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* Currently on leave from the Department of Biology and Institute of Molecular Biology, University of Oregon, Eugene 97403.

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Interaction of Calcitonin and Calcitonin Gene–Related Peptide at Receptor Sites in Target Tissues

Abstract. *Discrete receptor sites for calcitonin (CT) and calcitonin gene–related peptide (CGRP) were found in the nervous system and in peripheral tissues. Each peptide was capable of cross-reacting with the specific receptor of the other. In contrast to CT receptors, CGRP receptors were not linked to adenylate cyclase. However, CGRP could stimulate adenylate cyclase in CT target tissues apparently by interacting with CT receptors. The relative abilities of CGRP and mammalian CT to inhibit CT binding suggest that CGRP could serve as an endogenous ligand for CT receptors in the central nervous system.*

Distinct messenger RNA's encode the hormone calcitonin (CT) in the C cells of the rat thyroid, and a novel peptide, calcitonin gene–related peptide (CGRP) within the rat nervous system (*1*). Based on complementary DNA (cDNA) sequence analysis, CGRP was predicted to be a peptide (37 amino acids in length) with a 2,7-disulfide bridge and a phenylalanine amide at the carboxyl terminus (*1*). RNA blotting techniques and immunochemical studies have revealed the production of CGRP messenger RNA (mRNA) and CGRP in multiple locations within the nervous system (*1*). Calcitonin is a peptide (32 amino acids in length) with a 1,7-disulfide bridge and a proline amide at the carboxyl terminus. Although immunochemical studies have suggested that this peptide occurs within the nervous system (*2*), CT mRNA has not been revealed in neural tissue by hybridization with specific calcitonin cDNA probes (*1*). Despite the similar size of calcitonin and CGRP, and the presence in both peptides of a disulfide ring within the amino terminal region and an amide group at the carboxyl terminus, little sequence homology exists; furthermore, specific antisera to the CGRP sequence fail to recognize synthetic calcitonin (*1*). Specific binding sites have been demonstrated for CT in kidney, bone, other peripheral tissues (*3*), and the central nervous system (*4*); binding of CT to its receptor is associated with stimulation of adenylate cyclase. We now report on the distribution of receptor sites for CGRP, relationship of these receptors to the adenylate cyclase system, and the interaction of CGRP and CT at their respective sites in target tissues.

Male Sprague-Dawley rats (150 to 200 g), were decapitated and the brains, cervical and thoracic spinal cords, pituitary glands, adrenal glands, and kidneys were removed. The brains were frozen on dry ice, and discrete regions were macroscopically dissected. Pituitary glands, adrenal glands, and fractions of brain tissue were homogenized and prepared for analysis as described (*4*). Portions of each tissue containing 1 to 5 mg of protein were immediately frozen in dry ice and stored at −70°C. Purified renal membranes were prepared from dissected kidneys by differential centrifugation and ultracentrifugation (*5*), and skeletal tissue was prepared from the calvariae of 20-day-old Sprague-Dawley rat fetuses (*3*).

The salmon (s) CT and rat (r) CGRP (*6*) were each labeled with [^{125}I]NaI by the chloramine-T method (*7*) to a specific activity of 500 Ci/mmol, and purified (*4*). For binding studies, ^{125}I-labeled peptide (70×10^3 count/min) was incubated with or without unlabeled peptide in 0.5 ml of a buffer solution of 0.05*M* tris-HCl (*p*H7.5) and bovine serum albumin (20 g/liter) containing 200 μg of tissue protein (*8*). At the end of the incubation period, triplicate portions (100 μl) were each layered onto 200 μl of chilled assay buffer in a microcentrifuge tube kept at 4°C. Bound ^{125}I-labeled peptide was obtained after centrifugation (*4*), and the radioactivity was counted in a Packard Tricarb γ-spectrometer (Packard Corporation) at an efficiency of 50 percent.

