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Research Article

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The Regulation of Calcitonin in Normal Human Plasma as Assessed by Immunoprecipitation and Immunoextraction

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ABSTRACT We have studied the secretion of calcitonin in normal adults with a new procedure of increased sensitivity for the measurement of the hormone in peripheral plasma. In this method, endogenous calcitonin is immunoprecipitated with specific antibodies from a 10-ml plasma sample. The calcitonin is then dissociated from the antibodies and extracted into alcohol. The alcohol is evaporated, and the calcitonin is recovered in assay diluent for subsequent radioimmunoassay. The procedure produces a fivefold increase in immunoassay sensitivity and eliminates plasma proteins which can produce spurious immunoassay effects. This procedure was used to measure calcitonin in normal adults. The mean (\pm SE) plasma calcitonin in 43 subjects was 10 (\pm 2) pg/ml. At the end of a 3-h calcium infusion (12 mg/kg), mean plasma calcitonin in 10 subjects had risen to 114 (\pm 21) pg/ml. In 11 subjects, a 10-min infusion of 150 mg of calcium caused calcitonin to rise to a mean concentration of 28 (\pm 7) pg/ml at 20 min. EDTA infusion (50 mg/kg per 2 h) caused a slight decrease in plasma calcitonin.

These results are consistent with our previous reports of the low concentrations of calcitonin in adult plasma. Our data may underestimate calcitonin levels since not all of the heterogeneous species of hormone may be extracted by this method. In any case, this procedure has allowed us to determine that the low concentrations of plasma calcitonin in normal adults are responsive to perturbations of calcium homeostasis. The immunoextraction method may be applicable to other assays in which it is necessary to increase sensitivity or define specificity.

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INTRODUCTION

The secretion of calcitonin has been well studied in patients with medullary carcinoma of the thyroid (1-3). However, additional studies have been limited by the methodological difficulties encountered in the measurement of calcitonin in subjects other than those with this tumor (4-6). Artifacts have been demonstrated in radioimmunoassay methods which can give spuriously high or false positive estimations of plasma calcitonin (5-7). Accordingly, many (1-11), but not all (12, 13), of the laboratories studying this problem have not been able to definitively demonstrate calcitonin in the peripheral plasma of most normal subjects. To pursue this problem, we have made preliminary studies of the secretion of calcitonin in normal adults with a method for the immunoprecipitation and immunoextraction of calcitonin from plasma.

METHODS

40 subjects (36 males and 4 females ranging in age from 23 to 68) without evidence of calcium or skeletal disorders were studied after informed consent was obtained. Basal blood samples were collected after an overnight fast from each subject. 12 of the subjects received a 3-h infusion of 12 mg/kg of calcium, and 7 were given a 2-h disodium EDTA infusion (3, 5, 14). 11 received a 10-min infusion of 150 mg of calcium (15, 16). Blood samples were collected at the indicated time intervals (see Fig. 9-11) before, during, and after each infusion. All of the blood samples collected during these studies were assayed for calcitonin as described below, and calcium was determined by atomic absorption spectrophotometry or EGTA titration (Corning Calcium Analyzer Model 940, Corning Scientific Instruments, Medfield, Mass.).

Because of the difficulty in measuring calcitonin in normal subjects using standard radioimmunoassay procedures (5), we developed the following method (Fig. 1) to concentrate calcitonin from plasma. The preliminary details of the method were worked out by using 125 I-HCT (human

