

Characterization of specific receptors for calcitonin in porcine lung

(membranes/binding/specificity)

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ABSTRACT The binding of salmon calcitonin was investigated in subcellular fractions obtained from normal porcine lung. Only the membrane fraction (density, 1.14 g/cm³) showed specific binding for calcitonin. Specific binding of ¹²⁵I-labeled salmon calcitonin was competitively inhibited by concentrations of unlabeled homologous hormone in the range 0.01–1 nM. Half-maximal inhibition of binding was observed with 0.12 nM salmon calcitonin. Scatchard analysis of the data suggested the presence of one class of binding sites with a mean affinity constant of $0.9 \times 10^{10} \text{ M}^{-1}$ and a mean receptor number of $40 \times 10^8/\text{mg}$ of protein. The binding of salmon calcitonin was highly specific; half-maximal inhibition of binding was observed with 63.8 nM bovine calcitonin, the hormone corticotropin having no effect in this system.

In both higher and lower vertebrates, calcitonin has been shown to protect "the milieu intérieur" from an increase in calcium. In mammals, the hormone inhibits bone resorption (1). In fishes, it decreases the influx of calcium from calcium-rich sea water through the gill (2) and enhances the efflux out of the gill (3).

Specific binding sites for calcitonin have been demonstrated in rat bone and kidney membranes (4). The biological effect of this hormone has been shown to be mediated by adenylate cyclase in the plasma membranes of target cells: calcitonin causes an increase in the concentration of cyclic AMP in renal tubules (5) and fetal calvaria incubated *in vitro* (6). Its stimulation of cyclic AMP production in a cell line isolated from a human lung squamous cell carcinoma was reported (7). However, it was not expected that normal lung might also possess a calcitonin receptor.

In fishes, calcitonin receptors have been recently demonstrated in gill cells of the trout (*Salmo gairdneri*) (8), giving support to the hypothesis that this hormone may be involved in water balance and osmoregulation. This study pointed out the importance of the hormone in regulating ion or gaseous exchange in fishes and engendered the present investigation of the presence of binding sites for calcitonin in the similarly functional mammalian lung. These studies suggest a role for calcitonin in regulating the metabolic functions of the mammalian lung.

MATERIALS AND METHODS

Hormones and Chemicals. Synthetic salmon calcitonin, 2000 international units/mg, was a generous gift of Sandoz Laboratories. Bovine calcitonin (0.15 international unit/mg), porcine corticotropin (ACTH; 69 international units/mg), and sodium AMP were purchased from Sigma. Carrier-free Na¹²⁵I (17 Ci/mg; 1 Ci = 3.7×10^{10} becquerels) was obtained from New England Nuclear. Bovine serum albumin from Merck was heat-

inactivated before use. All other chemicals were of reagent grade.

Preparation of Lung Membranes. Membranes were prepared from fresh porcine lung that was obtained from a local slaughterhouse, transported to the laboratory on ice, and used immediately. The tissue was dissected free from all visible bronchi, and membranes were prepared according to the procedure of Sidhu and Michelakis (9). Purified membranes were suspended in 10 mM Tris·HCl buffer at pH 7.5, centrifuged, and stored at –80°C in small portions. Protein concentrations were determined by the method of Lowry *et al.* (10). The activity of 5'-nucleotidase, a usual membrane marker, was assayed according to the procedure of Emmelot and Bos (11). The inorganic phosphate liberated in this assay was determined by the method of Le Deaut *et al.* (12).

Analytical Procedure. Binding studies were carried out with salmon calcitonin labeled with ¹²⁵I at a specific activity of 200 Ci/g (7, 13). In a standard binding assay, membranes (0.2 mg of protein per ml) were incubated at 10°C with a fixed quantity of ¹²⁵I-labeled calcitonin (20 pM) with or without increasing amounts of native calcitonin (0.05–1.4 nM) in 0.5 ml of 50 mM Tris·HCl, pH 7.5/47 mM NaCl/2 mM KCl/0.48 mM MgSO₄/0.48 mM KH₂PO₄/4 mM Na₂HPO₄ containing 2% (wt/vol) bovine serum albumin and 1000 international units of kallikrein inhibitor (Zymofren, Specia) per ml. After 3 hr of incubation at 10°C, cell-bound peptide was separated by centrifugation (14) and quantified as described (8). Data are reported as specific binding; this is obtained by subtracting from the total that amount of labeled peptide that was not displaced by an excess of native peptide (2.8 nM).