For measurement of adenylate cyclase activity, the incubation mixture contained, in a 100-μl volume, 25 m*M* tris-HCl (*p*H 7.4), 0.1 m*M* adenosine triphosphate (ATP), [γ-^{32}P]ATP (0.8 to 2×10^6 count/min), 9 m*M* theophylline, 2 m*M* $MgCl_2$, bovine serum albumin (1 mg/ml),

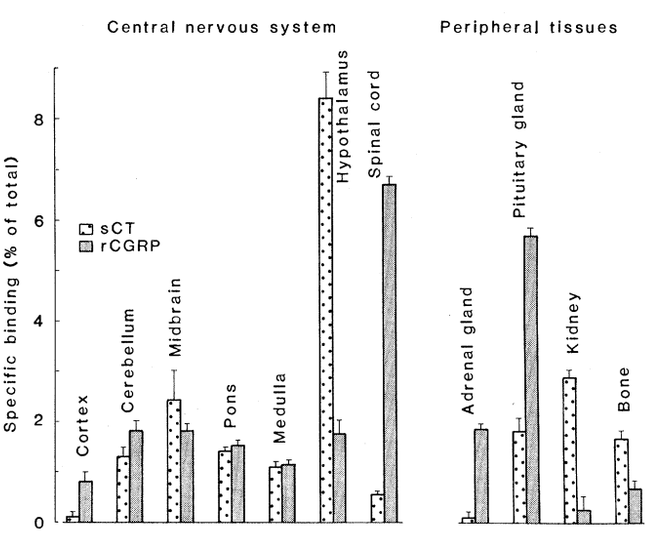

Fig. 1. Distribution of CT receptors and of CGRP receptors in the nervous system and in peripheral tissues of the rat. For assessment of CT receptors ^{125}I-labeled sCT was incubated with individual tissues in the absence or presence of excess unlabeled sCT (10^{-5}*M*). Specific binding represents the numerical difference between binding of the radioactive tracer in the absence of unlabeled sCT and binding not inhibited by excess sCT. Results are expressed as a percentage of added ^{125}I-labeled sCT specifically bound. For assessment of CGRP receptors similar studies were performed with ^{125}I-labeled rCGRP and excess unlabeled rCGRP. Each bar represents the mean ± standard error of triplicate determinations.

and an ATP-regenerating system consisting of 5 m*M* creatine phosphate (Boehringer-Mannheim), creatine phosphokinase (0.1 mg/ml), and approximately 20 to 50 μg of tissue protein. Peptides were added in 10-μl volumes and incubations were initiated by addition of tissue and continued for 30 minutes at 30°C. Reactions were terminated by addition of 100 μl of a solution containing sodium dodecyl sulfate (20 mg/ml), 1 m*M* unlabeled adenosine 3′, 5′-monophosphate (cyclic AMP), 10 m*M* ATP, and [^{3}H]cyclic AMP (about 15,000 count/min) in 50 m*M* tris-HCl *p*H 7.4, and by immediately boiling the mixture for 3 minutes. The ^{32}P-labeled cyclic AMP formed was isolated as described (*9*).

The distribution of specific binding of ^{125}I-labeled rCGRP and of ^{125}I-labeled sCT differed both in the nervous system and in peripheral tissues (Fig. 1). Thus, maximal specific binding of rCGRP was found in the spinal cord, with lesser amounts in the pituitary and adrenal glands. Significant binding was also observed in other regions of the brain, including the midbrain and cerebellum. In contrast, maximal binding of CT occurred in the hypothalamus with lesser amounts in the midbrain. Minimal, or no, sCT binding was demonstrable in the spinal cord or adrenal gland, whereas minimal, or no, rCGRP binding was seen in the bone and kidney, which are known calcitonin target tissues. In the kidney, which contained high-affinity binding sites for sCT (half-maximal inhibition of binding of ^{125}I-labeled sCT by $10^{-9}M$ sCT), rCGRP inhibited binding of ^{125}I-labeled sCT approximately as well as human (h) CT (half-maximal inhibition of binding by $10^{-6}M$ of each peptide) (Fig. 2, left). In the spinal cord, which contained high-affinity binding sites for rCGRP (half-maximal inhibition of binding of ^{125}I-labeled rCGRP by $10^{-9}M$ rCGRP), sCT produced half-maximal inhibition of binding of ^{125}I-labeled rCGRP at $10^{-6}M$ (Fig 2, right). No influence on adenylate cyclase was observed with either rCGRP or sCT in the spinal cord and adrenal gland which contained predominantly high-affinity CGRP receptors (Fig. 3). In the kidney, both CT and CGRP elicited the characteristic CT effects on adenylate cyclase (Fig. 3). The capacity of CGRP to alter adenylate cyclase activity was less than that of sCT, commensurate with its reduced ability, relative to sCT, to inhibit ^{125}I-sCT binding (Fig. 3).

