of about 3 μg/g (parathyroid hormone, 100 μg/g). It contains 109 amino acids (hormone, 84), each of which is present in equal or greater molar ratio than in the hormone. Its molecular weight, calculated from amino-acid composition, is 12,144; determined by dodecyl sulfate–polyacrylamide gel electrophoresis, it is 12,500 (hormone, 9563). Per mole, it reacts with antiserum to parathyroid hormone to an extent of 7–10% that of the hormone, and is about 50% as active in its hypercalcemic and bone resorptive properties in the appropriate assays. Time course and pulse–chase experiments with parathyroid gland slices, in which the incorporation of amino acid into isolated peptide and hormone were measured, indicate that the hormone is made from a protein precursor; the patterns of incorporation of radioactivity are those that would be predicted from a precursor–product relationship. When the large peptide was incubated with parathyroid gland extracts it was partially converted to a molecule that appeared to be the hormone, as based upon its coelution with marker hormone from ion-exchange columns. Finally, tryptic digestion of the peptide increased the immunoreactivity of the sample in accord with the known greater immunoreactivity of the hormone than the peptide. On the basis of these results, it is proposed that the peptide is a biosynthetic precursor of the hormone in bovine parathyroid gland.**

Parathyroid hormone (PTH) has long been recognized as a prime factor in the regulation of calcium metabolism in many animal species. As obtained from the bovine parathyroid gland, it is a simple peptide containing 84 amino acids (1). Its sequence has recently been determined (2, 3), and a chemically synthesized peptide corresponding to the N-terminal 34 amino acids of the molecule possesses biological activity (4). Biosynthesis (5, 6) and secretion (7) of PTH is inversely related to the concentration of calcium. The molecular mechanisms responsible for this response to calcium, however, are obscure. In the hope of elucidating these mechanisms, we have been studying the biosynthesis of PTH. These investigations have involved incubation of bovine parathyroid gland slices under various experimental conditions. In the course of this work we detected and isolated from the gland and gland extracts microgram quantities of a rapidly labeled radioactive peptide, with a specific radioactivity many-fold greater than PTH itself (6, 8, 9). Because the gland contains only about 3% as much of this peptide as it does PTH, its detection initially was dependent upon the use of the radioactive label; hence, earlier workers who did not use this methodology failed to note its existence. We refer to this new peptide as Calcemic Fraction-A (CF-A).

CF-A was biologically active in several assays. It was hypercalcemic in the rat, able to induce bone resorption and inhibit citrate metabolism in an organ culture assay, and was immunoreactive by radioimmunoassay. Relative to PTH, CF-A by weight was 5–7% as active in the radioimmunoassay and 30–50% as active in the other assays. Its biosynthesis in bovine slices was inversely related to calcium concentration, in the same manner as was PTH biosynthesis. Based on these data, we concluded that CF-A was related to PTH and speculated that it might serve as a biosynthetic precursor (8, 9).

The present report provides compositional and physical data on the CF-A molecule, includes the results of kinetic studies of its synthesis relative to PTH, and gives evidence that CF-A is convertible to PTH by parathyroid gland extracts. On the basis of our results, we propose that CF-A is a biosynthetic peptide precursor of PTH in parathyroid gland.

METHODS

[^{3}H]CF-A was prepared (9) from fresh bovine parathyroid glands or from commercial acetone powders (Wilson Laboratories). Briefly, this procedure included extraction of the tissue with 8 M urea–0.2 M HCl, organic solvent, and salt fractionation steps, and precipitation with trichloroacetic acid to yield a powder. This preparation was then processed sequentially by Sephadex G-100 gel filtration and carboxymethyl (CM)-cellulose chromatography. Since the preparation at this stage of purification was still heterogeneous, it was subjected to two additional chromatographic steps on longer CM-cellulose columns (0.9 × 33 cm) and shallower ammonium acetate gradients [0.1 M (pH 5.3)–0.25 M (pH 7.0)]; in the first step 8 M urea was included in the gradients (1) and in the second, urea was omitted. The product thus obtained was analyzed by polyacrylamide gel electrophoresis; it contained a single major band containing essentially all of the radioactive label and two slower-moving minor components. By this criterion, CF-A appeared to be about 90% pure. In two separate preparations, each beginning with 15 g of powder, we obtained a little over 800 μg of CF-A. These batches were tested by rat assay (10),

Abbreviations: PTH, parathyroid hormone; CF-A, calcemic fraction-A.