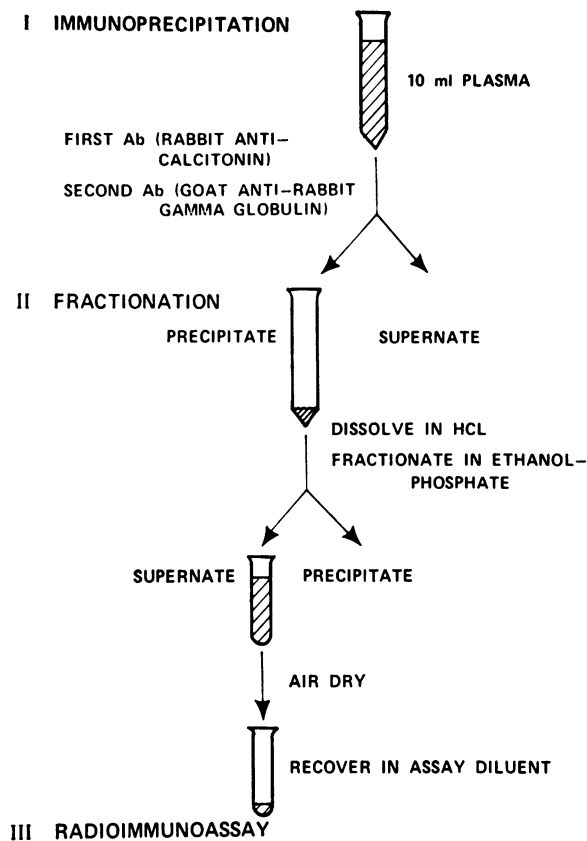


FIGURE 1 Outline of the procedure used for the immunoprecipitation and immunoextraction of calcitonin from human plasma. See text for details.

calcitonin)¹ and/or synthetic human calcitonin added either to bovine plasma that was nonreactive in the HCT assay (4) or to human plasma made calcitonin free by charcoal adsorption (5). 10 ml of plasma were placed in a 15-ml conical test tube (Falcon Plastics, Div. of Bioquest, Oxnard, Calif.). To this was added a known amount of ¹²⁵I-HCT (usually 100,000 cpm) or of synthetic human calcitonin with a trace amount of ¹²⁵I-HCT (usually 1,000 cpm). The calcitonin in each sample was concentrated by an immunoprecipitation procedure using rabbit antibody to synthetic human calcitonin (4), subsequently referred to as first antibody, and goat antibody to rabbit gamma globulin (Calbiochem, San Diego, Calif.), subsequently referred to as second antibody. To each 10-ml sample a varying volume (Fig. 2) of first antibody was added, the sample mixed by inversion, and the mixture incubated at 4°C for 18–24 h; no appreciable precipitation occurred at this step. Then a varying volume (Fig. 3) of second antibody was added, and the sample was mixed and incubated at 4°C for another 18–24 h. The immunoprecipitate (the first antibody-second antibody-calcitonin complex) which appeared was pelleted by centrifugation at 4°C at 2,200 rpm for 25 min. The supernate was decanted, and the precipitate was washed with 5 ml of distilled water, recentrifuged, and recovered as before.

¹ Abbreviation used in this paper: HCT, human calcitonin.

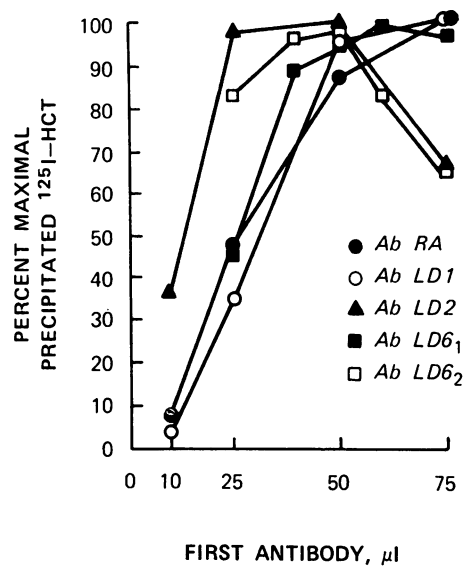


FIGURE 2 Effect of various concentrations of several antisera (RA, LD-1, LD-2, LD-6₁, LD-6₂) to HCT (first antibody) on the precipitation of added ¹²⁵I-HCT from 10 ml of plasma. The amount of second antibody (antirabbit gamma globulin) was 500 μl. Maximum precipitation was 84%.

