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Calcitonin Receptors of Kidney and Bone

Abstract. *Receptors for calcitonin, determined by activation of adenylate cyclase, were found in a distribution among zones of the kidney distinct from that of receptors for parathyroid hormone or vasopressin. Competitive binding studies showed that the receptors for calcitonin are similar in kidney and bone and that their high apparent affinity for salmon calcitonin accounts in part for the high biological potency in vivo of salmon calcitonin.*

Calcitonin, the polypeptide hormone discovered as a factor causing hypocalcemia in rats, has been identified throughout the vertebrate phylum (*1*).

In mammals, the hormone inhibits skeletal resorption (*1*) and influences renal function as well, causing natriuria, phosphaturia, and calciuria (*2*). We have compared the renal and skeletal receptors for the hormone by determining hormone-activated adenylate cyclase and binding of salmon calcitonin labeled with ^{125}I with cell membranes prepared from kidneys and bones of rats. The receptors for calcitonin were found to be similar in the two tissues. Salmon calcitonin, of all the species of the hormone tested, showed the highest apparent affinity for the receptors in both target tissues. This high affinity must account in part for the high potency of salmon calcitonin in vivo relative to the activity of mammalian species of the hormone.

The species of the hormone tested were: synthetic salmon calcitonin; synthetic human calcitonin M, the sulfoxide analog, and the (11-32)-amide fragment; purified porcine calcitonin [96 Medical Research Council units (MRCU) per milligram]; and bovine (60 MRCU/mg) and ovine (70 MRCU/mg) calcitonins, which were purified by published techniques (*3*). Renal plasma membranes were isolated from male Sprague-Dawley rats weighing 150 to 200 g by a method that has been described (*4*). Renal tissue was homogenized in a solution of 0.25*M* sucrose, 0.01*M* tris(hydroxymethyl)-aminomethane (tris), and 0.001*M* ethylenediaminetetraacetic acid (EDTA), at 4°C and *p*H 7.5, by using a loosely fitting Dounce homogenizer. A crude membrane fraction was obtained by differential centrifugation at 2200*g* in the same buffer and layered onto a continuous gradient of 32 to 42 percent sucrose (by weight) in 0.01*M* tris and 0.001*M* EDTA at *p*H 7.5. After centrifugation for 90 minutes at 100,000*g* at 4°C, the band appearing at a sucrose concentration of 38 percent was aspirated; aliquots were centrifuged and the pellets were stored under liquid nitrogen. Membranes from fetal rat calvaria were obtained by a modification of the method used to prepare skeletal adenylate cyclase (*5*). The tissue was powdered, while frozen, in a stainless steel mortar. The preparation was then homogenized in a solution containing 0.25*M* sucrose, 0.05*M* tris, and 0.02*M* EDTA at *p*H 7.5 by using an electrically driven rotating Teflon pestle. The homogenate was filtered through glass wool and centrifuged for 15 minutes at 2200*g* at 4°C. This crude membrane fraction was resuspended in 0.25*M* sucrose, 0.01*M* tris, and 0.001*M* EDTA at *p*H 7.5, centrifuged in aliquots, and stored as pellets under liquid nitrogen. Adenylate cyclase was assayed under the conditions given in the legend of Fig. 1. Synthetic calcitonin (salmon) was labeled with ^{125}I by a modification of the method of Hunter and Greenwood (*6*) to specific activities of 300 to 600 μc/μg. Binding studies were carried out for 4 hours at 22°C in 0.05*M* tris at *p*H 7.5; the solution contained 2 percent heat-inactivated albumin. Separation of the membrane-bound hormone was accomplished by

