Effects of Glucagon, Dibutyryl Cyclic 3′,5′-Adenosine Monophosphate, and Theophylline on Calcitonin Secretion In Vitro

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ABSTRACT The secretion of calcitonin by slices of porcine thyroid glands has been investigated. Calcitonin in the incubation medium was determined by radioimmunoassay. Secretion of calcitonin was diminished when calcium or magnesium was omitted and was increased stepwise as the concentration of calcium or magnesium in the incubation medium was increased. Calcitonin secretion was augmented substantially when either the quantity of thyroid tissue or volume of incubation medium was increased. Secretion of calcitonin was stimulated by glucagon, theophylline, and dibutyryl cyclic 3′,5′-adenosine monophosphate. It is concluded that calcitonin secretion is regulated by the concentration of calcium and magnesium, that secretion may be inhibited by calcitonin or a precursor and that secretion can be stimulated by increasing the concentration of cyclic 3′,5′-adenosine monophosphate in the parafollicular cells of the thyroid gland.

INTRODUCTION

Calcitonin is a polypeptide hormone secreted by the parafollicular cells of the mammalian thyroid gland (1–3). The rate of secretion is determined by the circulating serum calcium (4–6); increases in serum calcium enhance release and decreases diminish release of the hormone. Calcitonin lowers the serum calcium by inhibiting bone resorption (7, 8). This system involving the skeleton and thyroid gland provides a means for the negative feedback control of circulating serum calcium in response to hypercalcemia such as that, for example, which may occur after a meal (9). Calcitonin, whether endogenous or administered, also diminishes hypercalcemia induced in experimental animals with vitamin D (10) and parathyroid extract (10, 11).

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There is evidence that calcitonin secretion may be stimulated by glucagon (12, 13), a hormone which has also been used to treat hypercalcemia (14). Since the adenyl cyclase system is involved in the secretion of a number of polypeptide hormones including insulin (15), thyrotropin (16), renin (17), and adrenocorticotropic hormone (18), and since glucagon is known to stimulate the adenyl cyclase system in other tissues (19, 20), studies have been carried out to determine whether the adenyl cyclase system is related to the secretion of calcitonin. The effects of concentration of calcium, magnesium, and thyroid tissue and of volume of incubation medium have also been investigated.

METHODS

Incubation studies were carried out with slices of pork thyroid glands obtained from a local abattoir. Animals were killed by electric shock and the glands were removed within 15 min after death. Except where noted 50-mg slices (±10%) were incubated for 60 min under 100% O_2 at 37°C in a Dubnoff incubator (100 cycles per minute) in 2 ml Krebs-Ringer phosphate buffer, pH 7.4. Except where noted, in each study slices from a single thyroid gland were used. The standard concentrations of calcium and magnesium used were 2.5 and 3.08 mEq/liter, respectively. Glucose, 1 mg/ml, was also added. Calcitonin concentration in the incubation medium was determined at the end of incubation by radioimmunoassay. The rate of secretion of calcitonin was determined by multiplying the concentration of calcitonin (mμg/ml) by the volume of incubation medium and is expressed as millimicrograms per hour.

Radioimmunoassay. Pure porcine calcitonin[1] (21) was labeled with ^{131}I by a modification (22) of the method of Greenwood, Hunter, and Glover (23). Specific activity ranged from 100 to 300 μCi/μg. The labeled hormone was separated from damaged hormone and free iodine by adsorption on and elution from granules of silica, QUSO G-32[2] (24). Chromatoelectrophoresis (22) of the preparations of

[1] Kindly supplied by Dr. F. J. Wolf, Merck & Co., Inc., Rahway, N. J.

[2] Philadelphia Quartz Co., Philadelphia, Pa.