TABLE 1. *Aminoacid composition of bovine calcemic fraction-A; comparison with bovine PTH*

Amino acid	nanomol/fraction 20-hr Hydrolysate	70-hr Hydrolysate	Average or corrected*	Residues/mol†	CF-A mol integer	PTH mol integer	Difference CF-A–PTH
Lysine	92.72	93.80	93.26	14.14	14	9	5
Histidine	26.06	27.28	26.67	4.04	4	4	0
Arginine	48.74	48.59	48.67	7.38	7	5	2
Aspartic acid‡	58.95	61.52	60.24	9.13	9	9	0
Threonine	12.98	12.76	12.87	1.95	2	0	2
Serine	61.32	57.12	63.00	9.55	10	8	2
Glutamic acid‡	81.24	79.75	80.50	12.20	12	11	1
Proline	17.42	18.07	17.75	2.69	3	2	1
Glycine	42.33	44.47	43.40	6.58	7	4	3
Alanine	63.21	65.39	64.30	9.75	10	7	3
Valine	61.84	66.44	66.44	10.07	10	8	2
Methionine	9.56	5.62	11.09	1.68	2	2	0
Isoleucine	25.69	26.38	26.04	3.95	4	3	1
Leucine	62.91	65.64	64.28	9.75	10	8	2
Tyrosine	7.17	6.58	6.88	1.04	1	1	0
Phenylalanine	16.22	17.42	17.42	2.64	3	2	1
Tryptophan	—	—	—	1.0§	1	1	0
Total residues					109	84	25

Samples for aminoacid analysis were dissolved in constant-boiling HCl. Hydrolysis was conducted in an evacuated tube at 110° for the times indicated. Analysis was performed on a Beckman model 120 analyzer with 120-mm cells and accelerated flow rate.

* Values are averages of 20- and 70-hr analyses, except as follows: serine and methionine, extrapolated to zero time; valine and phenylalanine, 70-hr hydrolysis value used.

† Calculated on the assumption that 6.595 represents the one-residue figure, determined from the average of values of all amino acids except serine, methionine, and phenylalanine.

‡ Amide *N* not determined.

§ Determined by Ehrlich reagent and spectrophotometry (13).

bone culture assay (11), and radioimmunoassay (12). Values for biological and immunological activity were essentially the same as mentioned above.

In order to determine the initial glandular content of CF-A and PTH and to test the efficiency of the extraction procedure for each peptide, we added previously biosynthesized and purified [^{3}H]CF-A and [^{14}C]PTH of known specific radioactivities to urea–HCl homogenates of a batch of fresh bovine parathyroid glands and subjected the mixture to the standard procedure for isolation of CF-A and PTH. The dilution of specific radioactivity in the isolated products indicated that the fresh tissue contained about 3 and 100 μg/g of CF-A and PTH, respectively. In addition, the yields of the two peptides were monitored at each step of the isolation procedure through the powder stage. The procedure did not discriminate between PTH and CF-A at any step. 50% of the tissue content of CF-A and PTH was recovered.

RESULTS

The aminoacid composition of CF-A is given in Table 1. That of PTH is provided for comparison. The analysis indicates that CF-A contains 109 amino acids and has a minimum molecular weight of 12,144, compared to 9563 for PTH. It contains every species of amino acid in PTH, in an equal or greater molar quantity than exists in PTH. Like PTH, CF-A is devoid of cysteine but, in contrast, it contains two threonine residues, whereas PTH contains none. Of the 25 additional amino acids, seven are basic (lysine and arginine) and only one is acidic (glutamic acid). This excess of basic residues compared to PTH likely contributes to the high affinity of CF-A for CM-cellulose columns (for example, see ref. 9, Fig. 4).

