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On the Nature of Degradation of Calcitonin by Mammalian Cells (38243)

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(Introduced by R. M. Archibald)

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The importance of hormone inactivation as part of the mechanism for the termination of hormonal action has been increasingly recognized (1–3). However, the nature of this inactivation remains unclear. For example, it is not clear whether the target tissues necessarily possess a system to disintegrate hormones. Likewise, the specificity of this inactivation needs to be investigated. In the present study, evidence has been provided which indicated that porcine or salmon calcitonin competes with human calcitonin for a common degradation site in the kidney cells, and that there is a lack of hormonal degradation in terms of immunoreactivity in the bone cells.

Materials and Methods. Pure synthetic human calcitonin (HCT)[1] was kindly supplied by Ciba Inc. Purified salmon calcitonin (SCT)[1] (lot #K423119) and porcine calcitonin (PCT)[1] (lot #188579) were kindly supplied by Armour Pharmaceutical Company. Pure PTH was purchased from Wilson Laboratories. Pure glucagon was furnished by Eli Lilly and Co., Indianapolis and pentagastrin was a gift from Ayerst Chemicals Inc.

Crude collagenase was obtained from Worthington Biochemical Corp. Human albumin was a gift from Merck, Sharp and Dohme. Trasylol was obtained from FBA Pharmaceuticals Inc. Microfine silica Quso G32 was obtained from Philadelphia Quartz Company, Philadelphia, Pa. PBS (phosphate buffered saline) and CMRL 1066 (an enriched, chemically defined medium) were obtained from Grand Island Biological Company.

Cell suspensions of bone and kidney. The calvaria of one-week-old rats were fragmented and incubated at 37° for 90 min in crude collagenase solution, 0.3% in PBS, supplemented with glucose and antibiotics (4). The entire kidneys of adult Holtzman rats were similarly treated. At the end of incubation, the digested suspensions were filtered through a stainlesss-steel mesh of a pore size of 45 μm. The filtrate was then collected in 125 × 16 mm culture tubes. Upon standing at room temperature for 15 min, most of the aggregated cells settled down to the bottom. Then, and at each of fifteen 10 min intervals thereafter, a 0.5 ml aliquot of suspension was removed from the top of this tube and checked by microscopic examination for the absence of aggregated cells. All fractions which contain only single cell populations were pooled for the assay. The cells were counted with a haemocytometer. As determined by their ability to exclude trypan blue, more than 95% of the cells appeared viable. At least 60% of the bone cells maintained cytochemical staining of alkaline phosphatase after 2 days of growth in CMRL 1066 supplemented with 20% fetal calf serum.

Radioimmunoassay. Pure HCT was labeled with carrier-free ^{125}I according to the method of Greenwood *et al.* (5) except that the duration of the chloramine-T oxidative step was 60 sec. The antibody developed from rabbit at a serum dilution of 1:10,000 was sufficient to bind 50% of labeled HCT

[1] Abbreviations: HCT, human calcitonin; SCT, salmon calcitonin; PCT, porcine calcitonin; PTH, parathyroid hormone; PBS, phosphate buffered saline; TCA, trichloroacetic acid.

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(10,000 cpm). The radioimmunoassay was based on the nonequilibrium method (6). Its limit of detection was 20 pg HCT.

Inactivation experiments. The incubation was carried out in 13 × 75 mm flint glass tubes in a shaker bath at 37° for 30 min unless otherwise specified. The incubation mixtures consisted of varying numbers of cells and HCT in 0.5 ml of PBS, containing 1% human serum albumin with or without inhibitors or other peptide hormones. The reaction was initiated by the addition of cells. Immediately after the incubation cells were removed by centrifugation at 3,000 rpm for 1 min at 4° and aliquots of supernates were withdrawn for the determination of hormone levels by radioimmunoassay.

In each experiment, appropriate controls were prepared which were identical with respect to temperature, time, and additional peptide hormones, except that cells were omitted. As these controls contained 100% of the substrate available for degradation, the results were calculated as percentage of degradation as follows:

$$\% \text{ Degradation} = 100 - \frac{\text{Concentration of HCT in experimental tube}}{\text{Concentration of HCT in control tube}} \times 100$$

Results. The loss of immunoreactivity of human calcitonin was assumed to be an indication of hormone degradation in the present studies. The validity of this assumption was based on the observations that significant biological activity requires the entire sequence of 32 amino acids and that no biologically active peptide subfragment has so far been isolated or synthesized (7, 8).