TABLE I

Effect of Quantity of Thyroid Tissue on the Secretion and Concentration of Calcitonin

Thyroid	Calcitonin		n	*P* value*
	Concentration	Secretion		
mg	*mμg/ml*	*mμg/hr*		
50	0.9 ±0.1	1.8 ±0.2	8	
100	2.1 ±0.3	4.2 ±0.6	8	<0.01
200	4.7 ±0.6	9.4 ±1.2	8	<0.001
400	15.2 ±2.4	30.3 ±4.7	8	<0.001

Results are expressed as mean ±SE. n is number of observations. The volume of incubation medium was 2 ml.
* As compared to results with 50 mg thyroid.

labeled hormone used showed that 90% or more of total radioactivity was undamaged hormone and the remainder was damaged hormone and unincorporated iodine. Calcitonin-^{131}I was used within 2 wk after iodination. The assay was carried out with 0.3 ml calcitonin-^{131}I, 20,000–30,000 cpm, 0.3 ml guinea pig antiserum[3] 1:8,000, and 0.3 ml incubation medium or standard solution at multiple dilution. Assays were determined with undiluted incubation media since it was found in preliminary experiments that results were the same as those obtained after dilution with the buffer used to dilute the standard. All dilutions were carried out in 0.137 M sodium borate buffer, pH 7.5, containing 1% normal human albumin and 1 mM ethylenediaminetetraacetate (EDTA). Incubations were carried out for at least 48 hr at 4°C and for another 48 hr at 4°C after addition of rabbit anti-guinea pig serum (25). Bound/free ratios for standard and unknown samples were determined after centrifugation for 15 min at 2000 rpm by counting the supernatant and precipitate in an automatic gamma well counter. A total of at least 10,000 counts was obtained for each sample. Determinations of all standard and unknown samples were carried out in quadruplicate. Values as low as 0.1–0.2 mμg/ml could routinely be determined. Results are expressed as the mean ±SE. Statistical tests of significance were carried out with Student's *t* test.

RESULTS

Preliminary studies showed that thyroid slices were not capable of degrading calcitonin-^{131}I as determined by precipitation of the labeled hormone with 10% trichloroacetic acid (26). In addition, recovery of unlabeled calcitonin added to the incubating slices was quantitative. When calcitonin, 1 mμg/ml and 10 mμg/ml, was added, the net concentrations after incubation and correction for quantity of calcitonin secreted (a third set of eight thyroid slices showed a final mean concentration of 1.3 ±0.4 mμg/ml) were 1.1 ±0.2 mμg/ml and 10.2 ±0.7 mμg/ml, respectively, (n = 8). This indicates that the values for calcitonin represent actual secretion and not net production.

[3] Kindly supplied by Doctors E. L. Grinnan and W. O. Storvick, Eli Lilly & Co., Indianapolis, Ind.

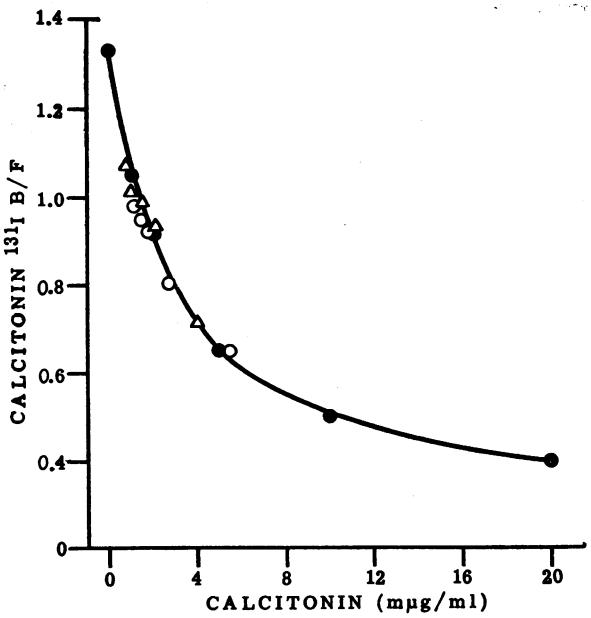

FIGURE 1 Radioimmunoassay curve for porcine calcitonin (black filled circles). Also shown are curves for two samples of media after incubation with a thyroid slice (open circle, open triangle) assayed undiluted and diluted 1:1, 1:2, 1:3, and 1:4. Note that the curves for the standard and unknown samples are identical.