The molecular weight of CF-A calculated from the amino-acid analyses agrees favorably with the value obtained by dodecyl sulfate–polyacrylamide gel electrophoresis. Fig. 1 shows the results of plotting the logarithm of molecular weight of several marker proteins against their electrophoretic mobilities. The average molecular weight of CF-A determined from its observed electrophoretic mobility was 12,500.

The incorporation of radioactive amino acids into CF-A and PTH isolated from parathyroid gland slices incubated for various periods of time is shown in Fig. 2. CF-A was rapidly labeled during the course of the incubation without a noticeable delay. After 1 hr, the incorporation of ^{3}H reached a near-maximum value. In contrast, there was an appreciable lag in the rate of aminoacid incorporation into PTH, after which there was a rapid increase that continued throughout the 2-hr period of incubation. Although at later times the total amount of radioactivity in PTH exceeded that in CF-A, the specific radioactivity of CF-A was at all times several-fold greater than that of PTH because of the much smaller amounts of CF-A than PTH present in the tissue.

Fig. 3 illustrates a study in which the tissue was "pulsed" with radioactive amino acids for a brief period, then "chased" in a fresh medium containing nonradioactive amino acids (with cycloheximide present to inhibit protein synthesis). The amount of radioactivity in CF-A rapidly decreased, whereas that in PTH increased for about 30 min, after which it also declined. This result, together with the rate study of Fig. 2,

suggests that PTH is synthesized from a peptide precursor, which could be CF-A.

That CF-A is convertible enzymatically to PTH is indicated in Fig. 4. When purified radioactive CF-A was incubated with homogenates of bovine parathyroid gland (Fig. 4*b* and *c*), a significant proportion of the material was converted to a species that coeluted with "marker" PTH from CM-cellulose columns. In addtion, an assortment of early-eluting and unidentified radioactive species appeared that may represent both primary and secondary cleavage products of the CF-A substrate. Control samples derived from a zero-time incubation (Fig. 4*a*), and from incubations with boiled homogenate and without homogenate (not shown), contained only a negligible amount of radioactivity in the PTH region of the elution profile.

In order to further test the likelihood that conversion of CF-A to PTH could occur, small amounts of CF-A were incubated with trypsin. In these experiments, advantage was taken of the 10- to 15-fold (per mole) greater reactivity of our 0–2 antiserum with PTH than with CF-A. If during trypsin incubation some PTH-like molecules were formed from the less-reactive CF-A, the immunoreactivity of the mixture would be expected to increase. Fig. 5 shows that this, in fact, did occur. A 4-fold increase in reactivity was noted after a few minutes reaction of CF-A with trypsin; this was followed by a gradual decline. This loss in immunological activity indicated that the products of the reaction were undergoing additional tryptic hydrolysis, so that achievement of the theoretical maximum increase in immunological activity in this type of experiment could not be anticipated.

DISCUSSION

The present data, together with our previous results, permit a closer examination of the relationship of CF-A and PTH. Calcemic fraction-A has a molecular weight and aminoacid composition that can accommodate the entire PTH molecule. Moreover, its hypercalcemic and bone resorptive activities and its reactivity with antiserum to PTH suggest that its chemical structure is similar to PTH. The conversion of CF-A by parathyroid gland extracts to a molecular species that appears to be PTH and the increase in immunoreactivity when CF-A is incubated with trypsin also attests to the likely similarity of structure. The results of the pulse–chase experiment (Fig. 3) clearly indicate that PTH is synthesized in this gland from a protein precursor. Moreover, the pattern of radioactive incorporation in this experiment and in the rate study (Fig. 2) are those that one would predict from a precursor–product relationship between CF-A and PTH. The foregoing observations, we believe, represent compelling