The integrity of the ¹²⁵I-labeled salmon calcitonin in the incubation medium after exposure to lung membranes was estimated by QUSO-G 32 adsorption (8, 15). Appropriate controls without membranes represented 100% of the substrate available for degradation.

RESULTS

Characteristics of Porcine Lung Membranes. The 12,000 × g pellet obtained from porcine lung was fractionated by non-linear sucrose gradient centrifugation (96,000 × g for 150 min). Three bands appeared (I, $\rho = 1.09 \text{ g/cm}^3$; II, $\rho = 1.14$; III, $\rho = 1.16$) plus a pellet ($\rho = 1.20$). When the binding of the biologically functional ¹²⁵I-labeled salmon calcitonin was measured in these three subcellular fractions, only band II was capable of high specific binding (Table 1). This band showed a 2-fold enrichment in 5'-nucleotidase activity compared to band III and corresponds to fraction PM-I (plasma membranes) (9).

Interaction of Calcitonin with Lung Membranes. The binding of ¹²⁵I-labeled salmon calcitonin to lung membranes was dependent on time and temperature (Fig. 1). The reaction was

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Abbreviation: ACTH, corticotropin.

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Table 1. Specific binding of ^{125}I -labeled calcitonin and 5'-nucleotidase activity in porcine lung fractions

Fractions from 12,000 \times g pellet	^{125}I -Labeled salmon calcitonin binding*	5'-Nucleotidase†
12,000 \times g pellet	0.94	—
Band 1	0.56	—
Band 2	10.50	0.40
Band 3	0.00	0.20
Pellet	2.20	—

Each value is the mean of triplicate determinations. Similar results were obtained with two other membrane preparations.

* Specific binding of ^{125}I -labeled calcitonin (0.02 nM) was measured after 2 hr of incubation at 20°C in the presence of 0.20 mg of protein per ml. Nonspecific binding, determined in the presence of 27.7 nM unlabeled salmon calcitonin, has been subtracted. Results are shown as (bound/total) per mg of protein.

† 5'-Nucleotidase activity was measured at 37°C after 15 min of incubation at 0.2 mg of protein per ml in the presence of 5 mM AMP (11, 12). Results are shown as $\mu\text{mol P}_i$ released in 15 min per mg of protein.

more rapid at 20°C, reaching a peak at 30 min; the decline that followed may be attributed to the inactivation of both the peptide and the receptor. At 10°C, an apparent equilibrium of bound calcitonin was reached in 2 hr of incubation and maintained for 2 hr. Therefore, subsequent experiments were performed after 3 hr of incubation at this temperature.

The binding of ^{125}I -labeled salmon calcitonin was a linear function of membrane protein concentration over a 5-fold range (Fig. 2). This included the concentrations used in the binding experiments (0.15 mg/ml) and permits normalization of the binding data on the basis of membrane protein concentration.

The binding of ^{125}I -labeled salmon calcitonin was competitively inhibited by native calcitonin in the range 0.05–1.4 nM (Fig. 3). Half-inhibition of the initial binding of ^{125}I -labeled salmon calcitonin (i.e., in the absence of native calcitonin) was observed with 0.12 ± 0.02 nM (SEM; $n = 4$) native calcitonin, indicating the high affinity of calcitonin to lung membranes.

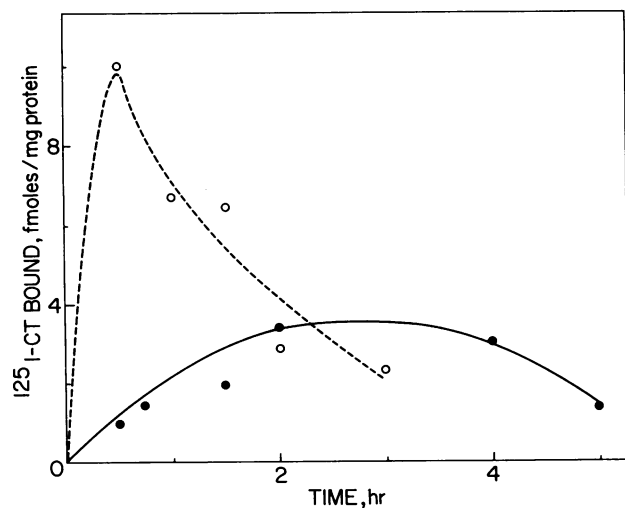


FIG. 1. Time-course of specific binding of ^{125}I -labeled calcitonin to porcine lung membranes. ^{125}I -Labeled calcitonin (0.02 nM) was mixed with protein (0.2 mg/ml) and incubated at 20°C (○) or 10°C (●) in the absence and in the presence of unlabeled homologous hormone to assess nonspecific binding. At indicated times, bound calcitonin was separated from the free hormone by centrifugation. Each point represents the mean of triplicate determinations.