A representative standard curve for the radioimmunoassay of porcine calcitonin is shown in Fig. 1. Serial dilution of the medium after incubation of a thyroid slice confirmed identity of the calcitonin.

Under standard conditions of incubation for 1 hr (2 ml incubation medium with a calcium concentration of 2.5 mEq/liter and a magnesium concentration of 3.08 mEq/liter) the mean secretion of calcitonin varied from one thyroid gland to another (range: 1.2–12.5 mμg/hr) when the glands were obtained on different days. Secretion rate varied very little, however, when the glands were obtained and incubations performed on the same day. Under these circumstances mean secretion for six glands (six slices per gland) varied from 7.8 ±0.5 mμg/hr to 9.8 ±0.5 mμg/hr and averaged 8.9 mμg/hr.

Effects of time, of quantity of thyroid, and of volume of incubation medium on calcitonin secretion. At concentrations of from 50 to 400 mg of thyroid tissue per 2 ml incubation medium, mean calcitonin secretion varied directly with the quantity of thyroid incubated (Table I). To determine the effects of time and whether the secretion of calcitonin is limited by the concentration of calcitonin or a precursor, incubations were carried out with volumes of 2 ml and 32 ml (Fig. 2). Most of the calcitonin secreted appeared in the incubation medium within 15 min. Concentration of calcitonin was almost identical with both volumes. To evaluate the ef-

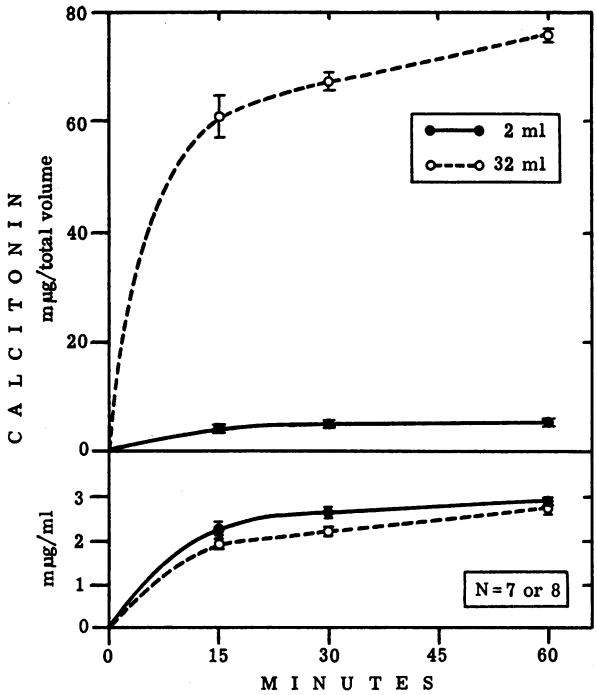

FIGURE 2 The effects of time and of volume of incubation medium on the concentration and secretion of calcitonin by thyroid slices. Tissue from a single thyroid gland was used. Whereas seven or eight flasks were incubated for each time interval for the 2 ml volume, eight flasks were used for the 32 ml volume and 2 ml of the medium was removed at each time interval for assay. Note that most of the secretion occurred within the initial 15 min period, that the concentration of calcitonin at each time interval was almost identical, and that the secretion rate was much higher with the larger volume.

fects of volume further, the volume of incubation medium was increased stepwise from 2 to 32 ml (Table II). As the volume was increased there was a stepwise augmentation in calcitonin secretion which was not accompanied by an increase in calcitonin concentration. Thus, calcitonin secretion can be increased by increasing either the quantity of thyroid tissue incubated or the volume of the incubation medium.