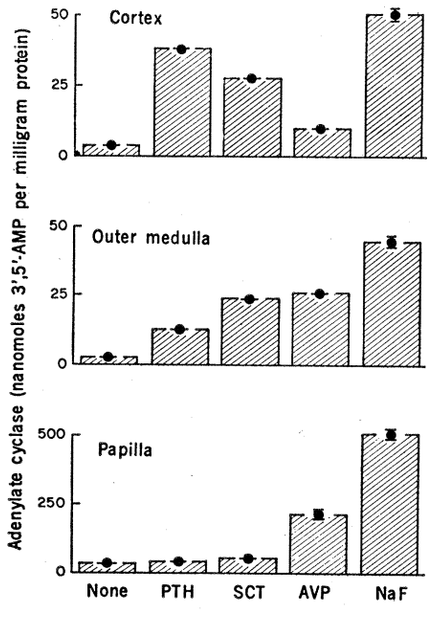

Fig. 1. Adenylate cyclase in membranes from three zones of the rat kidney. The kidneys were dissected into cortical, red (outer) medullary, and white medullary (papilla) zones and fractionated as described in the text. Adenylate cyclase was assayed at 22°C for 30 minutes in 50 m*M* tris, 0.013 percent bovine serum albumin, 30 m*M* KCl, 4.5 m*M* $MgCl_2$, 1.1 m*M* ATP, 4 m*M* creatinine phosphate, 10 μg creatine phosphokinase, and 9 m*M* theophylline, at *p*H 7.5; the total volume was of 70 μl. The ^{32}P-labeled cyclic 3',5'-adenosine monophosphate (AMP) that was formed was determined as described previously (*16*). The additives and their final concentrations were: parathyroid hormone (PTH) (1500 U.S. Pharmacopeia units per milligram), 20 μg/ml; salmon calcitonin (SCT) (4200 MRCU/mg), 200 ng/ml; arginine vasopressin (AVP) (60 international units per milligram), 800 ng/ml; sodium fluoride, 7 m*M*. The points represent the mean ± 1 S.E. of three determinations for the cortex and outer medulla, or the average and range of two determinations for the papilla. All three hormones and fluoride caused significant ($P < .001$) stimulation of the enzyme prepared from the cortex or outer medulla. In membranes from the outer medulla SCT caused greater activation ($P < .001$) than PTH, whereas the latter caused greater hormonal activation in the cortical preparation ($P < .001$).

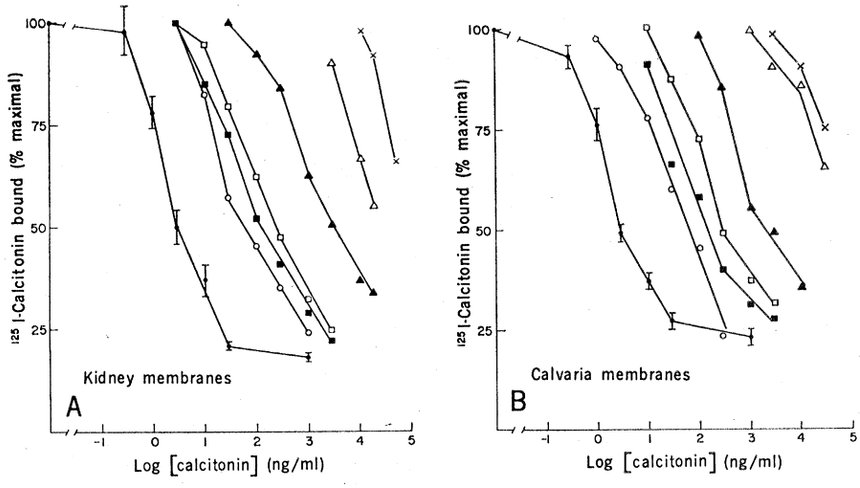

Fig. 2. Competitive binding experiments in which renal (A) or calvarial (B) membranes were used. [^{125}I]Calcitonin (salmon) was mixed with calcitonin analogs and added to membranes to give the final concentrations indicated in a volume of 500 μl. After a 4-hour incubation at 22°C the amount of [^{125}I]calcitonin bound was determined for each experiment with triplicate 100-μl aliquots; the data represent the means of two or more separate experiments. Each point for salmon calcitonin represents the mean ± 1 S.E. of four to ten experiments. (Closed circles) Salmon calcitonin, (open circles) porcine calcitonin, (closed squares) ovine calcitonin, (open squares) bovine calcitonin, (closed triangles) human calcitonin M, (open triangles) human calcitonin M (11-32)-amide, (crosses) human calcitonin M sulfoxide.

the method of Rodbell *et al.* [see *(7)*].