The calcitonin-containing precipitate was dissolved by adding varying amounts (Fig. 4) of 0.04 N HCl to each of the conical tubes and agitating for 1 h at room temperature on a laboratory rotator. This presumably dissociated the calcitonin-antibody complex. The calcitonin was then separated from the antibodies by precipitating the latter by adding to each sample 3.6 ml of cold 95% ethanol

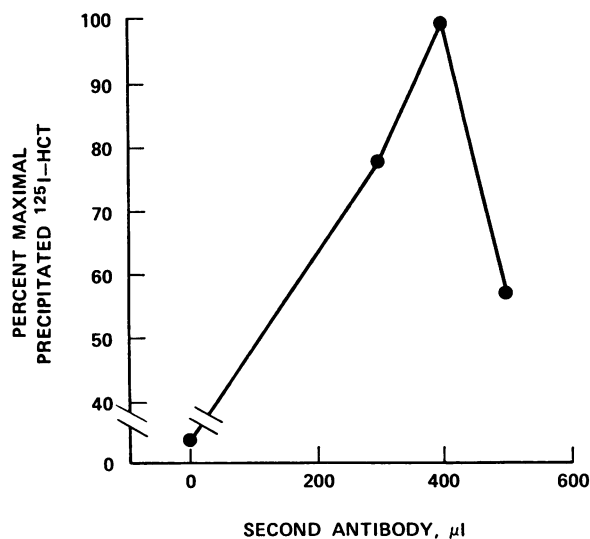


FIGURE 3 Effect of varying amounts of antisera to rabbit gamma globulin (second antibody) on the recovery by precipitation of added ¹²⁵I-HCT from 10 ml of plasma. The amount of rabbit antiserum to calcitonin (first antibody, LD-6) was 50 μl. Maximum precipitation was 75%.

(final concentration 70%) and 0.8 ml of 0.05 M dibasic phosphate buffer (final concentration 1 mM). After being agitated and incubated at 4°C for 1 h, each sample was centrifuged as before. The ethanol-phosphate supernate, presumably containing the calcitonin, was transferred to a 12 × 75-mm test tube. The liquid was volatilized by exposure to air pressurized through a manifold. The resulting calcitonin-containing residue of each test tube was then dissolved in 0.3 ml of 0.1 N acetic acid.

The recovery of calcitonin at each step was determined by either counting the sample for radioactivity in the case of the ¹²⁵I-HCT or by radioimmunoassay in the case of the unlabeled human calcitonin. For radioimmunoassay, the volume in each 12 × 75-mm test tube was adjusted to 1.1 ml, and the pH was adjusted to 7.5 by the addition of 0.8 ml assay diluent (5). Each sample was then assayed for calcitonin as previously described by us (4, 5, 14, 15). For each sample, a control incubation without added antibody was performed in the radioimmunoassay. Such controls consistently demonstrated no significant binding (less than 10%) of ¹²⁵I-HCT. This demonstrated that no functionally significant quantities of the calcitonin antibody were present in the samples.

To evaluate assay specificity, samples were charcoal adsorbed before immunoextraction and radioimmunoassay and also chromatographed on Bio-Gel P-30 (1 × 25-cm column) pre- and postimmunoextraction (Bio-Rad Laboratories, Richmond, Calif.). When the optimal conditions had been determined for the immunoextraction of added calcitonin, the procedure was applied to clinical samples. To each of the 10-ml plasma samples collected from the subjects studied, 1,000 cpm of ¹²⁵I-HCT was added to monitor calcitonin recovery. Noncalcitonin-containing samples such as bovine plasma or charcoal-adsorbed human plasma were used as blanks for the procedure.