Effects of volume and of concentration of calcium and magnesium on calcitonin secretion. Calcitonin secretion was considerably diminished when calcium was omitted from the incubation medium and showed very little change when the calcium concentration was increased above the normal physiological concentration of 2.5 mEq/liter (Table III). Because of the possibility that effects of calcium might be obscured by inhibition of calcitonin secretion just described, the effects of calcium were determined with an incubation volume of 32 ml. Under these circumstances, calcitonin secretion was greatly enhanced by increases in calcium concentration

TABLE II

Effect of Volume of Incubation Medium on the Secretion and Concentration of Calcitonin

Incubation medium	Calcitonin		n
	Concentration	Secretion	
ml	*mμg/ml*	*mμg/hr*	
2	1.3 ±0.1	2.6 ±0.2	8
4	0.4 ±0.1*	1.7 ±0.2	8
8	0.6 ±0.1*	4.5 ±0.6*	8
16	0.7 ±0.1‡	10.8 ±0.7*	8
32	0.9 ±0.1§	29.0 ±1.7*	8

Results are expressed as mean ±SE. n is number of observations. The concentration of calcium in the incubation medium was 5.0 mEq/liter.

* Represents a significant difference from 2 ml group (P <0.001).

‡ Represents a significant difference from 2 ml group (P <0.01).

§ Represents a significant difference from 2 ml group (P <0.05).

(Table III). The effects of magnesium were similarly studied. With the smaller volume of incubation medium, magnesium produced very little alteration in calcitonin secretion (Table IV). With the larger volume, calcitonin secretion was somewhat diminished without magnesium and significantly augmented only at a very high concentration of magnesium (Table IV). Both calcium and magnesium therefore appear to influence calcitonin secretion in vitro.

Effect of glucagon on calcitonin secretion. Because of

TABLE III

Effect of Calcium Concentration on the Secretion of Calcitonin

[Ca^{++}]	Calcitonin	n	P value*
mEq/liter	*mμg/hr*		
Experiment 1, incubation medium 2 ml			
0	0.9 ±0.1	8	<0.001
2.5	2.8 ±0.3	8	
5.0	3.0 ±0.2	8	NS
7.5	3.2 ±0.3	8	NS
Experient 2, incubation medium 32 ml			
0	13.4 ±1.3	8	<0.05
2.5	20.0 ±2.2	8	
5.0	42.4 ±4.2	8	<0.001
7.5	58.9 ±7.8	8	<0.001

Results are expressed as mean ±SE; NS is not significant; n is number of observations. The two experiments were performed on separate days with slices from two different thyroid glands.

* As compared to [Ca^{++}] of 2.5 mEq/liter.

TABLE IV

Effect of Magnesium Concentration on Secretion of Calcitonin

[Mg^{++}]	Calcitonin	n	*P* value*
mEq/liter	*mμg/hr*		
Experiment, incubation medium 2 ml			
0	9.1 ±1.2	11	NS
1.54	12.6 ±1.7	12	
3.08	12.6 ±1.3	11	NS
4.62	15.2 ±1.2	12	NS
Experiment 2, incubation medium 32 ml			
0	71.0 ±3.7	8	<0.01
1.54	110.1 ±9.6	8	
3.08	121.9 ±4.5	8	NS
4.62	237.4 ±11.2	8	<0.001

Results are expressed as mean ±SE; NS is not significant; n is number of observations. The two experiments were performed on separate days with slices from three different thyroid glands (results from two studies were pooled in experiment 1).

* As compared to [Mg^{++}] of 1.54 mEq/liter.

the observation that the hypocalcemic effect of glucagon is abolished by thyroidectomy (13), the effect of glucagon on calcitonin secretion was evaluated. Glucagon increased calcitonin secretion at concentrations of 10^{-7} mole/liter and above (Table V). Enhancement with glucagon occurred regardless of the initial rate of secretion of calcitonin.