As shown in Fig. 1, the degradation of HCT as measured by radioimmunoassay is dependent of the number of renal cells added. To investigate the possibility of inactivators released into the incubation medium, the indicated number of cells was preincubated with the reaction medium in the absence of substrate for 30 min at 37°. The reaction was then initiated by the addition of the substrate and processed for 30 min at 37°. The result showed that no inactivator was released into the medium (Fig. 1).

Bone cells isolated after crude collagenase treatment were incubated with HCT under the same condition and even as many as 9.5×10^5 cells were found to be incapable of degrading HCT (Fig. 1).

Within 5 min of exposure to 4.0×10^5 renal cells at 37°, approximately 15% of HCT was degraded (Fig. 2). However, the rate of degradation was slightly less after 5 min. When the same experiment was performed at 4°, degradation was not measurable. The addition of trypsin inhibitor (Trasylol) at concentrations of 500–1000 units per 0.5 ml did not appreciably affect the degradation (Fig. 2).

To investigate the specificity of HCT degradation by renal cells, it is essential that the competitive inhibitors added should not interfere with the radioimmunoassay. Therefore, several calcitonins and other small peptides were tested for crossreactivity with HCT radioimmunoassay. As shown in Fig. 3, PCT or SCT did not crossreact with anti-HCT antibody despite the use of a large excess of the heterologous calcitonins. This confirms the data of Byfield *et al.* (9). In addition neither insulin, pentagastrin, PTH nor glucagon interfered with the immunoassay for human calcitonin.

The effect of the relative ratio of the inhibitor to substrate concentration on the degradation of HCT is depicted in Fig. 4. When the ratio of PCT to HCT reaches 15, the protection by PCT begins to be observable. A higher concentration of SCT was required to achieve the same degree of inhibition of degradation of HCT. When the ratio reached 100, there was an almost complete protection of HCT degradation. On the other hand, several other peptide hormones such as PTH, glucagon, insulin, or pentagastrin caused no inhibition even at high concentrations (Fig. 4).

DeLuise *et al.* (10) demonstrated that PCT (130 units/mg) at a concentration of 10 μg/ml inhibited 60% of ^{125}I-PCT degradation by liver cytosol as measured by TCA precipitability. Using the same criterium,