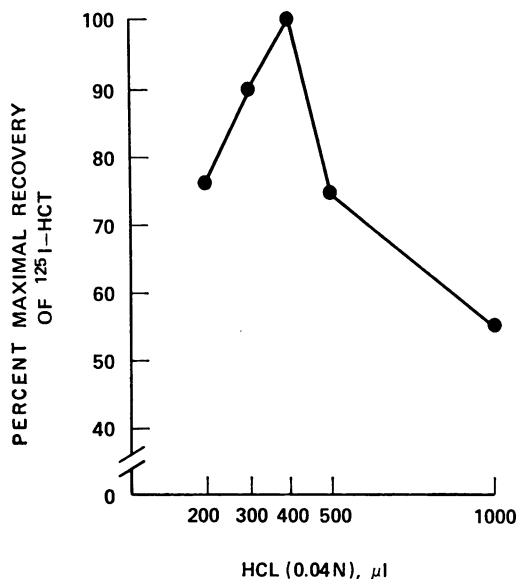


FIGURE 4 Effect of varying amounts of 0.04 N HCl on the dissociation and recovery of added ¹²⁵I-HCT from 10 ml of plasma after immunoprecipitation (Fig. 1). The amount of first antibody (LD-6) was 100 μl, and the amount of second antibody was 500 μl. Maximum recovery of total counts was 68%.

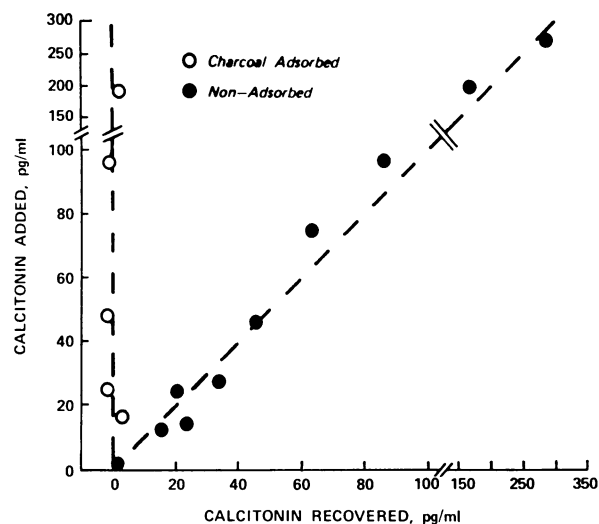


FIGURE 5 Final recovery (horizontal axis) by immunoextraction and radioimmunoassay of calcitonin added (vertical axis) to 10 ml of pooled human plasma. To evaluate assay specificity, each sample was measured before and after adsorption with charcoal, a procedure demonstrated to remove calcitonin from plasma (see text and reference 5). The recovery of calcitonin was linear to the amount added, and charcoal adsorption removed essentially all added calcitonin.

RESULTS

Calcitonin measurement. The recovery by immunoprecipitation of ¹²⁵I-HCT was a function of the concentration of both first and second antibody. Fig. 2 demonstrates that for a given volume of second antibody, 500 μl, the precipitation of ¹²⁵I-HCT was different for each of 5 antibodies (Ab) to calcitonin and also varied according to their final concentration. In addition, different bleedings of the same antibody (LD-6₁ and LD-6₂) precipitated different amounts of ¹²⁵I-HCT. For purposes of supply, we selected for subsequent studies a pool of antibody LD-6 which gave maximal precipitation of ¹²⁵I-HCT at 50 μl. Using this amount of LD-6, we determined that the concentration of second antibody also influenced the immunoprecipitation of ¹²⁵I-HCT (Fig. 3). This figure illustrates that maximal immunoprecipitation with 50 μl of first antibody occurred with 400 μl of second antibody.

We then determined the effect of the concentration of the 0.04 N HCl on dissociation and recovery of ¹²⁵I-HCT (Fig. 4). The recovery was a function of the concentration not only of both first and second antibody (Fig. 2 and 3) but the concentration of HCl as well. Although maximal recovery at this step occurred with 400 μl of acid, 300 μl of acid was ultimately used because it made final recovery greater by more completely dissolving the precipitate.