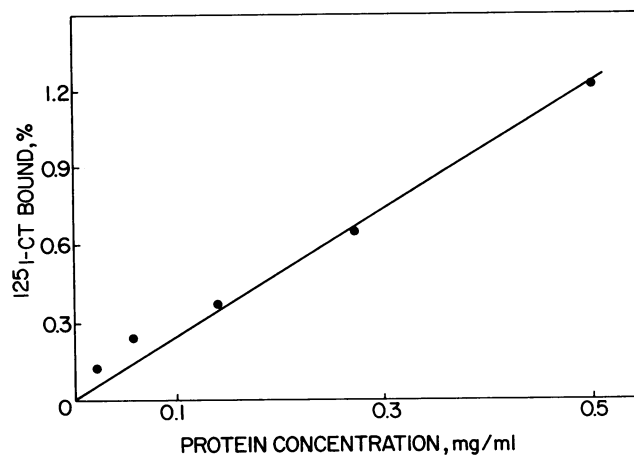


FIG. 2. Specific binding of ^{125}I -labeled calcitonin as a function of membrane concentration. Incubations were carried out for 3 hr at 10°C with ^{125}I -labeled calcitonin (0.02 nM) and varying membrane concentrations. Data represent the mean of triplicate determinations.

The interaction between calcitonin and its specific binding sites in lung membranes was quantitated. Calcitonin binding sites were saturable. At a hormone concentration sufficient for saturating the calcitonin binding sites [0.2 nM (Fig. 4 *Left*)], the amount of bound calcitonin [4.2 ± 2.1 fmol/mg protein ($n = 4$)] corresponded to about $25.3 \pm 12.6 \times 10^8$ molecules per mg of protein. When these data were analyzed by the method of Scatchard (16), a straight line was obtained, suggesting the presence of one class of binding sites (Fig. 4 *Right*). The affinity constant corresponds to $0.9 \pm 0.09 \times 10^{10} \text{ M}^{-1}$; the number of binding sites per milligram of protein is $40.0 \pm 16.8 \times 10^8$ ($n = 4$). The specificity of the calcitonin binding in lung membranes was investigated by testing the ability of bovine calci-

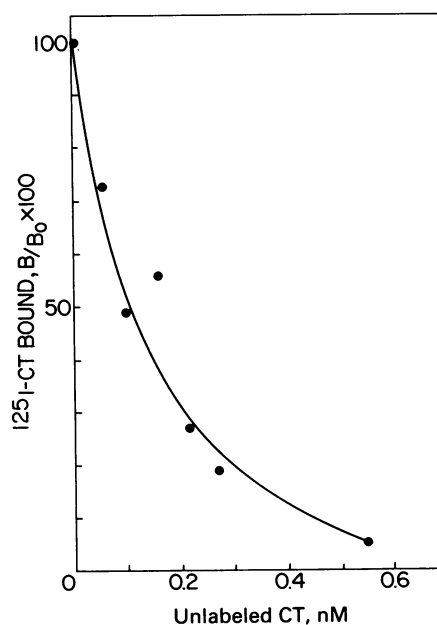


FIG. 3. Competitive inhibition of the specific binding of ^{125}I -labeled calcitonin to lung membranes. ^{125}I -Labeled calcitonin (0.02 nM) and unlabeled calcitonin at the concentrations indicated were incubated for 3 hr at 10°C with 0.2 mg of protein per ml. Results are represented as % of maximal binding—i.e., the binding of ^{125}I -labeled calcitonin in the absence of native calcitonin. Initial binding represented 4.9% of the total ^{125}I -labeled calcitonin. Each point is the mean of four separate experiments with triplicate determinations in each.

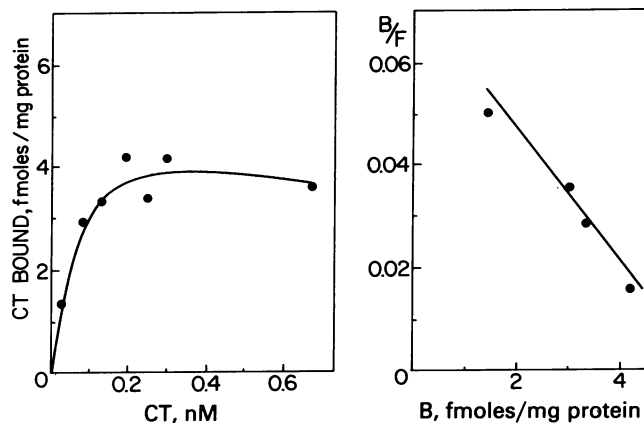


FIG. 4. Effect of unlabeled calcitonin on ^{125}I -labeled calcitonin binding to lung membranes. (Left) Data of Fig. 3 utilized for calculating the absolute amounts of bound calcitonin for each hormone concentration. (Right) Data expressed according to the method of Scatchard—i.e., the bound-to-free ratio of ^{125}I -labeled calcitonin (B/F) plotted as a function of the quantity of calcitonin bound (B) to membranes.