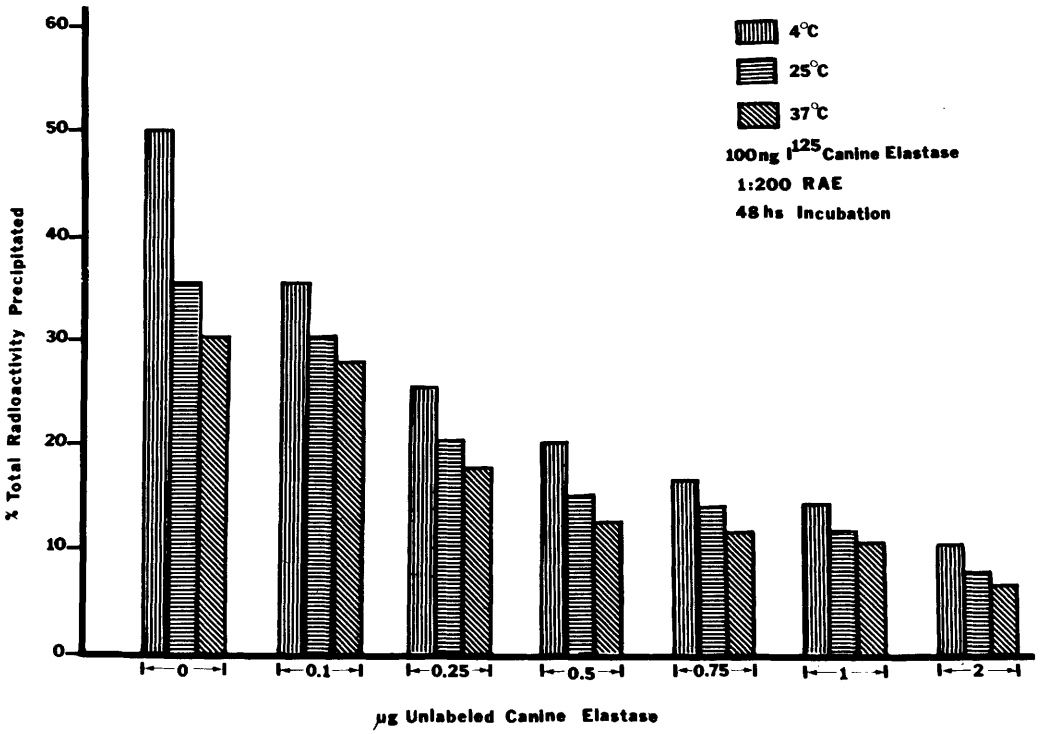

FIG. 2. Effect of temperature on the 2 antibody reaction by incubating the components at 4°, 25° and 37°.

ogous bile into the pancreatic duct, according to the method described by Elliot *et al.* (10).

Results. The effect of incubating ^{125}I-labeled elastase with RAE at different temperatures is shown in Fig. 2. Incubation at 25° and 37° resulted in less precipitation of ^{125}I-labeled elastase than incubation at 4°.

Figure 3 shows the results obtained with 4 different dilutions of antielastase serum in presence of varying amounts of unlabeled elastase. At a 1:100 dilution of antiserum, variation of the unlabeled elastase from 0 to 0.75 μg had little effect on the radioactivity precipitated. In this method a dilution of the antibody was chosen (1:200) to assure at least a 50% binding.

Figure 4 shows the mean performance of 20 standard canine elastase curves employing 8 separate lots of ^{125}I-labeled canine elastase over a period of 6 mo. All other components of this system were identical, and aliquots of the same lot of canine elastase were used throughout. The standard curve was plotted according to the method utilized by Morgan and Lazarow (8). Cross-reactions with other pancreatic enzymes were also studied in this method. No cross-reactivity has been observed with trypsin and chymotrypsin. Anti-canine elastase had a weak reaction against porcine elastase. Plasma containing elastase inhibitor was determined by radioimmunoassay after the addition of serum to standards and the same curve shape was obtained for both. Figure 5 represents the mean standard curve calculated by the method of Hales and Randle (11). The ratio of percent precipitated radioactivity in each standard in the curve (Ce), has been plotted versus the concentration of canine elastase. The plot is essentially linear and its equation has been calculated by linear regression. The correlation coefficient was found to be $r = 0.8869$. Figure 6 shows the results obtained in 10 dogs during experimental pancreatitis induced by the injection of autologous bile into the pancreatic duct. Within 15 min after the induction of pancreatitis the elastase levels as determined by radioimmunoassay increased to about 2½ times the con-

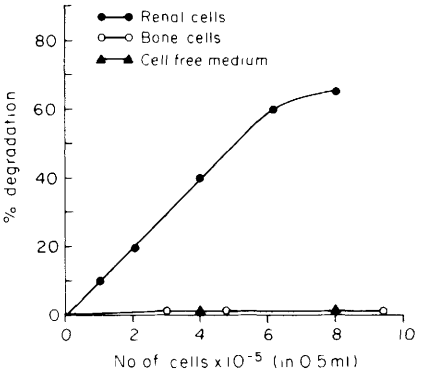

FIG. 1. Effect of increasing number of cells from rat kidney and calvaria on HCT degradation. Varying numbers of cells were incubated with 1×10^{-9} *M* HCT at 37° for 30 min. The experimental conditions, assay, and calculation of per cent degradation are as described under "Materials and Methods." The cell-free medium (▲—▲) was prepared as follows: Renal cells in varying concentrations as indicated were incubated with the medium in the absence of substrate for 30 min at 37° prior to the reaction. The cells were then removed by centrifugation (3000 rpm for 2 min). The reaction was then initiated by the addition of HCT to the supernates. Each point represents the mean of triplicate determinations.

Marx *et al.* (3) failed to find any protection of ^{125}I-SCT inactivation by as much as 1000 μg/ml of unlabeled SCT when kidney membrane was used.