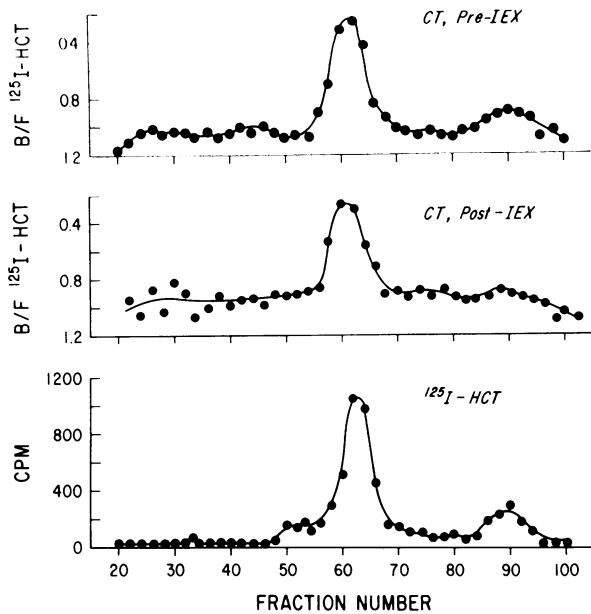


FIGURE 6 Chromatography of calcitonin on Bio-Gel P-30 (1×25 -cm column). The top panel shows the radioimmunoassayable pattern of synthetic human calcitonin. The middle panel shows the radioimmunoassayable pattern of synthetic human calcitonin after addition to human plasma and subsequent immunoextraction. The bottom panel shows the pattern for labeled HCT. The major peaks of calcitonin activity coeluted. The column was developed in immunoassay diluent; the void volume was at tube 30; the salt volume was at tube 90.

Using the condition of $50 \mu\text{l}$ first antibody, $400 \mu\text{l}$ second antibody, and $300 \mu\text{l}$ of HCl, we then immuno-precipitated and immunoextracted added amounts of synthetic human calcitonin from calcitonin-free plasma before and after charcoal adsorption. To control for degradation of calcitonin during incubation, identical aliquots of the hormone were incubated in parallel. The calcitonin extracted from all of the samples and the calcitonin in the incubation controls were then measured by radioimmunoassay. The results of this experiment are shown in Fig. 5. There was a linear relationship between added and recovered calcitonin. Charcoal adsorption removed essentially all added calcitonin.

Chromatography was performed on calcitonin added to plasma before and after immunoextraction. ^{125}I -HCT was used as a marker. The pre- and postextracted calcitonin migrated in indistinguishable patterns and coeluted with the ^{125}I -HCT marker (Fig. 6). Serial dilutions of extracted calcitonin were indistinguishable from the calcitonin standard in the immunoassay (Fig. 7).

Clinical studies. 40 subjects underwent 43 basal fasting calcitonin determinations by the immunoextraction and radioimmunoassay method described. Their

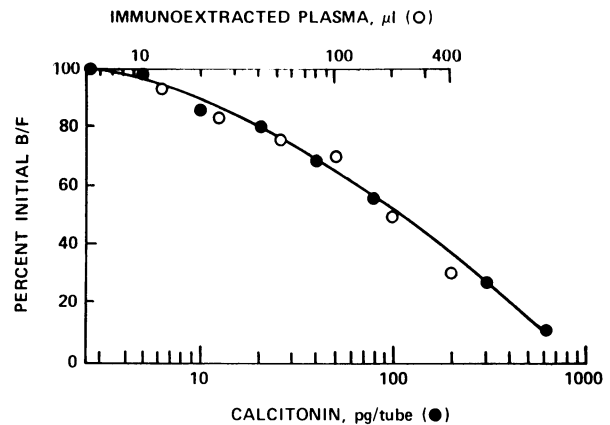


FIGURE 7 Linearity in the calcitonin radioimmunoassay of calcitonin immunoextracted from plasma (O) and standard human synthetic calcitonin (●). The two forms of calcitonin are indistinguishable in their assay reactivity.

calcitonin concentrations are plotted against simultaneous calcium concentration in Fig. 8. The parameters in pg/ml of the basal calcitonin concentrations were as follows: mean 9.9, SD 10.9, and SE 1.7. All of the samples produced an inhibition of tracer binding by antibody. However, when evaluated according to our previously described criteria (4, 5), 30% of the patients had undetectable ($< 4 \text{ pg/ml}$) levels of calcitonin. There were no apparent age or sex differences in plasma