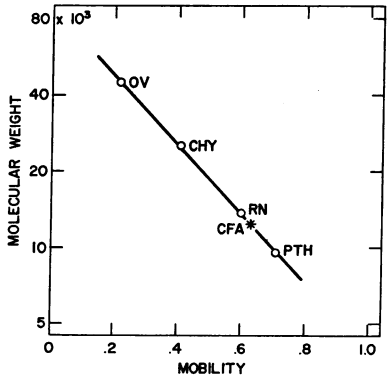

FIG. 1. Molecular weight determination of CF-A by dodecyl sulfate–polyacrylamide gel electrophoresis. The gels were prepared and analyzed by the procedure of Weber and Osborn (14). The current was 4 mA per gel for 4 hr. Mobility of each stained band (Coomassie Blue) was plotted against logarithm of molecular weight. The marker proteins used were ovalbumin (*OV*), chymotrypsinogen (*Chy*), ribonuclease A (*RN*), and parathyroid hormone (*PTH*). *CFA* refers to the unknown.

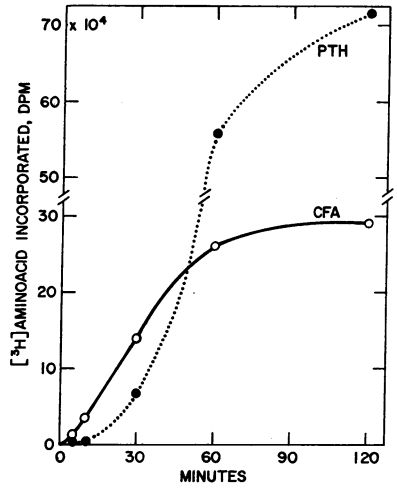

FIG. 2. Rate of biosynthesis of PTH and CFA. 4-g slices of parathyroid gland were incubated with [³H]leucine and [³H]-lysine under our standard (6) *low* calcium condition (0.5 mM calcium) for the times indicated. The tissue was then made into powders, from which PTH and CFA were isolated and purified as described, through the CM-cellulose chromatography stage. Nonradioactive powder was mixed with each sample before the Sephadex step to serve as a carrier and to limit adsorption.

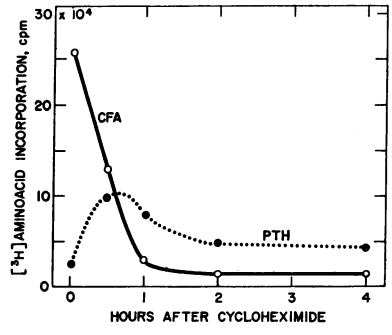

FIG. 3. "Pulse–chase" experiment to illustrate the labeling relationships between PTH and CFA. 6-g slices of parathyroid gland were incubated with [³H]leucine and [³H]lysine for 20 min. The tissue was transferred to fresh medium containing 50 mM leucine, 50 mM lysine, and 100 μg/ml of cycloheximide. Incubation was continued for the times indicated. Powders were then prepared from the tissue. These were mixed with nonradioactive powders as carrier, and the samples were chromatographed on Sephadex G-100 superfine columns. (With columns of sufficient length, it is possible to resolve mixtures of CF-A and PTH without resorting to subsequent CM-cellulose chromatography.) The appropriate fractions were then assayed for radioactivity. Control flasks indicated that the concentration of cycloheximide used inhibited aminoacid incorporation more than 98%.