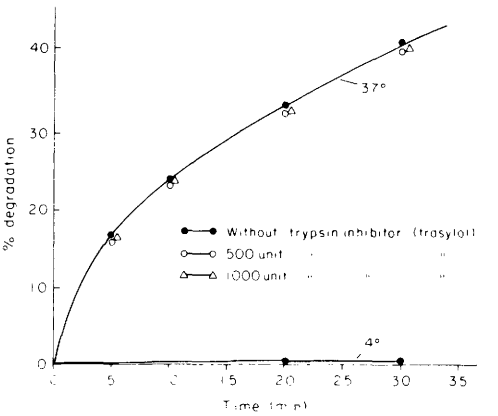

FIG. 2. Time course of HCT degradation by renal cells. The effects of varying temperature and proteolysis inhibitor are also depicted. HCT at 1×10^{-9} *M* was exposed to 4×10^5 cells per 0.5 ml for different time intervals. At the end of each interval, aliquots of supernates isolated by centrifugation were analyzed for the concentrations of immunoreactive hormone by radioimmunoassay. HCT incubated without addition of cells served as control. Each point represents the mean of triplicate determinations.

In the work presently reported, the assay was conducted according to the following improved procedure. The antigen–antibody complex had already been shown to be resistant to further inactivation (unpublished data), hence, the degradation was stopped

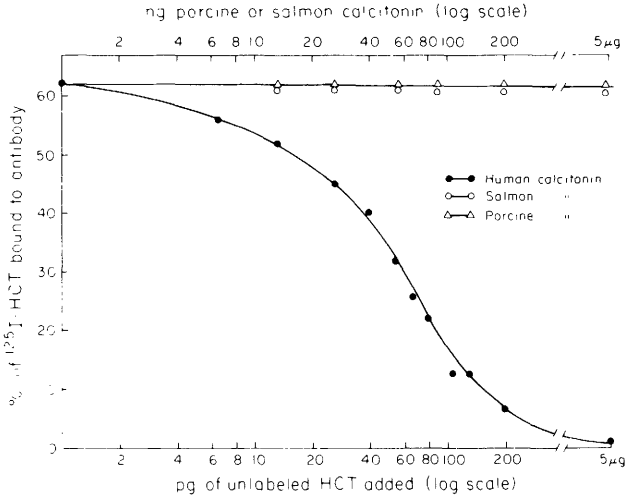

FIG. 3. The specificity of HCT radioimmunoassay. The lower scale of the abscissa indicates picograms of HCT. The upper scale of the abscissa gives nanograms of PCT, SCT or other peptide hormones.

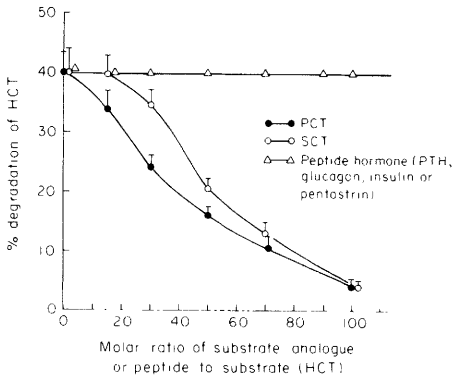

FIG. 4. Effect of heterologous calcitonins and other non-CT peptide hormones on the degradation of HCT. HCT at $1 \times 10^{-9} M$ was incubated with different amounts of other peptide hormones. The indicated molar ratios are those which existed at initiation of the incubation. The reaction was initiated by the addition of $4 \times 10^5/0.5$ ml of renal cells. Each point represents the mean value from four separate experiments; the vertical bars indicate the SD.

by addition of excess antibody. Dextran-coated charcoal was then used to separate antigen–antibody complex from the breakdown products. Precipitation with 5% TCA removed the remaining contaminants such as ^{125}I or small molecular weight compounds. The degree of inactivation was estimated as the loss of radioactivity associated with antigen–antibody complex. Data in Table I, in agreement with data obtained with unlabeled HCT, indicated that both SCT and PCT at a concentration of 2.5 μg/0.5 ml protected the breakdown of ^{125}I-HCT. PCT provided better protection than SCT whereas other small peptide hormones did not affect the degradation of ^{125}I-HCT.

Discussion. The kinetic data regarding degradation of HCT by cell suspensions are in general agreement with those from *in vivo* and *in vitro* studies which employ organ perfusion, slices, or subcellular fractions (10–12).

