In Vitro Biosynthesis of Calcitonin[1] (35198)

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Calcitonin is a hypocalcemic polypeptide hormone synthesized and secreted by a discrete group of cells in mammalian thyroid glands which, in evolutionary terms, correspond to cells of the ultimobranchial body of lower vertebrates (1, 2).

Enhanced secretion of calcitonin in hypercalcemic states has been well documented (3, 4). It has also been established that the level of blood calcium controls the level of circulating calcitonin (5). However, it has not yet been demonstrated whether or not serum calcium influences the synthesis of calcitonin, its release, or both.

In this paper, an *in vitro* calcitonin-synthesizing system in ovine thyroid slices is described which allows independent study of the synthesis or release of calcitonin. Using this experimental design, the incorporation of ^{14}C-glycine into calcitonin and the effect of high concentrations of calcium on the synthesis and release of calcitonin were studied.

Materials and Methods. Ovine thyroid glands were obtained from the slaughterhouse immediately after the animals were sacrificed. After removal of adhering tissue, the glands were cut with a Stadie-Riggs tissue slicer into slices approximately 0.5–1.0 mm thick.

The slices were incubated in 50-ml Erlenmeyer flasks (2.0 g/flask) containing 10 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) to which were added glucose (320 mg/100 ml) and all the amino acids of porcine calcitonin (20 mg/liter) except glycine. 2.5 μCi of uniformly labeled ^{14}C-glycine (Nuclear Chicago, 108 μCi/mmole, or Tracerlab, 70 μCi/mmole were added to each flask. The incubations were carried out in air in a shaker-water bath at 37° for 4 hr.

Incorporation of ^{14}C-glycine into calcitonin. Slices were incubated in Krebs–Ringer bicarbonate buffer containing a high (45.6 mg/100 ml) concentration of calcium. The incubations were terminated with the additon of 1.0 ml of 50% trichloroacetic acid to each flask. The fluid in each flask was decanted and the slices were minced and washed 10 times with cold acetone, 6 times with cold chloroform, again 10 times with cold acetone, dried at room temperature, and stored at 4° until used. The dried and defatted tissue from 220 g of incubated slices (110 flasks) was accumulated, pooled, and extracted with 10 vol of 8.0 *M* urea–0.1 *N* cysteine in 0.2 *N* HCl for 1 hr at room temperature (6). Calcitonin was isolated and partially purified by the method of Gudmundsson *et al.* (7). The mixture was filtered through cheesecloth and the residue was washed with doubly-distilled water (10 ml/g of dry powder). The washings were combined with the filtrate and were dialyzed against 0.1 *M* sodium acetate buffer (pH 4.6) for 24 hr at 4°. The dialysate was made up to 1.0 *M* with respect to sodium chloride with 3.0 *M* sodium chloride and the mixture was allowed to stand at 4° for 72 hr. The precipitate was then removed by centrifugation at 1400*g* for 3 hr at 4°. Solid sodium chloride was added to the supernatant to a final concentration of 2.0 *M*.

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After standing for a further 72 hr at 4°, the precipitate was collected by centrifugation at 1400*g* for 3 hr at 4° and dried by lyophilization. The lyophilized material was dissolved in 0.1 *M* formic acid (18 mg/ml) and applied to a 2.5 × 45-cm column of Sephadex G-100 and eluted with the same solvent. Fractions were collected with a Gilson fraction collector V[15] and the transmission of the effluent at 280 nm was monitored with a Uvicord absorptiometer and recorder (LKB Instruments). The fractions were assayed for hypocalcemic activity by the method of Kumar *et al.* (8). Male Holtzman rats, 140–170 g in weight and maintained on Rockland Mouse-Rat Diet, were fasted 18 hr before being used in the assays. The fractions containing hypocalcemic activity were pooled and lyophilized. The dried material was then dissolved in 1.0 *M* formic acid and applied to a 0.9 × 45-cm column of Sephadex G-50 Superfine and eluted with a solution of 0.1 *M* formic acid–1.0 *M* urea–0.2 *M* sodium chloride. The fractions from the Sephadex G-50 column were scanned for hypocalcemic activity. Fractions containing hypocalcemic activity were pooled, and the preparation was lyophilized. Two dimensional chromatography was carried out on the pooled fractions eluted from the Sephadex G-50 columns with the use of thin-layer cellulose sheets (Eastman Chromatogram Sheet 6065, cellulose with fluorescein indicator). The chromatogram was developed in one direction with a butanol:acetic acid:water (4:1:5) system and in the other direction with a propanol:water (70:30) system.