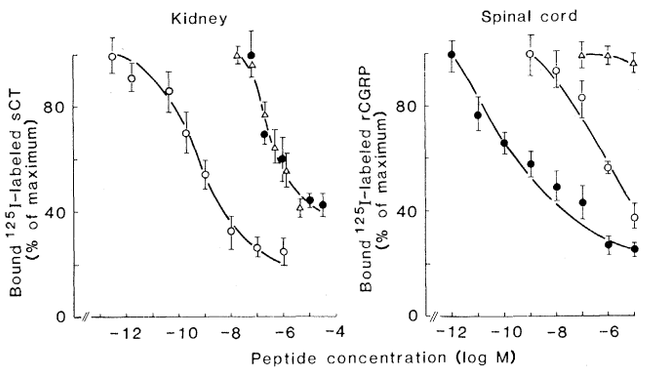

Fig. 2. Effect of increasing concentrations of unlabeled sCT (○), hCT (△), and rCGRP (●) on inhibition of ^{125}I-labeled sCT binding in kidney (left panel) and on inhibition of ^{125}I-labeled rCGRP binding in spinal cord (right panel). Binding studies were performed as described in the text. The ordinates represent ^{125}I-labeled peptide bound in the presence of each concentration of unlabeled peptide, divided by the maximal quantity of ^{125}I-labeled peptide bound × 100 (% of maximum). Maximal binding of ^{125}I-labeled sCT in kidney and of ^{125}I-labeled rCGRP in spinal cord was 4.4 ± 0.3 percent and 7.7 ± 0.4 percent, respectively. Each point represents the mean ± standard error of triplicate determinations. In each system, $10^{-5}M$ leucine enkephalin and methionine enkephalin (Peninsula Laboratories, Inc.), bovine parathyroid hormone (1-84) (Bachem Co.), and porcine insulin and human ACTH (1-39) (Sigma Chemical Co.) failed to inhibit binding of labeled peptide. Results similar to those in kidney were found in the hypothalamus (*14*).