A number of different peptide hormones are known which have species specific structures. For any given hormone other than calcitonin these heterologous analogues crossreact immunologically. Calcitonins on the other hand are the only hormones known to exist in a variety of structures with the same physiological role and which, in addition, produce antibodies which react only with the homologous calcitonin. The high specificity of calcitonins with respect to binding, as reported from other studies (3) and with respect to degradation here reported, probably reflects similarity of the 3-dimensional structure despite variations in

TABLE I. Effect of Various Peptide Hormones on the Degradation of Radioiodinated Human Calcitonin.[a]

Peptides (2.5 μg/0.5 ml)	% Degradation[b]
None	67.3 ± 5.4 (3)
PCT	19.0 ± 3.3 (3)
SCT	38.0 ± 5.5 (3)
Glucagon	66.2 ± 7.5 (2)
Insulin	73.1 ± 6.2 (2)

[a] Cells ($1 \times 10^5/0.5$ ml) were incubated with 10,000 cpm of ^{125}I-HCT for 30 min at 37°. The incubation medium with or without peptide hormones was the same as described under "Materials and Methods." At the end of incubation, an aliquot of 0.2 ml of excess anti-HCT serum was added to terminate the reaction. Cells were removed by centrifugation at 3000 rpm for 1 min at 4°. An 0.2 ml aliquot of dextran-coated charcoal suspension was then added to the supernates. After recentrifugation and addition of buffer washings of the charcoal precipitates, the supernates were treated with 5% TCA and recentrifuged. The TCA precipitates were washed twice with 5% TCA. The fraction of radioactivity in TCA precipitates represented antigen–antibody complex and was measured in a gamma counter.

$$\% \text{ Degradation} = 100 - \frac{\% \text{ of TCA precipitable radioactivity in experimental tube}}{\% \text{ of TCA precipitable radioactivity in control tube}} \times 100$$

[b] Mean ± SD. The number of experiments is indicated in the parenthesis.

amino acid sequences. The difference in the ability of SCT and PCT to protect HCT from degradation by renal cells may reflect difference in their affinities for the inactivation site.

The observation that high concentrations of unlabeled SCT that block the binding of ^{125}I-SCT to cell membranes fail to protect the breakdown of ^{125}I-SCT as determined by TCA precipitability may indicate that the site of binding for hormone degradation is separate from that of hormone action (3) In this present report, however, excess heterologous calcitonins were found to inhibit ^{125}I-HCT degradation. The discrepancy may be due to difference in either the assay method or the inactivation system chosen.

In addition to possible damage to hormonal structure resulting from the iodination procedure, the radioiodinated hormone may also be deiodinated without degradation to some extent during incubation with cells or membranes. Such possibilities may lead to difficulties in the evaluation of data from inactivation studies. For these reasons, the use of native (noniodinated) hormone seems to be preferable for the study of inactivation. In the competition experiments, the addition of excess amounts of PCT or SCT to the medium reduced the loss of immunoreactive forms which represent either biologically active intact HCT or biologically inactive subfragments. Therefore, the conclusion drawn from these studies of competition between several calcitonins can be only in terms of immunoreactivity rather than in terms of biological activity. It is also probable that the loss in immunoreactive HCT in the medium after the incubation is attributed to the selective adsorption of HCT to the kidney cells. To test this possibility, the washed cells after the inactivation experiment were exposed to 0.1 *M* acetic acid or propionic acid and 1 *M* NaCl (unpublished data). The results indicated that down to the limit of sensitivity of the radioimmunoassay and our experimental conditions none of HCT was released into the medium from the renal cells.

Finally, it is of interest to note under our experimental conditions as measured by immunological reactivity there is a lack of measurable degradation of human calcitonin by isolated bone cells. It is possible that HCT may have been degraded into immunologically active but biologically inactive products. This raises the question as to whether or not target cells must possess a degradation system for termination of hormone action. At least one study with TSH and the thyroid as a model system indicates that this condition may not be necessary (13).

Summary. By the use of immunological reactivity as an indicator, the degradation of synthetic human calcitonin was measured with cells isolated from rat kidney and bone after partial digestion with 0.3% crude collagenase. The degradation of the hormone by bone cells was undetectable (with up to 9×10^5 cells per 0.5 ml). In contrast, renal cells degrade HCT readily. The degradation activity in renal cells appear to be cell bound. Measurement by specific radioimmunoassay of the degradation of HCT in the presence of SCT or PCT suggests competition between three different species of calcitonins for a possible common site of degradation not shared by other peptide hormones tested.

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