Preliminary amino acid analysis showed that the glycine fraction of the calcitonin isolated was substantially labeled and the calcitonin appeared homogeneous upon two-dimensional thin-layer chromatography.

In experiments to determine the effect of calcium on the synthesis of calcitonin the slices were incubated in Krebs–Ringer bicarbonate buffer containing either 11.4 or 45.6 mg/100 ml of calcium. Three experiments were done, each with different glands. Following incubation, the slices were defatted, dried, and extracted as described above. Calcitonin was isolated from the extract through the second sodium chloride precipitation step of the method of Gudmundsson *et al.* (7). The calcitonin preparations were assayed for hypocalcemic activity.

In further experiments the release of calcitonin into the medium during the incubation was studied. After 4 hr of incubation, the slices were removed from the flasks and aliquots of the incubation medium were assayed for hypocalcemic activity.

Serum calcium was determined by a modification of the method of Ashby and Roberts (9) and total protein by the method of Lowry *et al.* (10) as modified by Oyama and Eagle (11).

Measurements of radioactivity were carried out in a Beckman liquid scintillation spectrometer, Model LS 250. The counting fluid was prepared by dissolving napthalene (100 g) and PPO (5 g) in 1 liter of dioxane.

Results. Isolation and purification of ^{14}*C-calcitonin.* The incorporation of ^{14}C-glycine into isolated calcitonin is demonstrated in Fig. 1, which shows the protein content, radioactivity, and elution protein pattern of NaCl-precipitated calcitonin preparation on a column of Sephadex G-100. Hypocalcemic activity was confined to fractions 6 through 10. Radioactivity was high in the fractions containing hypocalcemic activity with 18,000 dpm/mg of protein in fraction 6 and decreasing to about 8000 dpm/mg of protein in fraction 10.

The active fractions when combined and lyophilized, yielded a total of 1.3 mg. 0.56 μg of this material decreased the serum calcium of test animals 1.37 ± 0.09 mg/100 ml.

700 μg of the lyophilized material were dissolved in 1.0 *M* formic acid and applied to a column of Sephadex G-50 superfine and eluted with 0.1 *M* formic acid–1.0 *M* urea–0.2 *M* NaCl. The elution pattern of hypocalcemic activity and radioactivity are shown in Fig. 2. Hypocalcemic activity and radioactivity were confined to the main protein peak (Fractions 16, 17, and 18). These three fractions were pooled and lyophilized. The lyophilized material was dissolved in 0.1 *M* acetic acid containing 0.1% albumin and assayed for hypocalcemic activity. 0.18 μg of protein decreased the serum calcium 1.27 ±