The anatomic distribution of hormone-sensitive adenylate cyclase was analyzed by dissecting kidneys into cortical, red (outer) medullary, and white medullary (papilla) fractions. Membranes from each fraction were then isolated by the procedure described above. Anatomic separation of the parathyroid hormone and vasopressin-sensitive adenylate cyclases (*8*) was confirmed (Fig. 1). The calcitonin-sensitive adenylate cyclase was distributed differently from either of the above. These studies suggested that calcitonin acts on a cell population distinct from that sensitive to parathyroid hormone or vasopressin, but did not exclude partial overlap of cell types containing receptors for the three hormones. The relative specific activity (when compared to basal, parathyroid hormone, or fluoride-stimulated activity) was highest for the calcitonin-activated enzyme in membranes prepared from the outer (red) medulla. It is of interest that this anatomic area also shows the highest specific activity for Na,K-adenosine triphosphatase (*9*). This zone contains elements from the proximal portions of deep cortical nephrons, the thick portion of the ascending limb of the loop of Henle, and the collecting ducts, as well as interstitial, vascular, and neural cells (*10*). Further experiments would be required to determine whether the Na,K-adenosine triphosphatase and the calcitonin-activated adenylate cyclase are derived from the same cell population.

A series of calcitonins and calcitonin analogs were tested by competition against [^{125}I]calcitonin (salmon) for uptake by membranes prepared from undissected rat kidney. Salmon calcitonin was the most effective polypeptide tested; mammalian calcitonins were half-maximally effective at concentrations at least ten times higher (Fig. 2A). Competition studies in which membranes prepared from fetal rat calvaria were used produced dose-response curves similar to those obtained with renal membranes (Fig. 2B). Parathyroid hormone (30 μg/ml), adrenocorticotropin (30 μg/ml), growth hormone (30 μg/ml), and zinc-free insulin (60 μg/ml) had no effect on the binding of [^{125}I]calcitonin to renal or calvarial membranes. Fat cell ghosts and plasma membranes isolated from the adrenal gland or liver of the rat showed no specific binding of [^{125}I]calcitonin (*11*).

The effect of calcitonin M sulfoxide, virtually devoid of hypocalcemic activity in vivo (*12*), might be attributable to residual traces of unoxidized hormone. The effect of the (11-32)-amide synthetic fragment, however, was unexpected because it had appeared completely inert in vivo (*13*). Perhaps a shortened biological half-life accounts for the lack of effect in vivo; alternatively, this fragment might compete for receptors without activating the biological response. Similar discrepancies between binding in vitro and activity in vivo have been described for the hormone derivatives deshistidyl glucagon and desialylated gonadotropin (*14*).

The high biological effectiveness of salmon calcitonin relative to mammalian calcitonins has been related to a longer half-life in vivo and resistance of the hormone to degradation by plasma or tissue extracts (*15*). Our work shows that another factor, the high apparent affinity of salmon calcitonin for specific tissue receptors, must contribute importantly to the high potency of the hormone in vivo. The series of calcitonin analogs we studied gave relative affinities that were virtually the same with membranes from either kidney or bone. Thus, it is likely that the molecular structures of the membrane-receptor complexes in the two tissues are similar, if not identical.

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Thymus-Dependent and Thymus-Independent Lymophocyte Separation: Relation to Exposed Sialic Acid on Cell Surface

Abstract. *On preparative cell electrophoresis mouse lymph node lymphocytes separate into fast-moving (T, thymus-dependent) and slow-moving (B, thymus-independent) fractions. After treatment with neuraminidase all lymphocytes move as one very slow fraction, indicating that the difference in the mobility of the two kinds of cells is due to differences in the density of exposed sialic acid on their surfaces.*

Mouse lymphocytes from peripheral lymphatic organs can be separated by preparative free-flow electrophoresis into two populations (*1*, *2*). Zeiller *et al.* (*1*) studied the cooperation of separated cells in mounting an immune response in lethally irradiated mice and concluded that the low-mobility cells are B (thymus-independent) lymphocytes and the high-mobility cells are T (thymus-dependent) cells, but that B cells are present in the high-mobility fraction of spleen cells. We have characterized the separated cells both serologically with antiserum to theta (θ) antigen (*3*) and antiserum to mouse specific bone marrow derived lymphocyte antigen (MBLA), made in rabbits (*4*), and functionally by testing in vivo the graft-versus-host reaction and assaying in vitro for the presence of cytotoxic effector cells, plaque- and rosette-forming cells, and phytohemagglutinin-responding cells (*2*). Our conclusion was that the high-mobility cells are mainly T lymphocytes and the low-mobility cells mainly B lymphocytes, with no detectable overlap at the peaks (*2*). Furthermore, Bert *et al.* have shown that the mean electrophoretic mobility of mouse blood lymphocytes is reduced when the cell donor animal is first treated with antilymphocyte serum (*5*). Zeiller *et al.* (*6*) have demonstrated in the rat that the low-mobility cells are antibody-producing cells, whereas lymphocytes in the high-mobility fraction are able to induce graft-versus-host reactions.