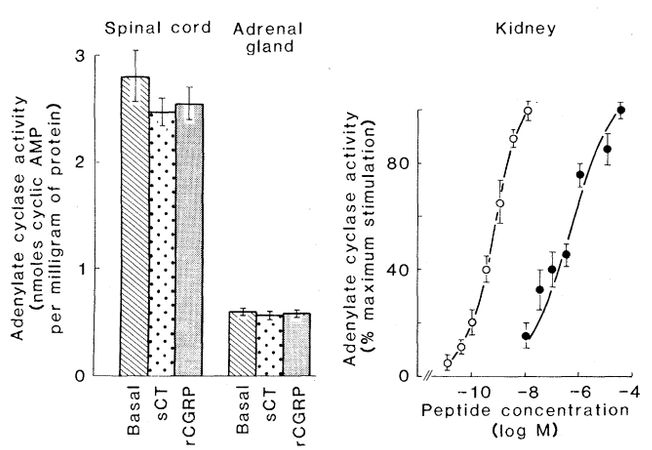

Fig. 3. Effect of $10^{-5}M$ sCT and of 10^{-5} *M* rCGRP on adenylate cyclase activity in spinal cord and adrenal gland (left panel) and in kidney (right panel). Each bar represents the mean ± standard error of triplicate determinations. Increasing concentrations of sCT and of rCGRP in kidney each produced increasing adenylate cyclase stimulation. Adenylate cyclase activity in kidney is expressed as the enzyme activity above the basal level stimulated by each concentration of peptide, divided by the maximal peptide-stimulated activity above basal × 100. Basal and maximal activity were 65 ± 19 and 600 ± 20 pmole of cyclic AMP per milligram of protein, respectively, for the 30-minute period of incubation. Each point is the mean ± standard error of triplicate determinations.

Despite this evidence for the existence of distinct specific binding sites for CGRP and CT, and despite the absence of immunochemical cross-reactivity (*1*), each peptide inhibited binding at the high-affinity receptor site of the other. Such cross-reactivity was not seen with unrelated peptides, and may have reflected, in part, conformational similarities of CT and CGRP imposed by the amino terminal disulfide bridge and carboxyl terminal amide group present in both molecules, as well as by the similar overall size of the two peptides. Previous studies in other systems (*10*) have also demonstrated cross-reactivity at the receptor level of peptides with only modest sequence homology and in the absence of immunochemical cross-reactivity.

Although rCGRP inhibited ^{125}I-labeled sCT binding in CT target tissues less well than sCT, its ability to inhibit binding was approximately equivalent to that of hCT which differs from endogenous rCT by only two residues (*11*). Consequently, rCGRP may activate CT receptors on an equimolar basis with rCT. Inasmuch as the biologically relevant comparison is between molecules isolated from rat, rather than with sCT which was employed as a conventional probe of CT binding (*2–5*), CGRP may be of importance in modulating calcitonin effects particularly in the nervous system.

Previous studies have confirmed the capacity of neural tissue to biosynthesize CGRP but have failed to provide corresponding evidence for neural CT biosynthesis (*3*); clearly, however, CT binding sites can be easily demonstrated in the nervous system (*4*) and effects on central nervous system function have been described after intracerebroventricular injections of CT (*12*). Although binding sites in circumventricular organs of the nervous system may be accessible to

circulating levels of CT (*13*), binding sites in other neural regions would predictably not be able to interact with circulating CT of peripheral origin. In view of our evidence for equivalent competition by mammalian CGRP and mammalian CT at CT binding sites, CGRP could serve as an endogenous ligand for the CT receptors demonstrated in the central nervous system. The CGRP might carry out central nervous system actions previously ascribed to CT (*12*), in addition to altering neural function itself.

No evidence of CGRP effects on adenylate cyclase was found in tissues containing high-affinity binding sites for CGRP, but lacking high-affinity binding sites for CT. Consequently, CGRP and CT may function via separate second messenger systems. Nevertheless, CGRP was capable of influencing the adenylate cyclase system apparently through the CT receptor in tissues containing predominantly high-affinity CT binding sites. Consequently, each peptide may initiate cyclic AMP-dependent or -independent events depending on the initial binding site with which it interacts.

Therefore, CT and CGRP appear to have close biosynthetic links related to a single genomic locus (*1*), to have discrete target-tissue receptors with the potential for heterologous interactions, and to have different second messenger systems.

DAVID GOLTZMAN*
JANE MITCHELL
Department of Medicine
McGill University
Montreal, Canada H3A 1A1

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* To whom correspondence should be addressed at McGill University, Allan Research Building, Room 117, 1033 Pine Avenue West, Montreal, Canada H3A 1A1.