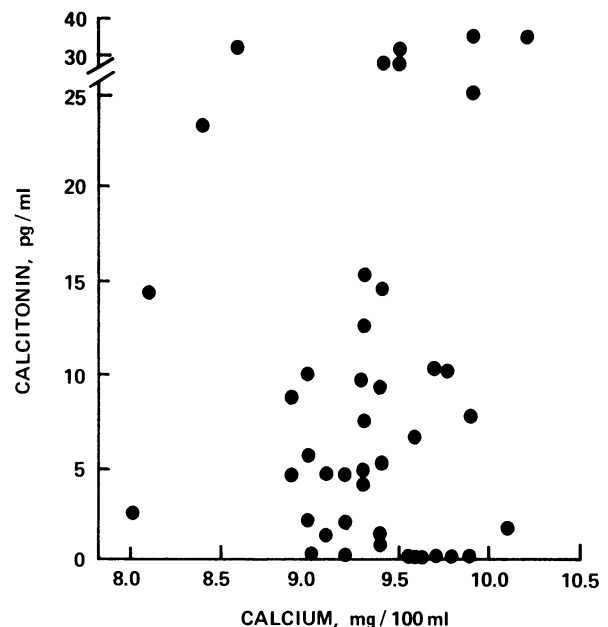


FIGURE 8 Basal plasma calcitonin plotted as a function of total plasma calcium concentration in normal adults. 43 determinations were performed in 40 subjects. There is no correlation between calcitonin and calcium. See text for further discussion.

calcitonin. But, the limited number and ages of subjects, especially female, do not permit firm conclusions regarding these points. There was no correlation between basal plasma calcitonin and calcium in this population. The data include two subjects postthyroidectomy who had undetectable calcitonin.

To study the effect of calcium on plasma calcitonin, a 3-h calcium infusion of 12 mg/kg was performed in 12 subjects (Fig. 9). In every patient there was a significant increase in calcitonin during the calcium infusion. Mean plasma calcitonin for all subjects at the beginning of the infusion was 12.5 (SE±3.0) and at the conclusion was 113.8 (±21.0) pg/ml; this represented a ninefold increase in plasma calcitonin. The calcium rose from 9.2 (±0.1) to 12.6 (±0.3) mg/dl.

In addition to the 3-h calcium infusion, a shorter calcium infusion was used to study calcitonin secretion in 11 subjects (Fig. 10). In all but one case, the calcitonin at 20 min had increased significantly over basal. In the subject whose calcitonin did not increase, there

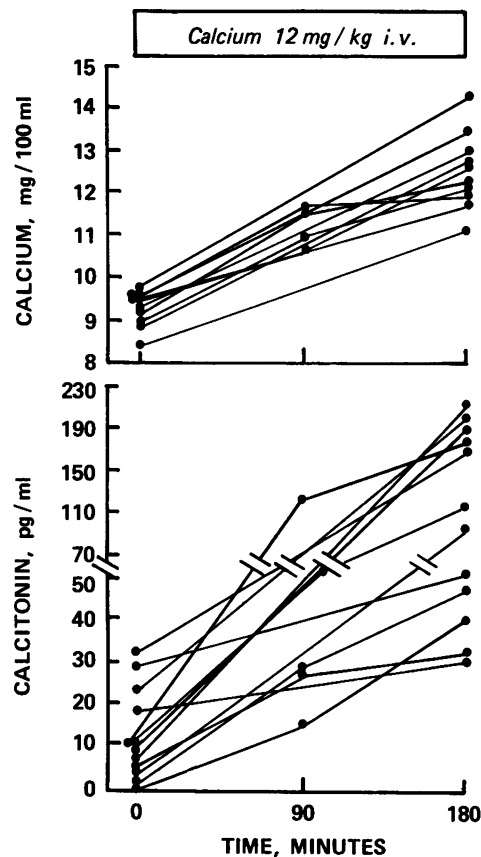


FIGURE 9 Effects of calcium infusion on plasma calcitonin in 12 normal subjects. Calcium was infused at a rate of 4 mg/kg per h. In each patient there was a significant increase in plasma calcitonin as well as calcium during the infusion.