Effect of theophylline, imidazole, and dibutyryl cyclic 3′,5′-adenosine monophosphate on calcitonin secretion. In view of the stimulatory effects of glucagon on adenyl cyclase in other tissues (19, 20), the effects of theophylline, imidazole, and dibutyryl cyclic 3′,5′-adenosine monophosphate were investigated. Imidazole (1×10^{-2} mole/liter), an activator of the enzyme phosphodiesterase which regulates the conversion of cyclic 3′,5′-adenosine monophosphate to adenosine monophosphate (27), did not alter calcitonin secretion significantly (Table VI). Theophylline (1×10^{-3} mole/liter), an inhibitor of phosphodiesterase (27) produced a twofold increase in calcitonin secretion. The increase with theophylline was modestly diminished when imidazole was added to the incubation medium, but the difference was not statistically significant.

Dibutyryl cyclic 3′,5′-adenosine monophosphate at a concentration of 5×10^{-3} mole/liter did not increase calcitonin secretion (Table VI). When the concentration of dibutyryl cyclic 3′,5′-adenosine monophosphate was increased to 1×10^{-2} mole/liter, there was a significant increase in secretion of calcitonin. In both studies calcitonin secretion was augmented by theophylline (1×10^{-3} mole/liter) both alone and in the presence of dibutyryl cyclic 3′,5′-adenosine monophosphate. Thus, secretion of calcitonin can be increased by theophylline, an inhibitor of phosphodiesterase, by dibutyryl cyclic 3′,5′-adenosine monophosphate and by the two together.

TABLE V

Effect of Glucagon on the Secretion of Calcitonin

Treatment	Calcitonin	n	*P* value*
	mμg/hr		
Experiment 1			
Control	1.6 ±0.2	8	
Glucagon, 1×10^{-8} mole/liter	1.6 ±0.1	6	NS
Glucagon, 1×10^{-7} mole/liter	2.6 ±0.2	6	<0.01
Glucagon, 1×10^{-6} mole/liter	2.6 ±0.3	6	<0.05
Glucagon, 1×10^{-5} mole/liter	3.6 ±0.7	6	<0.05
Experiment 2			
Control	12.5 ±0.8	8	
Glucagon, 1×10^{-5} mole/liter	35.6 ±2.7	8	<0.001

Results are expressed as mean ±SE; NS is not significant; n is number of observations. The two experiments were performed on separate days with slices from two different thyroid glands.

* As compared to the control value.

TABLE VI

The Effect of Imidazole, Theophylline, and Dibutyryl Cyclic 3′,5′-Adenosine Monophosphate (DB-CAMP) on the Secretion of Calcitonin

Treatment	Calcitonin	n	*P* value*
	mμg/hr		
Experiment 1			
Control	2.4 ±0.3	8	
Imidazole, 1×10^{-2} mole/liter	3.2 ±0.3	8	NS
Theophylline, 1×10^{-3} mole/liter	6.0 ±0.5	8	<0.001
Imidazole, 1×10^{-2} mole/liter + theophylline, 1×10^{-3} mole/liter	5.3 ±0.3	8	<0.001
Experiment 2			
Control	5.6 ±0.3	8	
DB-CAMP, 5×10^{-3} mole/liter	5.8 ±0.3	8	NS
Theophylline, 1×10^{-3} mole/liter	7.2 ±0.3	8	<0.01
DB-CAMP, 5×10^{-3} mole/liter + theophylline, 1×10^{-3} mole/liter	8.0 ±0.3	8	<0.001
Experiment 3			
Control	2.0 ±0.2	8	
DB-CAMP, 1×10^{-2} mole/liter	4.2 ±0.2	8	<0.001
Theophylline, 1×10^{-3} mole/liter	3.4 ±0.4	8	<0.01
Theophylline, 1×10^{-3} mole/liter +DB-CAMP 1×10^{-2} mole/liter	4.7 ±0.3	8	<0.001

Results are expressed as mean ±SE; NS is not significant; n is number of observations. The three experiments were performed on separate days with slices from three different thyroid glands.

* As compared to the control value.