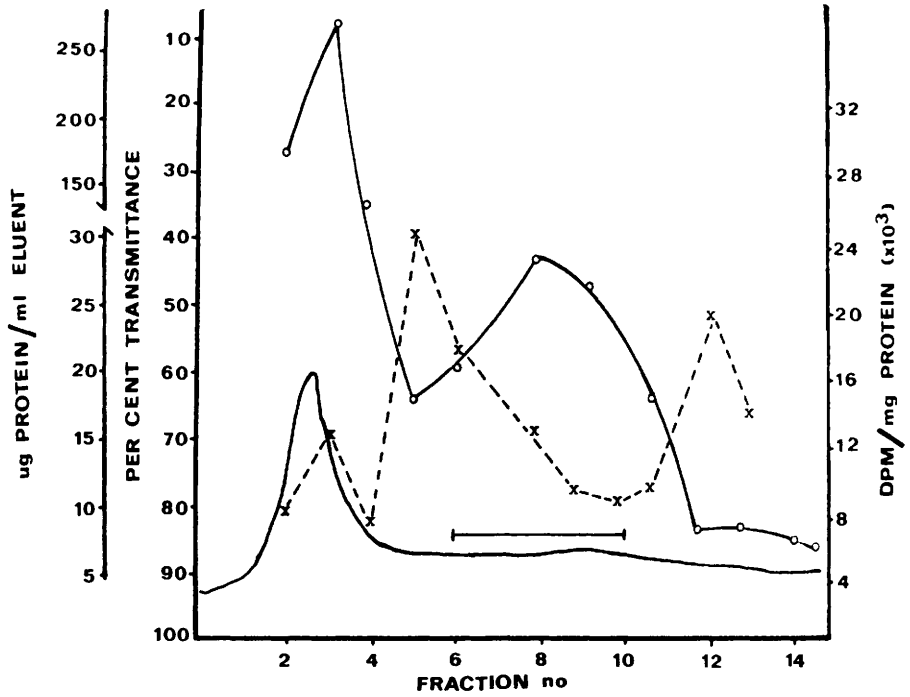

FIG. 1. Protein content (o); radioactivity (x); and elution pattern (—) of NaCl-precipitated calcitonin preparation on a column of Sephadex G-100. The first 60 ml were discarded. Flow rate: 34.5 ml/hr; 4.0-ml fractions were collected. Hypocalcemic activity (—).

0.07 mg/100 ml. Fraction 16 contained 4900 dpm/100 μg of protein; fraction 17, 4770 dpm/100 μg; and fraction 18, 3580 dpm/100 μg of protein. Two-dimensional chromatography of an aliquot of the combined fractions (16, 17, and 18) showed only one spot.

Effect of calcium on synthesis of calcitonin. Comparison of the decrease of serum calcium in test animals after the administration of the calcitonin preparation obtained from fresh, unincubated glands, with the decrease following the administration of the calcitonin preparation, obtained from slices incubated in physiological Krebs–Ringer bicarbonate buffer shows no significant difference (Table I).

However, the decrease in serum calcium obtained by administration of calcitonin preparations from slices incubated in Krebs–Ringer bicarbonate buffer containing high concentrations of calcium is greater than the decrease obtained by administration of calcitonin preparations from slices incubated in buffer containing low concentrations of calcium (Table II). The differences between these hypocalcemic activities are significant.

Effect of calcium on the release of calcitonin into the incubation medium. The decrease

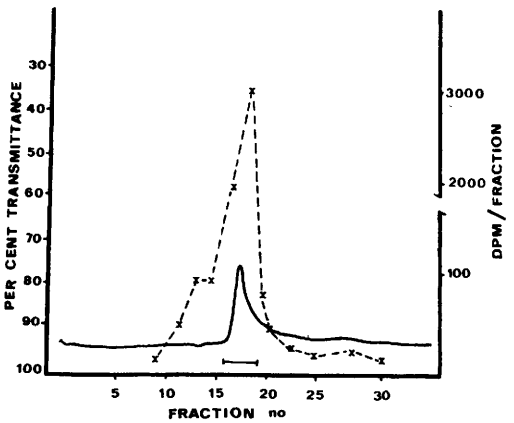

FIG. 2. Elution pattern of hypocalcemic activity from a Sephadex G-50 superfine column. Flow rate: 6 ml/hr; 1.0-ml fractions were collected. The hypocalcemic activity was eluted as a single peak at fractions 16, 17, and 18; hypocalcemic activity (—); 2× expansion of the % *T* scale; radioactivity (- - -).

TABLE I. Hypocalcemic Activity of Calcitonin Preparations Obtained from Incubated and Unincubated Thyroid Gland Slices.[a]

Preparations	ΔCa (mg %/10 μg of protein)[b]
Vehicle (0.1 *M* acetic acid)	+0.55 ± 0.11
Unincubated glands	−0.65 ± 0.17
Incubated glands	−0.76 ± 0.15

[a] Calcitonin was isolated from the extract through the second sodium chloride precipitation step of the method of Gudmundsson *et al.* (7).

[b] Each value represents mean and standard error for a group of five test animals.

in serum calcium following the administration of incubation media containing high calcium was 1.25 mg %/25 μg of protein (Table III). The decrease in serum calcium consequent to the administration of incubation media containing physiological concentrations of calcium was 0.75 mg %/25 μg of protein (Table III). In control animals, which received buffer containing high or physiological concentrations of calcium, the serum calcium increased by 0.49 and 0.62 mg/100 ml, respectively. The net effect on serum calcium is obtained by adding the changes in serum calcium of the control to that of the experimental animals consequent to the assay of buffer and incubation media. The net changes are −1.74 ± 0.08 and −1.37 ± 0.08 mg/100 ml for the high calcium and low calcium media, respectively. The fall in serum calcium is attributed to the calcitonin released from the thyroid slices into the media. The difference of the means of the serum calcium changes is significant.