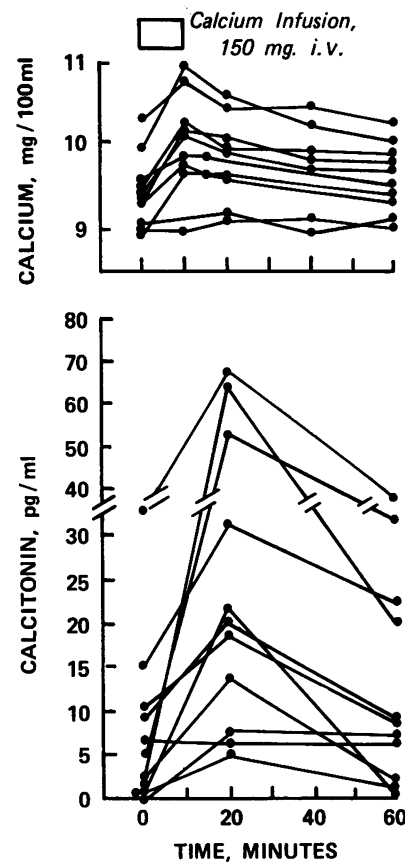


FIGURE 10 Effect of short calcium infusion on plasma calcitonin in 11 normal subjects. 150 mg of calcium were infused over 10 min. In each subject except one there was a significant increase in plasma calcitonin over basal by 20 min. In the one subject showing no increase in calcitonin there was no detectable change in serum calcium.

was additionally no detectable change in serum calcium. While the mean calcium increase for all subjects at 20 min was only 0.3 mg/dl, the calcitonin at 20 min had increased from 8.3 (±3.4) to 28.2 (±7.0) pg/ml, a threefold rise.

Finally, the effect of EDTA infusion on plasma calcitonin was evaluated in seven normal subjects. In these studies, EDTA did not consistently suppress the calcitonin level, but the overall trend was a slight diminution in hormone concentration. Fig. 11 illustrates the mean changes in this study; calcium fell from 9.3 (±0.1) to 7.2 (±0.2) mg/dl; calcitonin fell from 12.5 (±3.7) to 10.4 (±2.7) pg/ml.

DISCUSSION

Because of the difficulties encountered in the measurement of plasma calcitonin by radioimmunoassay in patients other than those with medullary thyroid carci-

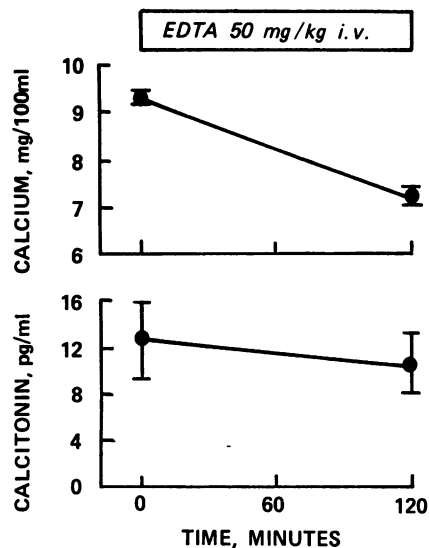


FIGURE 11 Effect of EDTA infusion on plasma calcitonin in normal subjects. The mean (\pm SE) changes in plasma calcium and calcitonin are shown for seven subjects. Although some subjects demonstrated a decrease in plasma calcitonin during the infusion, there was no significant change for the group.

noma, the secretion of this hormone has not been extensively studied in normal subjects (1-11). Accordingly, to pursue such studies, we have developed an immunoprecipitation and immunoextraction procedure which was used to concentrate the calcitonin contained in peripheral blood samples (Fig. 1). This method immunoprecipitated 60-80% of calcitonin from plasma and achieved a nine-fold reduction of the volume of the original plasma sample. An effective four- to fivefold concentration of the original amount of calcitonin in each sample was thereby accomplished. The calcitonin in the concentrated samples was then measured by radioimmunoassay (3-5). This procedure has allowed us to sufficiently increase the sensitivity of our radioimmunoassay to study the secretion of this hormone in normal subjects (Fig. 8-11).