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Measuring Gene Expression with Light

Abstract. *Light is produced by recombinant* Escherichia coli *that contain* lux *genes cloned from the marine bacterium* Vibrio fischeri. *The bioluminescence phenotype requires genes for regulatory and biochemical functions, the latter encoded by five* lux *genes contained in a single operon. These* lux *genes were disconnected from their native promoter and inserted into the transposon mini-Mu. The resulting transposon, mini-Mu*lux, *could induce mutations by insertional inactivation of a target gene, and the* lux *DNA was oriented to align target gene transcription with that of the* lux *genes. Genes in* Escherichia coli *and* Vibrio parahaemolyticus *were mutagenized, and mutants containing transposon-generated* lux *gene fusions produced light as a function of target gene transcription. Light production offers a simple, sensitive, in vivo indicator of gene expression.*

Transposon mutagenesis and gene fusions have been used to study gene regulation. Transposon insertions result in a null mutant phenotype (total loss of function), are convenient to map, and physically associate a selectable marker such as a drug resistance gene with the target gene (*1*). Gene fusions, constructed by aligning indicator structural genes to the controlling elements of other genes, link the transcription of a given gene to that of indicator genes such as the *lac* operon. Thus, a manageable and selectable phenotype is substituted for one that may be difficult to analyze (*2*). By inserting *lac* genes into transposon Mu, Casadaban (*3*, *4*) combined the transposon and gene fusion technologies. One particular construction, transposon mini-Mu, causes mutation by insertional inactivation, fuses target gene transcription to the *lac* genes, carries a selectable marker, and can be transduced by helper phage Mu. We have used mini-Mu to construct a transposon that generates gene fusion mutants that produce light.

Seven *lux* genes contained on one DNA fragment from *Vibrio fischeri* encode the regulatory and biochemical activities necessary for light production in *Escherichia coli* (*5*) (Fig. 1A). The genes *luxR* and *luxI* control the transcription of one operon containing *lux* genes *I*, *C*, *D*, *A*, *B*, and *E*. The subunits of luciferase

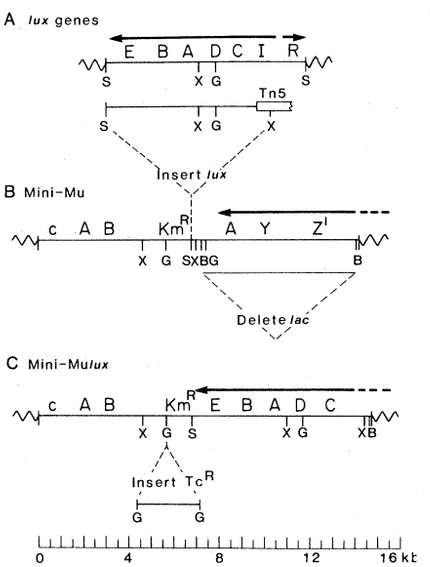

Fig. 1. Construction of mini-Mu*lux* transposons. Restriction sites are S, Sal I; B, Bam HI; X, Xho I; and G, Bgl II. Arrows indicate the extent and direction of transcription of operons. (A) The *lux* genes and operons in a Sal I fragment from *V. fischeri*. (B) The mini-Mu (MudII1681) which contains transposase genes (*A* and *B*), the gene for repressor of transposition (*c*, a temperature-sensitive allele), the kanamycin resistance gene (Km^r), and part of the *lac* operon with genes *Z'*, *Y*, and *A*. This particular mini-Mu generates protein fusions containing the functional carboxyl terminal portion of β-galactosidase and was chosen because the *lac* genes could be deleted by Bam HI digestion. Mini-Mu was transposed to plasmid pKO-1 to generate plasmid pJE101 with which the various recombinant DNA manipulations could be achieved. (C) After deletion of the *lac* genes, the Sal I–Xho I *lux* gene fragment was ligated into the Sal I site of mini-Mu producing mini-Mu*lux*(Km^R). The DNA end produced by Xho I digestion ligated with the Sal I end of mini-Mu but could not be recleaved by Sal I or Xho I. A mini-Mu*lux*, mini-Mu*lux*(Tc^R), which encodes tetracycline resistance, was constructed by addition of a Bgl II fragment from transposon Tn10.