tonin and ACTH to displace bound ^{125}I -labeled salmon calcitonin (Fig. 5). ACTH was inactive; bovine calcitonin had about 1/500th the activity of salmon calcitonin. The dose for 50% inhibition was 63.8 nM for bovine calcitonin and 0.12 nM for salmon calcitonin. ^{125}I -Labeled salmon calcitonin (0.02 nM) exposed to the membranes at 10°C was recovered from the incubation medium and tested for degradation by using QUSO-G 32. No degradation of labeled calcitonin occurred during the 3-hr incubation at 10°C (Table 2).

DISCUSSION

This work establishes the presence of receptors for calcitonin in normal lung. The binding is time- and temperature-depen-

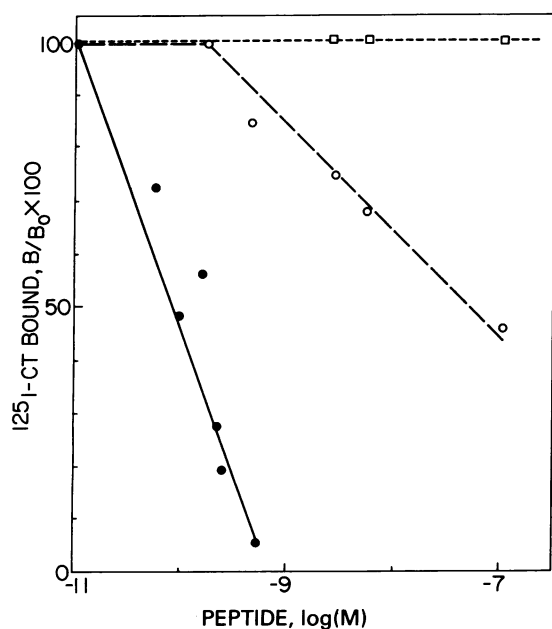


FIG. 5. Effect of unlabeled salmon calcitonin (●), bovine calcitonin (○), and ACTH (□) on the binding of ^{125}I -labeled calcitonin to lung membranes. Experimental conditions were as in Fig. 3. Results expressed as % of initial binding (B_0) of the labeled hormone were plotted as a function of unlabeled peptide concentration on a semilogarithmic scale. Each point represents the mean of triplicate determinations.

Table 2. Behavior of ^{125}I -labeled calcitonin present in the medium after exposure to porcine lung membranes

Incubation, $^\circ\text{C}$	% undegraded calcitonin				
	30 min	60 min	120 min	180 min	240 min
20	101.6	102.3	104.2	99.1	99.1
10	106.2	102.1	99.2	94.6	91.8

^{125}I -Labeled calcitonin (0.02 nM) was exposed to 0.20 mg of protein per ml at 20°C or 10°C . Membranes were collected by centrifugation and aliquots of the supernatant were analyzed by QUSO adsorption (15). Results are expressed as % of controls—i.e., ^{125}I -labeled hormone incubated in the medium without membranes.

dent, specific, and saturable according to the usual criteria for hormone-receptor interaction. Our results are consistent with the recently reported (7) presence of calcitonin binding sites in a human lung cancer line where only one type of binding site also was observed. Nevertheless, the calcitonin behavior toward its receptor sites in lung appears somewhat different from that observed in other higher vertebrate tissues—namely, rat and calvaria membranes (17) and human lymphocytes (18)—in which Scatchard analysis of the data suggests the presence of two classes of receptor sites. However, the affinity constant for the interaction of calcitonin with its specific receptors in lung appears to be identical to that reported for the high-affinity low-capacity site in rat kidney membranes (17) and 1 order of magnitude lower than that in rat calvaria membranes (17).

When the results obtained in the present study are compared to those reported for gill (8), a tissue with the same function as lung, the same differences were obtained in the Scatchard analysis of the binding data. However, the affinity constant, lower in lung membranes (10^{10} M^{-1}) than in gill cells (10^{11} M^{-1}), suggests the importance of this hormone in regulating gill function.

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