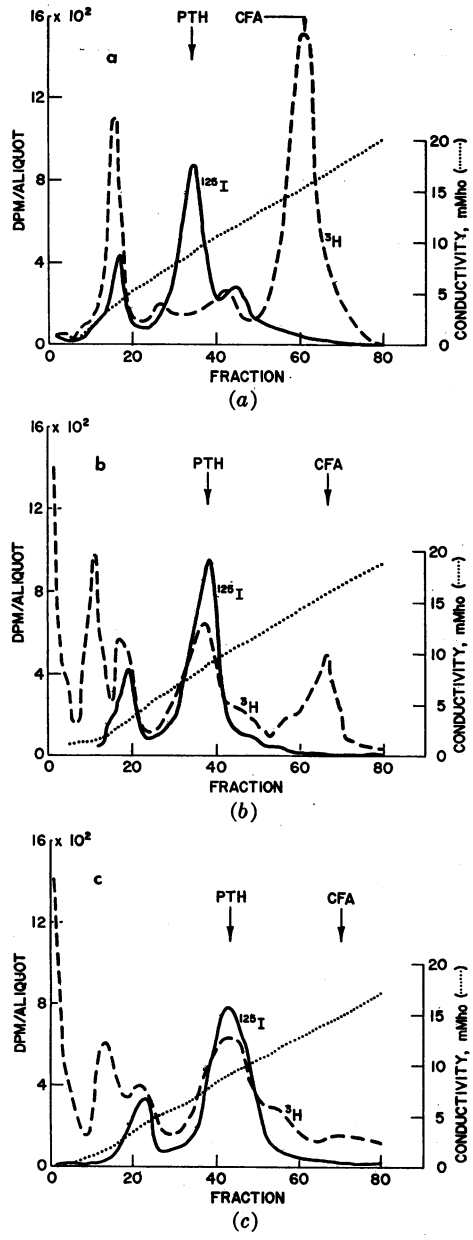

FIG. 4. Incubation of radioactive CF-A with parathyroid gland extracts. Flasks containing 5 μg of [^{3}H]CF-A, 0.3 ml of Solution A (see below), and 0.6 ml of tissue supernatant were incubated at 37°. The tissue supernatant was prepared from a 10% (w/v) homogenate of fresh parathyroid gland in Solution A (350 mM sucrose–25 mM KCl–10 mM magnesium acetate–50 mM Tris·HCl, pH 7.5). The crude homogenate was centrifuged at 10,000 × *g* for 15 min and the filtered supernatant was used; the reactions were terminated by addition of an equal volume of 0.8 M acetic acid containing 30 mg of a gland powder at the trichloroacetic acid precipitate stage of purification, mixed, and immediately frozen. (The powder was present as a carrier to minimize losses of [^{3}H]CF-A on CM-cellulose.) The samples were later mixed with a small amount of ^{125}I-labeled PTH to serve as a marker, and applied to 0.5 × 5-cm CM-cellulose columns and eluted (9). Aliquots of each tube were taken for measurement of radioactivity. Although not shown, additional controls with a boiled enzyme preparation and without enzyme were performed. The elution profiles of these samples were similar to that shown for the zero-time point. The peaks occurring at tubes 18–20 in each profile are not identified, but have nothing to do with incubation since similar peaks were observed in all of the control profiles. *a*, 0 min; *b*, 60 min; *c*, 120 min.

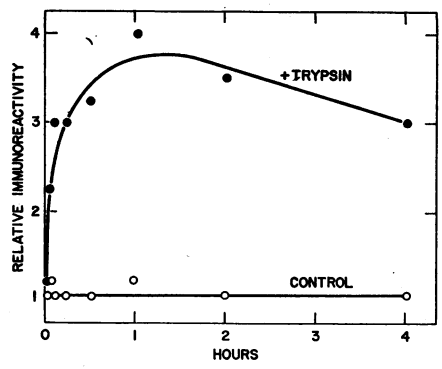

FIG. 5. Flasks containing 15 μg of [^{3}H]CF-A, 50 mM Tris·HCl buffer (pH 7.0), and 5 μg of trypsin (Worthington), in a total volume of 1.0 ml, were incubated with shaking at 37°. The control flask contained no trypsin. Aliquots were withdrawn at the times indicated and immediately mixed with the diluent used for radioimmunoassay (12).

evidence that CF-A is a biosynthetic peptide precursor of PTH.

This relationship seems to be directly analogous to the situation in islet tissue, where proinsulin has been established as the protein precursor of insulin (15), and possibly also to the case of glucagon synthesis, in which, recently, the existence of a "proglucagon" has been reported (16). In this light, it is likely that within the parathyroid gland, CF-A is cleaved proteolytically to PTH, which is then either stored or secreted§. Based on the lower immunoreactivity of CF-A compared to PTH, it is likely that some of the additional aminoacid structure partially covers or modifies the immunological determinants of the PTH portion of the CF-A molecule. The nature of the cleaved segment(s), whether a major moiety is formed equivalent to the C-peptide of proinsulin (15), and whether such a molecule or molecules plays a physiological role in calcium homeostasis, must be determined.