Table 1. Effect of antiserum to θ and antiserum to MBLA on electrophoretically separated neuraminidase-treated C-SSI lymph node lymphocytes. The antiserums were tested in the presence of complement.

Lymphocyte	Peak	Cytotoxic index* with antiserum to	
		θ	MBLA
Unseparated		0.63	0.24
Untreated	Fast	0.91	0
Untreated	Slow	0	0.60
Neuraminidase treated	†	0.58	0.28

* The cytotoxic index = (% killed with antiserum − % killed with control serum)/(100 − % killed with control serum). The trypan blue dye exclusion test was used. Antiserums were assayed at "plateau" level (antiserum to θ, 1 : 8; antiserum to MBLA, 1 : 4). Guinea pig complement (C′) was used at a dilution of 1 : 10. For controls, AKR serum plus C′ was used for antiserum to θ and normal rabbit serum plus C′ was used for antiserum to MBLA. † Only peak.

Sialic acid is quantitatively the most important identified anionic group on the surface of most types of cells (*7*). We now report that differences in the number of sialic acid groups exposed on the surface of lymphocytes are responsible for their different electrophoretic mobilities.

Cell suspensions of lymphocytes were prepared from the lymph nodes of random-bred C-SSI white mice (*2*). Without further treatment, one lot of lymphocytes was separated by preparative electrophoresis. A second lot was treated with *Vibrio cholera* neuraminidase (Behringwerke, Marburg am Lahn, Germany) at 37°C for 30 minutes prior to electrophoresis. The lymphocytes were exposed at a concentration of 10^8 cells per milliliter to the enzyme solution, which contained 50 units of enzyme per milliliter in RMPI (Roswell Park Memorial Institute) 1640 medium, *p*H 7.2. Since this *p*H is well above the optimum (*p*H 5.5) for neuraminidase activity, we made the tests with bovine submaxillary mucin as substrate, and found that at *p*H 7.2 at least 50 percent of the neuraminidase activity is retained. The neuraminidase preparation was also tested for possible contamination with proteolytic enzymes (*8*) and with ribonucleases (*9*), which are known to alter the electrophoretic mobility of some cells (*10*). Contamination could not be detected. Prior to electrophoresis, a third lot of lymphocytes was treated in RPMI medium only. Electrophoretic separation was effected on a free-flow electrophoresis apparatus (*11*) (also commercially available as Model FF4 Desaga, Heidelberg, Germany) in a buffer of low ionic strength [as described by Zeiller *et al.* (*6*) with minor modifications (*2*)] in an electric field of 100 volt/cm.

Figure 1 shows the distribution profiles of the three lymphocyte preparations. The curves have been resolved into Gaussian distributions (shaded areas) by a semigraphic method (*12*). The mean mobilities and standard deviations were also determined by this method. Both untreated lymphocytes

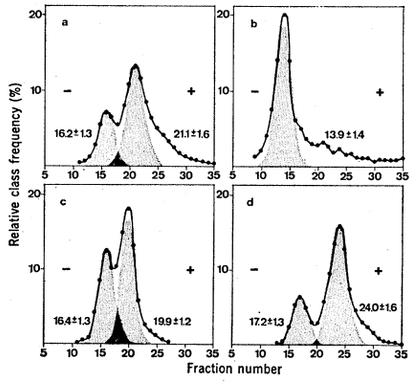

Fig. 1. Distribution profiles of electrophoretically separated lymph node cells from C-SSI mice: (a) untreated lymphocytes, (b) lymphocytes treated with *Vibrio cholera* neuraminidase, (c) lymphocytes treated with RPMI 1640, and (d) lymphocytes treated with *V. cholera* neuraminidase and EDTA at 4°C. Shaded areas represent the Gaussian distributions. The numbers indicate the mean mobility values ± standard deviations. The numbering of the fractions starts from the injecting point of the cell suspension and increases toward the anode (+).