DISCUSSION

The results of the present studies show that secretion of calcitonin in vitro is regulated in part by the concentra-

tion of calcium and magnesium in the incubation media. The stimulation with magnesium occurred at high and unphysiological concentrations and it is possible that the effects of magnesium may be less important than those of calcium. The enhancement of calcitonin secretion with glucagon, an activator of the adenyl cyclase system in other tissues (19, 20), theophylline, an inhibitor of phosphodiesterase (27), and dibutyryl cyclic 3′,5′-adenosine monophosphate suggests that secretion can be augmented by increasing the concentration of cyclic 3′,5′-adenosine monophosphate in the parafollicular cells of the thyroid gland. These studies with regard to the adenyl cyclase system and calcitonin secretion are in agreement with preliminary findings carried out in vivo (28).

The results of the influence of calcium concentration on calcitonin secretion by thyroid slices support a number of observations obtained in studies carried out in vivo. Production of hypercalcemia in rabbits resulted in a fivefold increase in the concentration of calcitonin in peripheral blood as determined by radioimmunoassay (4, 5). In pigs, hypercalcemia brought about a much more substantial increase in calcitonin concentration in the thyroid venous effluent (29). This discrepancy in the magnitude of the increase in calcitonin secretion with increments in calcium concentration between these in vivo studies and the present findings when an incubation volume of 2 ml was used suggested a limitation in secretion related to concentration of calcitonin in vitro. This possibility was supported by the observation that secretion could be markedly enhanced in vitro without increasing the concentration of calcitonin by enlarging the volume of incubation medium. The effects of calcium and magnesium on calcitonin secretion also became more readily apparent when the volume of incubation medium was increased. It should be noted that inhibition of insulin secretion in vitro by insulin has been demonstrated (30).

On the other hand, both the concentration as well as the secretion of calcitonin could be augmented when the quantity of thyroid tissue was increased. This phenomenon has also been demonstrated with insulin secretion in vitro (31). These observations suggest that there is a minimum rate of hormonal secretion which is related to the quantity of tissue and provide a possible explanation for enhanced hormonal secretion by hyperplastic glandular tissue. For example, persistent hypercalcemia can be produced in rats by implantation of large numbers of isologous parathyroid glands (32).

Glucagon has been shown to lower the serum calcium in both patients (14) and laboratory animals (13, 33, 35). As noted earlier, there is evidence that this effect of glucagon may be mediated in part by stimulation of calcitonin secretion. In dogs, hypocalcemia induced with glucagon is abolished by thyroidectomy (13). In preliminary experiments, glucagon has been shown to stimulate calcitonin secretion (as determined by bioassay) in the pig (12) and in patients with medullary carcinoma of the thyroid gland (34) which secretes calcitonin. On the other hand, glucagon also produces hypocalcemia in rats after thyroparathyroidectomy (33, 35). In tissue culture experiments, glucagon, like calcitonin (7), inhibits the release of ^{45}Ca from embryonic bone induced with parathyroid extract (35). Thus, glucagon appears to act to lower the serum calcium both by stimulation of calcitonin release as well as by direct inhibition of bone resorption.

The mechanism by which calcium stimulates calcitonin secretion has not been determined by the present experiments. It should be noted that insulin secretion which is related to the adenyl cyclase system (15), is inhibited by magnesium and stimulated by calcium (36). However, in other tissues such as bone, for example, calcium at high concentrations is an inhibitor of adenyl cyclase (37). It is, therefore, possible that the stimulatory effects of calcium on secretion of calcitonin may be independent of the adenyl cyclase system. This possibility can only be clarified when effects of calcium on the components of the adenyl cyclase system in the cells which secrete calcitonin are determined.

There was some variability between thyroid glands in the rate of calcitonin secretion when the glands were obtained on different days but not when they were obtained on the same day. These findings suggest that it is the physiologic status of the animal at the time of sacrifice rather than the number of parafollicular cells which determines the rate of secretion. In any event when stimulation occurred with a given stimulus, glucagon, for example, it was elicited at both low and high initial rates of calcitonin secretion.

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