As mentioned earlier, CF-A was biologically active when tested *in vivo* and *in vitro*. Whether the intact molecule is inherently active, or instead must be converted to PTH or some other active fragment, is not known. If biological activity requires an initial cleavage, then our estimates of potency are dependent upon several factors, which include rate of cleavage and removal or inactivation of the active fragments. Examples of such dependency for other hormone-related peptides are abundant. Thus, the chemically synthesized peptide corresponding to the N-terminal [1–34] aminoacid sequence of PTH is much-less potent relative to PTH *in vivo* than *in vitro* (4). The 15-fold greater potency of salmon calcitonin compared to porcine calcitonin is believed due to its slower rate of metabolic destruction *in vivo* (20). As a final, and perhaps more pertinent, example, it is believed that proinsulin must be converted proteolytically to an active molecule for it to exert a biological effect (21).

§ Whether, in fact, the 84-aminoacid molecule is secreted as such is a matter of controversy. Sherwood *et al.* (17) and Arnaud *et al.* (18) propose that the tissue hormone is a precursor of a smaller secreted species. Habener *et al.* (19), on the other hand, reported that PTH itself and not a smaller peptide is secreted *in vivo* [and this secreted PTH is then said (19) to be cleaved to a smaller species while circulating in the blood].

On the other hand, the question of whether or not CF-A possesses inherent biological activity assumes physiological importance only if the intact molecule enters the circulation. In the case of proinsulin there is evidence that the precursor is released by the pancreas and circulates in the blood, where its concentration changes under various physiological stresses (22). In this regard, Habener *et al.* (19) stated that certain adenomas appear to secrete an immunoreactive peptide larger in size than the 84-aminoacid molecule. This suggests that CF-A, like proinsulin, may be directly released by the gland. If this proves to be the case, specific tests to determine the concentration of circulating CF-A might provide useful insights into disturbances of mineral metabolism.

An awareness of the CF-A–PTH relationship may also bear importantly on the search for biochemical levels of control of parathyroid gland function by factors such as calcium and magnesium. For example, we reported that the rate of synthesis of CF-A was inversely related to calcium concentration to the same degree as was the synthesis of PTH (8, 9). In the course of the present studies, moreover, we have observed that the relative degree of incorporation of radioactive amino acid into CF-A and PTH varied markedly in batches of glands obtained at widespread intervals throughout the year (e.g., [^{3}H]CF-A/[^{3}H]PTH ratios ranged from 30 to 0.5), whereas batches of glands gathered at closer time intervals showed smaller variations. This type of result may mean that the prior history of the animal, such as diet and age, affects both the biosynthesis of the prohormone and its conversion to PTH. In preliminary studies in rats, we have found that the concentration of dietary calcium can, in fact, influence the synthetic rate of CF-A and the relative incorporation of radioactive amino acid into CF-A and PTH.

Finally, it is important to determine if the CF-A–PTH relationship is unique to cattle or, instead, is a general one. We find in preliminary studies that there exists in human parathyroid adenoma, and in normal rat and chicken parathyroid glands, a rapidly labeled peptide fraction with physical properties similar to bovine CF-A.

NOTE ADDED IN PROOF

Subsequent to the submission of this manuscript, Kemper *et al.* [(1972) *Proc. Nat. Acad. Sci. USA* **89**, 643–647] reported that a precursor to parathyroid hormone was synthesized in bovine parathyroid gland slices *in vitro*. Based upon migration of the proposed precursor during polyacrylamide electrophoresis, they indicated that the precursor might be 15–20 amino acids larger than parathyroid hormone and have a molecular weight of 11,500. It appears likely that this substance detected by Kemper *et al.* is similar to Calcemic Fraction-A.

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