Discussion. The data show that slices of ovine thyroid glands are capable of synthesizing calcitonin when incubated in a suitable medium. After incubation with ^{14}C-glycine, a fraction containing calcitonin with a specific biological activity of approximately 67 MRC units/mg and high specific radioactivity was isolated. The radioactivity in the fraction having the highest hypocalcemic activity was 4900 dpm/100 μg of protein.

Calcitonin preparations obtained from the slices incubated in buffer containing low concentrations of calcium decreased the serum calcium on the average of 12%/10μg of protein. Preparations obtained from the slices incubated in buffer containing high concentrations of calcium decreased the serum calcium by an average of 17%; thus the slices incubated in high calcium buffer either synthesized more of the hypocalcemic agent, or catabolic processes were inhibited by the high calcium concentrations.

The high calcium medium contained 46.5 mg/100 ml of calcium, a concentration far above any physiological level. This, of course, introduced the complicating factors of nonspecific effects of calcium and ionic strength. The increased hypocalcemic activity induced by the high concentration of calcium in the incubation medium seems to be a specific property of the calcium and not of the increased ionic strength of the medium. In studies now being conducted, the magnesium concentration of the medium was increased to give the same ionic strength as that of the high calcium medium and no change in hypocalcemic activity has been observed.

The results obtained in this investigation

TABLE II. Hypocalcemic Activity of Calcitonin Isolated from Ovine Thyroid Slices Incubated in Krebs–Ringer Bicarbonate Buffer Containing Physiological and High Concentrations of Calcium.

Preparation	ΔCa (mg %/10 μg of protein)[a]	*p*	% Decrease in serum calcium
Expt. 1			
Physiological buffer (4)	−1.02 ± 0.18		8.7
		<.06	
High calcium buffer (4)	−1.61 ± 0.41		14.1
Expt. 2			
Physiological buffer (3)	−1.55 ± 0.18		15.7
		0.05	
High calcium buffer (3)	−2.55 ± 0.29		20.7
Expt. 3			
Physiological buffer (5)	−1.31 ± 0.15		11.0
		<.01	
High calcium buffer (5)	−2.06 ± 0.18		17.2

[a] Each value represents mean and standard error. The number of animals used for each assay is indicated in parentheses.

TABLE III. The Effect of Calcium on the Release of Calcitonin from Thyroid Slices.

Preparation	ΔCa (mg/100 ml)	ΔCa (mg %/25 μg of protein)	Net effect[a] (mg %; mean ± SE)
Low calcium buffer (5)[b]	±0.62 ± 0.12		
Low calcium incubation medium (10)		−0.75	−1.37 ± 0.08
High calcium buffer (5)	±0.49 ± 0.10		
High calcium incubation medium (10)		−1.25	−1.74 ± 0.08
p			<0.05

[a] Net effect is the summation of the change in serum calcium of control and experimental animals consequent to the assay of buffer and incubation media.

[b] Number of animals.

support the hypothesis that calcium exerts a direct effect on the release of calcitonin from the thyroid glands, since an increase in the concentration of calcium in the incubation media led to an increase in the release of hypocalcemic activity. From the data, it can be calculated that the glands incubated in Krebs–Ringer buffer containing a high calcium concentration release the equivalent of at least 500 MRC milliunits of calcitonin/g of glands/4 hr while glands incubated in buffer containing physiological concentrations of calcium released only the equivalent of 440 MRC milliunits of calcitonin/g of glands/4 hr.

Hamilton and Cohn (12), using a similar system, showed that the synthesis of parathyroid hormone is stimulated by low concentrations of calcium. Their results and those reported here support the *in vivo* observations that calcium levels of the blood control the titers of parathyroid hormone and calcitonin.

Summary. The hypocalcemic activity of calcitonin preparations isolated from ovine thyroid slices incubated in Krebs–Ringer bicarbonate buffer containing low and high concentrations of calcium was studied. Biosynthesis of calcitonin was found to be stimulated by high calcium concentration in the incubation medium. The secretion of calcitonin from the ovine thyroid slices was enhanced by calcium.

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