In addition to the resulting increase in sensitivity, the immunoextraction procedure also offers several other methodological advantages. Plasma proteins are excluded by this procedure thereby eliminating one of the factors known to have nonspecific effects in the calcitonin radioimmunoassay (5, 7). Radioimmunoassay measurements can also be made in the more sensitive part of the standard curve thereby circumventing the variability in assay performance that is encountered near the zero point of the standard curve (5, 17). This was especially true for the samples collected during calcium infusion. These samples usually produced a greater than 50% depression of initial B/F (bound to free ratio). Even most basal samples produced a sig-

nificant depression of initial B/F. Therefore, these samples could be read off the reliable portion of the standard curve (5).

However, this method also has several disadvantages. A large amount of sample is needed, 10 ml of plasma. This will limit the application of this method in certain instances, such as in the study of infants, in serial studies in the same patient, or in measurements of thyroid venous calcitonin. Another disadvantage of this method is the large amount of first and second antibody consumed. In addition, because of the documented immunochemical heterogeneity of plasma calcitonin (18, 19), it is likely that the one peak of calcitonin of this immunoextraction procedure (Fig. 6) does not represent all species of circulating calcitonin. Accordingly, not all forms of plasma calcitonin may be present in the final extraction product, and we may be underestimating total plasma calcitonin with this procedure. Of course, similar problems of immune specificity also pertain to the antibodies used in standard radioimmunoassay procedures (19). In fact, it is likely that the immune specificity of different assay systems in combination with the recently demonstrated (18, 19) immunochemical heterogeneity of plasma calcitonin could explain some of the differences encountered among different laboratories in the measurement of plasma calcitonin (2, 12, 13, 19-22).

Using the extraction procedure, we have determined that the basal levels of calcitonin can be detected in most normal adults (Fig. 8). The concentration of hormone is low and consistent with our previous estimations and the estimations of several other laboratories that have used either standard immunoassay procedures or extraction procedures for calcitonin measurement (1-11, 20, 21). However, some laboratories have reported significantly higher basal levels of calcitonin (12, 13). We do not find in our limited number of subjects any obvious age or sex difference in calcitonin concentration or any correlation between basal levels of plasma calcium and calcitonin. This is in contrast to some but in agreement with other published data (2, 13, 21, 22). The latter is not surprising to us since it is most likely that there is interaction between calcium and calcitonin in the basal state and that they regulate each other. Under such circumstances, one would not expect a correlation between them.

In acute studies we have found that calcium is a reliable stimulus to calcitonin secretion. A standard 3-h calcium infusion causes a ninefold increase in plasma calcitonin (Fig. 9), an effect greater than that noted by others (2, 8-10, 12, 13). In fact, even alterations of plasma calcium which have a minimal effect on blood calcium concentration still stimulate plasma calcitonin up to threefold (Fig. 10). Accordingly, plasma calci-

tonin is very sensitive to perturbations in calcium homeostasis in normal subjects.

The effect of EDTA-induced hypocalcemia on plasma calcitonin is not conclusive. Although some patients did exhibit a drop in plasma calcitonin during EDTA infusion, the mean data of the patients studied (Fig. 11) did not demonstrate as significant a decrease in concentration of the hormone as has been reported by others (13). This may be due to the small number of patients studied or to the fact that in these EDTA studies we are approaching the limits of detection of our immunoassay system.

The immunoextraction procedure has allowed us to conduct studies of calcitonin secretion in normal subjects. These studies indicate that calcitonin does circulate in peripheral plasma and that its concentration is sensitive to induced changes in blood calcium. This procedure should be applicable to studies in other conditions where there may be either primary or secondary disorders of calcitonin secretion, such as malignancy and hypercalcemia, hypergastrinemia and renal disease. In addition, the principles used in this procedure may also be applicable to the measurement of other hormones where it is desirable to have assays of increased sensitivity and defined specificity. However, with an appreciation of the immunochemical heterogeneity of calcitonin (18, 19) and of its low peripheral concentrations, it is likely that even more sophisticated methods will be necessary for a thorough study of the secretion of this hormone in humans.

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